Comparative study on the response of marine bivalves to temperature stress

by

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 2

Chapter 4

Abstract

Suspension-feeding bivalves are the key species in estuaries and are commonly farmed in coastal areas. As the marine environment is experiencing numerous biochemical and physiological perturbations due to the change of temperature, salinity and dissolved oxygen, these changes have significantly impacted bivalve growth, survival and distribution. Among environmental factors, temperature is an important factor that regulates physiological responses in aquatic poikilotherms. As a result of global warming, temperature can increase animal metabolism and activities, resulting in the changes in growth, development and immunological responses of marine bivalves. In most situations, filter-feeding bivalves can clear food particles from the water column and high-water temperature promotes feeding activity. Hence, it is necessary to investigate temperature-dependent responses in grazing rate, growth, metabolism and immunity to improve our understanding on the environmental impact in bivalve aquaculture. This thesis consists of three data chapters (2, 3, and 4).

Chapter 2 addresses the effect of temperature elevation on physical, metabolic and growth of oyster *Crassostrea gigas*, mussel *Mytilus galloprovincialis*, and cockle *Katelysia rhytiphora*. All three bivalve species were separately exposed to three temperatures (15 °C, 20 °C, and 25 °C) in tanks for 30 days with three replicates. Temperature significantly impacted seston clearance, food absorption efficiency, oxygen consumption, excretion, pyruvate kinase enzyme activities, and growth performance. Oysters had the highest clearance rate and food absorption efficiency regardless of temperature when compared to other species. The best growth occurred at 15 ^{o}C, whereas the growth rate was negative at 25 ^{o}C in all species. At 25 °C, the growth rate of each species became negative due to a significant reduction in food

clearance, high metabolism and high excretion. Cockles had the lowest growth rate, ranging from -8.4 to -15.7 J $g^{-1}h^{-1}$ compared to oysters and mussels, suggesting that cockles have the poorest adaptability to thermal stress.

In Chapter 3, the impact of temperature change on the defence system of bivalves was investigated by measuring immunological parameters and antioxidant enzymes relevant to stress response. Each species was exposed to three temperatures, 15 °C, 20 °C and 25 °C for 14 days. The total haemocyte count, phagocytosis, reactive oxygen species and the activity of antioxidant enzymes such as superoxide dismutase and catalase were used as indicators to measure the response of each species to different temperatures. Water temperature significantly affected immune functions in molluscs and led to oxidative stress and reduction of immunosurveillance in all three species of marine bivalves. Temperature also affected the survival of these molluscan species with highest mortality at 25 °C. Cockles were mostly affected by temperature elevation. This chapter demonstrates that the intertidal species like oysters have a greater tolerance to extreme thermal stress than subtidal species (e.g. mussels) and demersal species buried in sand (e.g. cockles).

As temperature has a significant impact on biology and growth performance in marine bivalves, Chapter 4 compares the seasonal food selectivity and feeding capacity of three bivalve species. Seston abundance varies significantly across spatial and temporal scales due to water transport processes, climate and environmental fluctuations, and has important applications to the success of food filtration of marine bivalves. This study demonstrates that oysters and mussels selectively fed on large food particles (e.g., diatom, dinoflagellate and large picoplankton >2 μm) regardless of season, but mussels could access a wider size spectrum of food particles compared to oysters. Cockles on the other hand, selected for both large and small food particles

(e.g., *Synechococcus* and small picoeukaryotes <2 µm) and fed more efficiently on small particles than both oysters and mussels.

Overall, temperature is an important environmental factor that needs to be taken into account in shellfish aquaculture and management as it significantly alters most functions in the biological process of aquatic organisms, which in turn can impact growth, survival and productivity. The results of this thesis can improve our knowledge on how temperature-dependent stress modulates marine bivalves that have different habitats in nature. These findings would help to choose locations for marine bivalve farming to counteract the possible negative effect of temperature elevation due to global warming in future.

Declaration

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

Maziidah Ab Rahman

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CHAPTER 1

GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1. Marine Bivalve: Fishery and mariculture production

Global fishery resources are at alarming rates of decline, with 85% of marine stocks including pelagic, demersal fish, and shellfish species reported as overexploited (FAO 2005; Urquhart et al., 2013). The resource decline is related to the growth of human population experienced in last a few decades. The application of modern techniques to facilitate harvesting, transport, storage, and packaging also have accelerated this trend.

Bivalves fisheries including oysters, mussels, scallops and clams continue to decline every year (Lutz et al., 2012). Several factors attribute to the major landing declining in recent decades, including overfishing, degradation of reefs by commercial fishing, deteriorating water quality, high sediment loads, and emergence of molluscan diseases. In response to problems associated with this immense situation, research projects, policy developments, and conferences have been established with the aim of delivering sustainable fishing industry to combat overexploitation and promote resource recovering and projection (Urquhart, 2013). Furthermore, in Europe, a reform of European Common Fisheries Policy (CFP) also emphasizes the important of thriving coastal communities which integrate most of the policy and research on socioeconomic, biological and ecological aspects in order to achieve sustainable fisheries.

Aquaculture practice is the fastest growing sector in the food-producing industry and helps to compensate with the depleted stock of wild caught oysters (Cranford et al., 2012). The Pacific oyster *Crassostrea gigas* has become the major marine aquaculture bivalve species in most part of the world such as Japan, China, Australia, France, North-western Europe and North America to meet the gaps

between market demand and declined fisheries landings. The success in hatchery production has opened opportunities for the aquaculture of *C. gigas*. The international production of *C. gigas* through aquaculture has been shown that activities have increased from the 1950s (initiation of statistic collections by the FAO) until the mid-1980s when production remained constant. Worldwide annual production today of *C. gigas* is approximately 662 500 tonnes. The international value has followed this increase and is currently valued at USD1.263 billion (FAO, 2005).

Bivalve farming is one of the oldest aquaculture industries in Australian history (Nell, 2005). The increase in domestic demand for shellfish species coupled with the potential export markets has renewed the interest in promoting and developing to assist the industry. Oyster farming has become the fourth largest aquaculture industry and includes three species, the Sydney rock oyster *Saccostrea glomerata*, the Pacific oyster *C. gigas*, and the flat oyster *Ostrea angasi* (Troost, 2010). Most Australian oysters are cultivated in the hatchery environment before being transported in trays, baskets and rafts where they grow to market size. In recent years, selective breeding programs have been established in Sydney rock oysters and Pacific oysters to improve the key traits such as growth rate, body shape and disease resistance. The industry has been growing rapidly in the states where oyster farming exists, especially in South Australia (SA), New South Wales (NSW), and Tasmania, with South Australia being the biggest Pacific oyster producer with a farm gate value of $40M (~6,250,000 dozen) in 2011/12 (Eco Search, 2013). Currently there are over 500 oyster aquaculture license holders in Australia (Urquhart et al., 2013). Besides oyster, mussels farming dominated by *Mytilus galloprovincialis* and *Mytilus edulis* also significantly contribute to bivalve aquaculture in Australia. Moreover, currently

the State government also showed interest to maximise the existing lease via new potential aquaculture entrants such as mud cockles and clams (FRDC-PIRSA Initiative II, 2013).

1.2. Bivalve habitat and role in the ecosystem

Bivalves are key indicators of the health and performance of an aquatic ecosystem and they play significant roles in ecosystem processes, reorganising the structure and function of communities. Molluscan species can mediate energy and nutrient flux, recycle biogenic silica, and contribute to bioturbation and sediment resettling (Chauvaud et al., 2000; Navarro and Thompson, 1997; Newell, 1988). Large amounts of nutrients linked to different components of seston in the environment are taken up and recycled by bivalves thereby influencing the condition in their habitats. The loss of bivalves from a system due to overexploitation, disease outbreak, pollution or reduction of food supply may result in alterations of coastal ecosystem functions (Ruesink et al., 2005).

Cultivation of filter-feeding bivalves occurs mainly in coastal bays and estuaries. These habitats possess high primary productivity, support a high load of filter-feeding biomass and promote rapid bivalve growth. Many estuarine bivalves can tolerate a wide range of environmental changes since coastal zones are commonly subject to unique patterns of environmental change. The biochemical composition of body tissues and growth rates are strongly influenced by environmental factors such as temperature, food, salinity, dissolved oxygen, particulate organic matter and bacterial abundance. Each factor plays a significant role influencing the ecosystem function in a complex way. It has been reported in several studies that the change of temperature and food availability are the two most

important factors that could influence bivalve growth performance (Rico-Villa et al., 2009; Sara and Mazzola, 1997).

Within Australia, shellfish aquaculture industry is virtually made up of oysters producing 14,800 tonnes worth $100 million, mussels with 3,100 tonnes and a value of $10 million, and indigenous clam species which are currently sold at $18-22/kg on local market (Duthie, 2012). Although the mussel *Mytilus galloprovincialis* has already contributed to molluscan aquaculture, the mud cockle *Katelysia* sp. is a new entrant and has a great potential for aquaculture. Mussels and cockles are both filter feeders, but mussels obtain food in water column while cockles filter food in the substrate and can greatly influence energy and nutrient fluxes between pelagic and benthic communities.

1.3. Bivalve food sources and physiological processes

Phytoplankton measured as chlorophyll *a* in water is the principal food source for marine bivalves. Filter-feeding bivalves have long been known as herbivores, consuming mainly phytoplankton and organic compounds (Lehane and Davenport, 2002), and this perhaps is warranted with phytoplankton dietary contribution as high as 89% (Xu and Yang, 2007). However, recent studies have revealed that bivalves can ingest a substantially wider range of other material in seston such as detritus, bacteria, faecal pellets, microzooplankton and mesozooplankton (Webb et al., 2013; Ezgeta-Balic et al., 2012, Peharda et al., 2012, Davenport et al., 2011). Since bivalve molluscs are actively filter feeders, food availability in water column and environmental variables in water contribute the most to the survival and growth of cultured species (Rico-Villa et al., 2009; Saxby, 2002). Their dietary assimilation is also dependent on the habitat where they live (Xu and Yang, 2007). A wide range and variation in density of available food in cultured sites eventually determine the

success and sustainability of bivalve culture. In order to maintain continuous growth and productivity and attain benefit from it, sustainable development and management plan are needed to meet ongoing culture challenges.

Research in recent years has attempted to establish relationships between food supply and environmental factors with reference to the production of marine bivalves. Through the introduction of advanced technologies (e.g. flow cytometry, video endoscopy, confocal microscopy and multi-factor analysis), the understanding of particle feeding and selection process by bivalves has improved markedly (Review in Cranford et al., 2005; Yahel et al., 2005). However, many knowledge gaps still exist, as each species performs differently and has various adaptations and strategies to cope with changes in particle size. Bivalve feeding physiology is influenced by physical and environmental factors. It has been reported that temperature and salinity are the key factors affecting the physiological and metabolic response in marine aquatic poikilotherms (Anestis et al., 2010, Anestis et al., 2007, Berthelin et al., 2000). Comprehensive identification of the mechanisms of thermal limitation and adaptation is important for organism performance, especially for acquiring food and temperature-dependent metabolism.

In South Australia (SA) waters, there is little knowledge on bivalve food sources especially in marine bivalves. For instance, in a recent Fisheries Research Development Corporation (FRDC) project, bivalves could not be included in the modelling of carrying capacity in the Spencer Gulf due to the lack information on the trophic function and feeding biology of bivalves. Based on the previous studies, it seems that bivalves primarily oysters, cockles and mussels in SA water are deriving their nutrients from a diverse range of food sources. Hence, the information on bivalve diets in relation to spatial and temporal variation is needed to elucidate

carrying capacity models of the southern Australian ecosystem and may aid design of a holistic management framework for sustainable bivalve aquaculture.

1.4. Factors limiting shellfish growth

The relationship between shell and flesh growth and environmental factors is complex as these factors may fluctuate rapidly. As for the fact that the marine environment is experiencing numerous biochemical and physiological perturbations, such as changes in temperature, salinity and oxygen, these changes would impact the performance of bivalve production and product quality, and distribution of organisms that exist in the system (Malham et al., 2009; Newell, 2004). The changes or alteration of those exogenous factors might impose structurally and functionally changes in the bivalve immune system. One of the environmental aspects that affect immune function of marine bivalve is temperature. In recent years, oyster growers worldwide have encountered the phenomenon of summer mortality on their cultured organisms with almost 15% losses in summer (Berthelin et al., 2000). On some occasions, the low food quantity could also contribute to the suppression of immunological and physiological functions, resulting in high mortality of some bivalve species (Li et la., 2009).

1.5. Management of shellfish production

There are many management approaches that have been implemented around the world to promote sustainable bivalve aquaculture, especially in areas where over-exploitation of resources has become apparent. The European Monitoring and Regulation of Marine Aquaculture Concerted Action reviews the licensing, regulations, monitoring programs and scales of marine aquaculture production for all European countries. It aims to establish scientific guidelines for Best Environmental Practice (Fernandes et al., 2000). In South Australia, all aquaculture practices are

required to be licensed. The Primary Industries and Regions SA (PIRSA) is a responsible organization to manage and assist each individual application for the leasing of areas of water and for what species may be cultured as outlined by the Aquaculture Act (SA) (2001). Additionally, the SA Environment Protection Authority (EPA) is the mandatory referral agency for all aquaculture practices. Although this is largely focussed on impacts on farming of expelled waste and the direct effects that human activity has made, it does not have any direct requirements addressing ecological effects. The code of practice for the SA oyster farming industry is recognised as a key management tool and has been used for communicating management among growers.

In order to achieve high productivity and adequate management of bivalve farming, managers have raised an important issue to implement the carrying capacity model to assist the industry development. An important component of the carrying capacity model is food availability which could limit growth and survival. The estimate of molluscan carrying capacity involves a wide variety of factors that need to be considered such as temperature and temporal variation of food supply (Hui, 2006). Appropriate location and stocking densities of organisms are also important to take into account to ensure that adequate food is available, and growth is sustainable. Furthermore, a whole ecosystem approach is necessary to achieve truly sustainable management. The application of carrying capacity aided with more holistic management approaches that seek to resilience of bivalve populations is needed to account for all differential effects of environmental parameters as this scenario may lead to the change in species production and productivity. Together, a holistic approach will overcome the limited knowledge on the relationship and parameters that might lead to greater scientific uncertainty (Filguire et al., 2013). Here in SA,

bivalve aquaculture management is still considered in a data-poor environment, therefore this study on diet and feeding of marine bivalves is expected to fill this knowledge gap for information on ecosystem variability at different scales to meet the need for important consideration to design the management framework.

1.6. Need for this thesis research

There is an increasing need worldwide to improve the understanding for ecologically sustainable aquaculture development and ecosystem-based resource management. A recent report on modelling and performance monitoring of SA's Spencer Gulf in relation to aquaculture and carrying capacity could not include the component of marine bivalves due to the lack of understanding on the nature of food and feeding in major economically important molluscan species (Middleton et al., 2013). Additionally, the South Australian Oyster Research Council (SAORC) has recently identified the nutrition factor as an essential area to address the ongoing mortality issue and the need for research is recognised to assist in further development. It has been found that the competitive overlap in food sources will result in the decrease of overall species richness (Compton et al., 2007), therefore the effects of dietary overlap extend into the consequences of population decline in existing species. Furthermore, environmental factors especially temperature have direct impact on the growth performance as these filter-feeding animals derive their food from the water column. Hence, further understanding of diets of SA oysters and other key bivalve species such as mussels and mud cockles is pivotal to assist in development and diversification of bivalve culture in Australia and to develop a framework for designing whole ecosystem management techniques, encapsulating and further understanding bivalve diets.

1.7. Main research hypotheses

There is a need to understand the relationships between environmental conditions such as temperature, food availability and growth patterns of marine bivalves. Additionally, the ecosystem dynamics of suspension-feeders need to be further understood so that temporal and spatial variability can be managed. The main hypothesis to be tested in this study is that water temperature is a driving force regulating growth, physiology, metabolic activity and immunological response in bivalves and the response to temperature is species dependent, especially between species that have different habitats in nature. There is often a close relationship between bivalve's population declination and extreme temperature that can reduce organism's performance, growth and reproduction (Anestis et al., 2010, Portner and Knust, 2007). The secondary hypothesis is that the overlap of food resources exists between bivalve species and the food partitioning between species allows co-existence in the same ecosystem, providing the basis for diversification of molluscan farming in the same area.

1.8. Overall study objectives

The main objective of this comparative study is to improve the knowledge of three marine bivalves, the Pacific oyster *C. gigas*, Mediterranean mussel *M. galloprovincialis,* and mud cockle *Katelysia rhytiphora* pertaining to the physiological, metabolic and immune responses and feeding competition at different temperatures. To achieve these objectives will contribute to improving marine bivalve farming strategies and management and therefore help in developing methods to identify potential farming areas for sustainable aquaculture. Specifically, this study aims to achieve the following three objectives:

1) to investigate the impact of water temperature on the physiological

parameters, metabolism and growth of Pacific oysters, mussels, and mud cockles.

2) To evaluate the effect of temperature stress on immune responses of Pacific oysters, mussels and mud cockles.

3) To compare the seasonal filtering capacity of three bivalve species Pacific oysters, mussels and mud cockles.

In this study, three trials were conducted to address the above aims. Specifically, these three experiments include:

1) Effect of temperature on physiological, metabolism, and growth performance of three marine bivalves, which addresses aim 1 and is presented in Chapter 2.

2) The impact of temperature on immune response of three marine bivalve species, which addresses aim 2 and is presented in Chapter 3.

3) Analysis of the seasonal impact of three marine bivalves on seston particles in water column, which addresses aim 3 and is presented in Chapter 4.

Thesis structure and content organisation

This thesis is presented in five chapters: a general thesis introduction in Chapter 1, three data chapters, and a general discussion in Chapter 5. Chapter 3 and Chapter 4 have been published, and Chapter 2 is currently under review.

Chapter 1 is a general introduction to the thesis that outlines the research background and major knowledge gaps in molluscan biology and shellfish aquaculture. While there is a demand to fully utilise the resources in shellfish farms by introducing potential bivalve's species, several factors need to be considered to ensure food availability and resource partitioning among farming species. The

temperature-dependent impact on the performance of major economically important molluscan species warrants further investigation.

In Chapter 2, the effect of water temperature on physiological and metabolic parameters of Pacific oysters, mussels, and mud cockles were investigated. Temperature is the most important factor that regulates physiological and metabolic responses and growth in aquatic poikilotherms. Comprehensive identification of thermal limitation on their growth performance is of pivotally importance in choosing a location for molluscan farming. This chapter has been submitted to Journal of Biochemistry and Physiology, and is currently under review.

Chapter 3 has been published in Fish and Shellfish Immunology (2019, 86, 868 - 874). As the temperature has a significant impact on physical and metabolic assessments of bivalves, this chapter investigates the immune response to temperature change. The total haemocyte count (THC), phagocytosis, reactive oxygen species (ROS) and the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were used as indicators to measure the response of each species to different temperatures.

In Chapter 4, the study aims to understand the size range of seston particles that major bivalve species could consume in the water column at different seasons and compares food selectivity of these three bivalve species. Seston varies significantly across spatial and temporal scales due to the water transport processes, climate and environmental fluctuations and has important applications to the success of food filtration of marine bivalves. Understanding seston dynamics is important to tackle several key issues related to the growth of marine bivalves. This chapter has been published in Journal of Experimental Marine Biology and Ecology (2020, 522, 151251).

Chapter 5 carries general discussion of this thesis where all major research findings are summarised and discussed. Final recommendations to the marine bivalve industry to improve production are also provided. Further research based the research findings of this thesis is recommended.

1.9 Publications

1.9.1 Co-authorship of chapters

The manuscripts (here referred to as Chapters 2, 3, and 4) have either been submitted (Chapter 2) or published (Chapter 3 and 4) to peer-reviewed journals. Although I am the principal contributor for each manuscript, some people who made substantial contributions including experimental design, data analysis and interpretation are listed as co-authors. My principal supervisor, Professor Jian G. Qin and co-supervisor, Professor Xiaoxu Li are co-authors of all manuscripts, due to their major contributions to experimental design, sample analysis and writing of each manuscript. Shaun Henderson and Dr. Penny A. Miller are also listed as co-author in all manuscripts due to their contribution towards running the experiments and assisting in sample collection, analysis and reviewing manuscripts.

1.9.2 Thesis publications

***Chapter 2*:** Maziidah A. Rahman, Shaun Henderson, Penny A. Miller, Xiaoxu X. Li, Jian G. Qin. (Under review). Assessment on the physiological and metabolic adaption of three marine bivalves under temperature stress. *Journal of Biochemistry and Physiology*. (Submitted)

***Chapter 3*:** Maziidah A. Rahman, Shaun Henderson, Penny A. Miller, Xiaoxu X. Li, Jian G. Qin. (2019). Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas,* Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora. Fish and Shellfish Immunology*, 86, 868-874.

***Chapter 4*:** Maziidah A. Rahman, Shaun Henderson, Penny A. Miller, Xiaoxu X. Li, Jian G. Qin. (2020). Analysis of the seasonal impact of three marine bivalves on seston particles in water column. *Journal of Experimental Marine Biology and Ecology*, 522, 151251.

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Chapter 2

Physiological, metabolic and growth responses to thermal stress in Pacific oyster *Crassostrea gigas*, mussel *Mytilus galloprovincialis*, and cockle *Katelysia rythiphora*

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2.1. Abstract

The continuing increase in seawater temperature has significantly affected estuarine and coastal organisms such as bivalves that have poor ability of locomotion. This study assessed the effect of temperature elevation on suspension feeding, physiology, metabolism and growth of three bivalve species *Crassostrea gigas* (Thunberg, 1973), mussels *Mytilus galloprovincialis* (Lamarck, 1819), and cockles *Katelysia rhytiphora* (Lamy, 1937). All three bivalve species were separately exposed to three temperatures (15°C, 20 °C, and 25 °C) in tanks for 30 days with three replicates. Clearance rate, food absorption efficiency, oxygen consumption rate, excretion rate, pyruvate kinase enzyme activities, and the scope for growth (SFG) were significantly decreased by increasing temperature in all three species. At 25 °C, the values of SFG in these bivalve species became negative due to a significant reduction in clearance rate, high metabolism and high excretion rate. The best growth occurred at 15 ^{o}C and the growth rate showed a negative value at 25 ^{o}C in all species. Cockles had the lowest SFG value, ranging from -8.4 to -15.7 J $g^{-1}h^{-1}$ compared to oysters and mussels, suggesting that cockles have the poorest adaptability to thermal stress. The response of these three bivalves to temperature elevation became more obvious after 30-day exposure though oxygen consumption was not influenced by temperature at the beginning. Oysters had the highest clearance rate and food absorption efficiency regardless of temperature. This study indicates that climate warming has significant impacts on physiology, metabolism, and growth in coastal and estuarine bivalves, and growth cannot be sustained at 25 ^{o}C in all three bivalve species due to low clearance rate, high metabolism and high excretion rate.

Keywords: Temperature stress, molluscs, physiological, metabolism, scope for growth

2.2. Introduction

Global warming has impacted the performance of organisms in the marine and estuarine environments (Belkin, 2009). The mean global temperature has increased by approximately 0.7 °C in the last century and is expected to further increase by 1.8 – 4.0 °C by the end of the 21st century (Mann et al., 2008; Pachauri et al., 2007). The marine ecosystem has been adversely affected by the climate-driven environmental change and high temperature in the marine ecosystem can alter primary productivity, biochemical composition, and metabolism in marine organisms (Wernberg et al., 2013). Temperature is a vital factor that influences the performance of an organism in growth and health status (Wang et al., 2015; Anestis et al., 2010; Wang and Overgaard, 2007). The current rate of climate warming has caused thermal stress to a wide range of aquatic organisms as the limits of their temperature tolerances have been approached or exceeded (Talmage and Gobler, 2011). Beyond the borders of the thermal window, marine invertebrates and vertebrates have developed internal hypoxia even in fully oxygenated waters, and a significant reduction in aerobic capacity and metabolic rate (Portner, 2002a; Portner, 2002b; Portner et al., 2005). In addition, higher temperatures can make marine organisms more vulnerable to other environmental stressors (Aagesen and Hase, 2014).

Marine bivalves have physiologically evolved to live within a specific range of environmental condition, and the extreme environment outside the optimal range can cause physical stress or even mortality (Denise et al., 2013). Temperature not only limits the spatial distribution of bivalves, but also greatly influences physiological and metabolic processes that are relevant to various biological functions such as enzymatic activities, oxygen consumption and growth (Anestis et., 2007; Pernet et al., 2012). Thermal adaptation relies on the capacity of glycolytic and mitochondrial metabolism such as the respiratory chain and the tricarboxylic acid

cycle. The thermal response and the ability of adaptation determine the scope of physiological activities and the range of tolerance. Thermal stress can also impact the ecological functions through which the animals can change their mode of life and cause behavioural and physiological adaptation (Anestis et al., 2010; Portner, 2002a).

Recently, mass summer mortality of marine bivalves has caused a great economic loss in aquaculture worldwide (Duthie, 2010; Garnier et al., 2007; Samain et al., 2007). There is a close relationship between the animal response and the duration of thermal stress on growth, immune function and reproduction as most marine animals can adapt to the change of environmental temperature over time (Aagesen and Hase, 2014; Dutertre et al, 2010; Portner and Krust, 2007). The Pacific oysters *Crassostrea gigas*, Mediterranean mussels *Mytilus galloprovincialis*, and mud cockles *Katelysia rythiphora* are common bivalve species that serve as ecosystem engineers in coastal area of southern Australia to regulate the energy flux in the system. Oysters, mussels and cockles live in their respective habitats of the intertidal, sedentary and underground zones, but these animals are often exposed to thermal stress in the environment due to temperature elevation in summer. The thermal limit and adaptation have been studied in various bivalve species (Matoo et al., 2013; Alcamo et al., 2007), such as immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora* (Rahman et al., 2019), but the pattern of temperature response has not been experimentally compared in a laboratory condition between bivalves that live in different habitats in nature and at different ambient temperatures over time.

More studies are necessary to better understand how temperature affects both individual organisms and the whole ecosystem. Measurements of the different

physiological rates of bivalves (clearance, ingestion, absorption, respiration, excretion) can be integrated to determine the net energy balance (difference between energy obtained from food and the energy loss in respiration and excretion), which is commonly referred to the "Scope for Growth" (SFG) (Wang et al., 2015; Anestis et al., 2010; Gazeau et al., 2014). Besides being a good indicator of an organism's condition and growth rate, this physiological index is also a precise and sensitive index of the environmental condition (Albentosa et al., 2012). The SFG has been successfully used in a working model in a variety of bivalve species exposed to various environmental conditions (Albentosa et al., 2012; Duarte et al., 2014).

In this study, we aim to evaluate the change of scope for growth (SFG) by comparing the response to water temperature change among three marine bivalves at different time periods of thermal exposure. The SFG represents the energetic response of an individual by determining the energy gain from ingested food minus the energy loss through respiration and excretion (Sara et al., 2008). The SFG approach is a well-accepted measure to quantify the impact of environmental stress through the change of temperature (Sara et al., 2008; Helson and Gardner, 2007). In addition, we also compared the metabolic response of these bivalve species to temperature change by determining the activity of pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK). These two enzymes control the glycolytic flux to anaerobic end products, while their ratio (PK/PEPCK) is an indicator of anaerobic capacity (Anestis et al., 2010). A low value of this ratio indicates a shift from aerobic to anaerobic metabolism in marine bivalves (Anestis et al., 2010; Greenway and Storey, 2000). This study provides a comprehensive evaluation on the pattern of how three bivalve species with different natural habitats cope with thermal stress over time. The results have revealed the importance of

thermal exposure duration and the adaptability of different bivalve species living in the coastlines to thermal stress due to climate change in future.

2.3. Materials and Methods

2.3.1. Animal collection and management

A total of 120 Pacific oysters (*C. gigas*, mean shell length = 63.7 ± 1.9 mm), 120 Mediterranean mussels (*M. galloprovincialis*, mean shell length = 58.4 ± 1.7), and 180 mud cockles (*K. rhythiphora*, mean shell length = 40.5 ± 1.4 mm) were collected from the Coffin Bay, South Australia. Animals were dry transported to the marine laboratory at Flinders University in foam boxes within 48 h of collection. Prior to the experiment, animals were acclimatised for 14 day in a flow through seawater system at 20 ^{o}C. The range of water quality parameters was kept at 18 - 20 ^{o}C, 7.8 - 8.4 mg L^{-1}dissolved oxygen, pH 8.0-8.4, and 35.5-37.5‰ salinity. During acclimatisation, animals were daily fed *ad libitum* with monoalga *Isochrysis galbana*. Dead animals were removed from the tanks and replaced with a similar sized animal and 50% of seawater in the tank was daily replaced through a continuous water flow.

After two weeks of acclimation at 20 ± 0.28 °C, animals were randomly distributed among three groups at 15 ± 0.53 ^{o}C, 20 ± 0.30 ^{o}C, and 25 ± 0.72 ^{o}C, respectively. The water temperature in the experimental tanks was monitored every day and maintained at a constant value using a thermostat device. These temperatures were chosen because they are within the range of temperatures encountered by the three species in their natural habitats. The experiment was conducted in 25-L aquaria with aeration in triplicate. Animals were fed with *I. galbana*, and the mean concentration of cells in each aquarium was 30 000/ml and this was achieved by daily adding a concentrated culture of *I. galbana*. Animals were tested on day 0

(samples were collected right before starting the experiment), 15 and 30 and various physiological parameters such as clearance rate, respiration rate and absorption efficiency were determined individually at their corresponding temperature to detect the trend of thermal response of animals over time. To determine the activity of pyruvate kinase and phosphoenolpyruvate carboxykinase, animals were sampled on the above days, respectively. The mantle tissue and posterior adductor muscle were removed and ground in liquid nitrogen, and the tissue was stored at −80 °C for determination of enzymatic activities.

2.3.2. Determination of physiological parameters and SFG

Clearance rate

For each group, clearance rate, food adsorption efficiency and respiration rate were measured according to the procedures by Widdows and Staff (2006). At each sampling time, 12 animals were used to measure the physiological responses at each temperature. Clearance rate, i.e., the volume of water cleared with suspended particles per hour, was measured in a closed system. Individuals were placed in separate glass beakers containing 2 L of filtered seawater (0.45 μm) positioned on multi-stirrer base plates to keep the water thoroughly mixed and oxygenated. After the acclimation period of 30 min in the beaker when animals opened valves for normal filtering, algal cells *Isochrysis galbana* were added to each beaker to give an initial concentration of 24 000 cells mL^{-1}. Three 5-ml samples were collected each time during the 1-hour period. The microalgal cell concentration was determined on a microscope using a Neubauer haemocytometer. The beakers without animals were used as a control to monitor the change of algal cell concentration over the experimental period. The clearance rate (CR) was then calculated using the following equation:

$CR = 2L\ (\log_e C_1 - \log_e C_2)/\text{time interval (t)}$

where C_1 and C_2 are the cell concentrations (cell/ml) between two sampling times.

Respiration rate

Respiration rate was determined by placing an individual animal in glass respirometers (500 ml) containing oxygen-saturated seawater stirred by a magnetic stirrer bar beneath a perforated glass plate supporting the animal. The respirometer was sealed and the decline in oxygen concentration was measured by using the Strathkelvin oxygen electrode connected to a Strathkelvin oxygen meter (Model 781).

Absorption efficiency

Food absorption efficiency was measured by comparing the proportion of organic matter in the algal cells and the animal faeces according to the method of Conover (1966). Algal food and faecal samples were collected on washed, ashed and pre-weighed Whatman grade GF/C filters. Salts were washed out of the filters with 10 ml of deionised water for three times and the filters were dried at 90 °C for 24 h. The filters were then weighed before ashing in a furnace at 450 °C for 6 h and re-weighed. Faeces were collected from the acclimation beakers at the time of respiration measurements.

Excretion rate

Animals were placed individually in water jacketed chambers containing 0.8 L of filtered (0.45 μm) seawater and one additional chamber without animal was served as the control. After incubation for 1 h at the temperatures where the animals acclimated, samples from the water containing the animals and from the control were analysed for ammonia with a spectrophotometer (Spectroquant® NOVA 60, Merc

Company) according to the standard method of Solorzano (1969). The excretion rate (U, mg NH_4–N/h) was calculated as follows:

$U = (C_{test}–C_{control}) \times V/ t$

where C_{test} is the NH_4–N concentration in the beaker with the animal; $C_{control}$ is the NH_4–N concentration in the control; V is the volume of incubation seawater (L) and t is the incubation time (h). Values for excretion rate were transformed to J/h using the conversion factor: 1 mg NH_4–N/h = 24.8 J/h (Elliot and Davison, 1975).

2.3.3. Determination of pyruvate kinase activity (PKA) and phosphoenolpyruvate carboxykinase (PEPCK) activities

For the determination of enzymatic activity, samples of frozen mantle and posterior adductor muscle tissue powders (200–500 mg) were rapidly weighed and homogenized (1:5, w/v) in ice-cold 50 mM imidazole–HCl (pH 7.0) containing 100 mM sodium fluoride, 10 mM EDTA, 10 mM EGTA, 30 mM 2-mercaptoethanol, 40% glycerol (v/v) and 0.1 mM phenylmethylsulphonyl fluoride, which was added just prior to homogenization. After centrifugation at 25 000 × *g* for 20 min at 4 °C, the supernatant was removed and passed through a 5 mL column of Sephadex G-25 equilibrated in 40 mM imidazole–HCl buffer (pH 7.0) containing 5 mM EDTA, 15 mM 2-mercaptoethanol and 20% glycerol to remove metabolites of low molecular mass. All enzymes were assayed according to Churchill and Livingstone, (1989) in 100 mM triethanolamine–HCl pH 7.6 in a final volume of 1 ml. Assays were carried out in duplicate to detect the change in absorbance at 340 nm using a FLUOstar Omega microplate reader (BMG Labtech, German).

2.3.4. Calculation of the scope for growth (SFG)

After physiological measurement, animal tissues were removed from the shells and dried at 90 °C for 24 h to obtain the dry weight of tissues. The individual

clearance rates (L h^{-1}) and oxygen consumption rates (μ mole O_2 h^{-1}) were standardised to 1 g dry weight using the standard weight exponent (b = 0.67, [27]). Each physiological rate was then converted to energy equivalents (J h^{-1} g^{-1}) in order to calculate the energy budget and the scope for growth (SFG), which represents the difference between the energy absorbed from food (food consumption × absorption efficiency) and the energy loss in excretion (the energetic equivalent of ammonia excreted).

2.3.5. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analysed using two-way analysis of variance (ANOVA). Three temperature treatments vs three grazer species were tested at three intervals to examine the effect of temperature and molluscan species on all dependent variables. Data were tested for the normality of data distribution and homogeneity of variances before proceeding with the analysis. When significant interactions between main factors were observed, pairwise comparisons were used to determine significant differences between treatment combinations. If the interaction between the season and species was not significant, then the main effect was considered and the post-hoc Tukey's HSD was used for multiple comparisons. The level of significant difference was set at $P < 0.05$. All data were analysed using the statistical package IBM SPSS Statistics 20.

2.4. Results

2.4.1. Clearance rate (CR)

Either temperature or grazer species had significant influence on the CR after the experiment had lasted 30 days (two-way ANOVA; $P < 0.05$, Fig 1), but there was no interaction between these two fixed factors (Table 1, $P = 0.987$).

"On day 0, the CR was marginally affected by temperature ($P = 0.055$) and significantly different among species ($P = 0.007$). At 15 ^{o}C, the CR of oysters and cockles were significantly higher than mussels ($P < 0.05$), at 20 ^{o}C, the CR of oysters was significantly higher that of mussels and cockles. However, at 25 ^{o}C, cockles had significantly the lowest CR ($P <0.05$) compared to oysters and mussels."

On day 15, significant differences were observed in all temperature levels and species ($P < 0.05$). The CR was significantly higher in oysters and mussels than in cockles. On day 30, the increase of temperature from 15 ^{o}C to 25 ^{o}C significantly reduced CR ($P < 0.05$) in all three species. The CR of oysters was always higher than either of mussels or cockles and the cockles always had the lowest CR regardless of temperature ($P < 0.05$). The increase of temperature significantly impaired the molluscan clearance rate especially after exposure to the temperature of 25 ^{o}C for 30 days. Post hoc analysis showed that a significant reduction of CR was observed in all species especially in cockles by 35% after a 30-day exposure.

2.4.2. Food absorption efficiency (FAE)

The absorption efficiency of food particles (Fig. 1) were significantly affected by temperature regardless of species ($P < 0.05$) or by species regardless of temperature ($P < 0.05$) at the end of 30 days. There was a significant interaction between temperature and species in absorption efficiency of food particles ($P < 0.05$).

On day 0, temperature significantly impacted in food absorption efficacy. However, post hoc comparison showed that unlike mussels and cockles, the FAE of oysters were not significantly affected by temperature. After 15 days, significant difference in FAE was observed in all temperature regardless of species. Again, when temperature increased to 25 ^{o}C, FAE was significantly increased in all species.

On day 30, FAE was significantly varied by the increase of temperature in all species. At 25 ^{o}C, FAE in oysters was significantly higher than in cockles (P = 0.025), but not significantly different from mussels (P = 0.692).

2.4.3. Oxygen consumption rate (OCR)

The oxygen consumption rate significantly differed with temperature in all species ($P < 0.05$, Fig. 1) and was also significantly different between species ($P < 0.05$) after thermal treatments for 30 days. Significant interaction was found between temperature and species in oxygen consumption ($P < 0.05$).

On day 0, OCR was not significantly affected by the temperature treatment except between 15 ^{o}C and 25 ^{o}C (P = 0.033). Post hoc test revealed that OCR in oysters was significantly higher than in mussels and cockles, but there was no significant difference between mussels and cockles (P = 0.99). On day 15, significant difference was observed in all temperature regardless of species. On day 30, OCR was increased by increasing temperature. Post hoc test showed that cockles were significantly different from oysters and mussels ($P < 0.001$), but there was no significant difference between oysters and mussels (P = 0.160).

2.4.4. Excretion rate (ER)

Excretion rate in bivalves were significantly affected by temperature in all species tested ($P < 0.05$, Fig. 1d). There was significant interaction between temperature and species ($P < 0.001$) at the end of 30 days.

On day 0, excretion rate in all molluscan species was significantly affected by temperature. Post hoc showed that the excretion rate increased significantly when animals were exposed to 25 ^{o}C (P = 0.030). On day 15, significant difference was observed in all temperature regardless of species. On day 30, a similar pattern was

observed for all species as on day 15 in the excretion rate. The exertion rate significantly increased by 20% - 24% between at 15 ^{o}C and 25 ^{o}C of all species.

2.4.5. Pyruvate kinase activity (PKA)

PKA in the posterior adductor muscle (PAM)

The pyruvate kinase activity in the posterior adductor muscle (Fig. 2) significantly varied among temperature treatments (two-way ANOVA; $P < 0.05$) and was also significantly different between species regardless of temperature ($P < 0.05$) at the end of 30 days. There was no significant interaction between temperature and species on the PKA value ($P = 0.307$).

On day 0, there was no significant difference ($P = 0.680$) of temperature effect on the enzyme activity. However, post hoc comparison showed that, compared to oysters and mussels, PKA in cockles did not significantly vary ($P > 0.05$) between temperatures. On day 15, significant difference of PKA was observed at all temperatures regardless of species. At 15 ^{o}C, PKA significantly reduced in all species, while the elevation of temperature to 25 ^{o}C significantly increased the enzyme activity. On day 30, the PKA was significantly varied by the change of temperature in all species. At 15 ^{o}C, cockles had lower PKA ($P < 0.05$) than oysters and mussels. However, at 25 ^{o}C, the PKA in oysters was significantly higher than that in cockles ($P = 0.039$), but not was significantly different from that in mussels ($P = 0.542$).

PKA in the mantle

The enzyme activity in the mantle tissue was significantly affected by temperature in all species ($P < 0.05$, Fig. 2) and was also significantly different between species ($P < 0.05$) at the end of 30 days. No significant interaction was found between temperature and species on PKA ($P = 0.233$).

On day 0, the was no significant variation of the enzyme activities between temperatures ($P = 0.812$) or among species ($P = 0.570$). On day 15, significant difference was observed in all temperatures regardless of species. The PKA in the mantle at 15 ^{o}C was significantly reduced in all species ($P < 0.05$) while cockles had the lowest enzyme activity relative to other species. The escalation of temperature to 25 ^{o}C increased PKA in all three species in the mantle. On day 30, a similar pattern was observed on day 15 with a further increase at 25 ^{o}C. Post hoc test showed that the PKA in cockles was significantly escalated by increasing temperature ($P < 0.001$) compared to oysters and mussels, and there was no significant difference between oysters and mussels ($P = 0.160$).

2.4.6. Phosphoenolpyruvate carboxykinase (PEPCK) activities

PEPCK in the posterior adductor muscle

The phosphoenolpyruvate carboxykinase activity in the posterior adductor muscle significantly varied among temperature treatments (two-way ANOVA; $P < 0.05$; Fig. 3) but there was no significant difference between species ($P = 0.063$) at the end of 30 days. There was no interaction between temperature and species on the PEPCK value either ($P = 0.512$).

On day 0, there was no significant difference ($P = 0.650$) of temperature effect on this enzyme activity. However, post hoc comparison showed that compared to oysters and mussels, PEPCK in cockles significantly varied ($P < 0.05$) between temperatures. On day 15, significant difference of PEPCK was observed at all temperatures regardless of species. At 15 ^{o}C, PEPCK significantly reduced in all species, while the elevation of temperature to 25 ^{o}C significantly increased the enzyme activity. On day 30, the PEPCK was significantly varied by the change of temperature in all species. At 15 ^{o}C, cockles had a significant lower PEPCK ($P <$

0.05) than oysters and mussels. However, at 25 ^{o}C, the PEPCK in oysters was significantly higher than that in cockles (P = 0.048), but not was significantly different from that in mussels (P = 0.633).

PEPCK in the mantle

The PEPCK activity in the mantle tissue was significantly affected by temperature in all species (P < 0.05, Fig. 3) and was also significantly different between species (P < 0.05) at the end of 30 days. No significant interaction was found between temperature and species on PEPCK (P = 0.321).

On day 0, the was no significant variation of the enzyme activities between temperatures (P = 0.734) or among species (P = 0.490). On day 15, significant difference was observed in all temperatures regardless of species. The PEPCK in the mantle at 15 ^{o}C was significantly decreased in all species (P < 0.05) while oysters had the lowest enzyme activity relative to other species. The escalation of temperature to 25 ^{o}C increased PEPCK in all three species in the mantle. On day 30, a similar pattern was observed on day 15 with a further increase at 25 ^{o}C. Post hoc test showed that the PEPCK in oysters was significantly escalated by increasing temperature (P < 0.001) compared to mussels and cockles.

The pattern of changes in the ratio PK/PEPCK is shown in Fig. 4. Specifically, the ratio remained constant in the adductor mussels at the beginning of the experiment. Warming to 25 °C decreased the ratio of PK/PEPCK after a period of 15 days. Thereafter, the ratio further decreased in both adductor mussels and the mantle of all species.

2.4.7. Scope for growth (SFG)

Calculation of the scope for growth (SFG) showed the positive values at 15 °C for all three species (Fig. 3). Oysters showed higher SFG than mussels and

cockles ($P < 0.05$). At 20 °C, although it remained positive, SFG was lowered by 40% compared to that at 15 °C. The SFG values followed the same pattern at 15 °C where the SFG in oysters and mussels were higher than that in cockles. The further increase of temperature to 25 °C lowered the SFG or turned it to a negative value, indicating a significant loss of animal's ability to retain energy from ingested food at high temperature. At 25 °C, cockles showed the lowest SFG value ($P < 0.05$), ranging from -8.4 to -15.7 J $g^{-1}h^{-1}$ (-6.057±1.988 SD) compared to oysters and mussels.

2.5. Discussion

Over the last decade, there have been growing evidence showing temperature related disease incidence or mass mortality in marine bivalves due to climate change and concomitantly physiological, metabolic and immunological depression in marine organisms (Dickinson et al, 2012). This comparative study on three marine bivalve species exposed to three temperatures has illustrated that water temperature plays a significant role in regulating growth performance at physiological and metabolic levels. However, the rates of change associated with temperature in physiology and metabolism of marine bivalves are subject to the history of adaptation to the ambient temperature. This study clearly demonstrates that all three species could not sustain their energy gain at 25 ºC due to low clearance rate, high metabolism and high excretion rate.

2.5.1. Effects of temperature on the physiological activities

In bivalves, the ability of water clearance rate (CR) is related to ambient temperature and its concomitant increase with temperature plateaus at optimum temperatures and then declines (Wang et al;. 2005; Matoo et al., 2013;Newel et al., 1977). However, the CR can decrease rapidly with the increase in temperature above

the optimum threshold. Previous studies showed that CR in bivalves increased when organisms exposed to temperatures of 15 ^{o}C – 18 ^{o}C, and significantly reduced when temperature extended beyond 25 ^{o}C (Sara et al., 2008; Zhang et al., 2009; Bayne et al., 1976). The present study shows that the increase of temperature significantly suppressed clearance rate in all three bivalve species. Oysters had the higher clearance rate than other two species, and cockles had the lowest clearance rate. Physiological mechanisms underlying the difference in thermal tolerance of cockles and oysters are presently unknown, but may be related to the difference in ecological adaptation of these species. The cockle is a typical infaunal species and are buried in the sediment in a relatively constant condition. In contrast, oysters and mussels are intertidal and subtidal species and are exposed to highly variable environments (Matoo et al., 2013; Portner, 2010). The study clearly shows the difference in thermal adaptation between species that inhabit in different environments.

In line with the data of the present study, Sobral and Widdows (1997) demonstrated that the increase of temperature beyond 20 °C can result in lower clearance rates, leading to a marked reduction of growth in the infaunal clam *Ruditapes decussatus*. These authors also reported that high temperatures (>27 °C) are stressful to the clam, as shown by the low and negative values of the scope for growth. While working on *Perna perna*, Resgalla et al (2007) observed that under a chronically thermal stress condition, this species shows a capacity to compensate the CR between 15 ^{o}C and 30 ^{o}C, but with a tendency of increasing up to 25 ^{o}C and then a rapid reduction occurs beyond 25 ^{o}C. These results are similar to the findings with Goulletquer et al [41] who reported the optimal temperature for *Ruditapes philippinarum* was 12 - 20 °C, and the filtration rate was nearly constant during this temperature range. However, there was evidence that temperature significantly

increased the CR at 25 °C but then significantly decreased at 30 °C in *Modiolus barbatus* (Ezgeta-Balic et al., 2011). The reduction in CR beyond 25 °C is probably due to reaching the upper limit of the optimum level in this species. Additionally, there is evidence that *M. galloprovincialis* keep the valves closed for longer at 24 °C than at 10 – 17 °C (Anestis et al., 2010). Recently, Wang et al (2005) also reported a CR performance curve showing an increase at 25 °C and a decrease at 30 °C in *Mytilus coruscus* in a thermal stress experiment, suggesting that the range of thermal tolerance differs between marine bivalves.

In the present study, temperature elevation significantly increased food absorption efficiency (FAE) and oysters had the highest absorption efficiency ranging from 70 to 80% followed by mussels from 65 to 75% regardless of temperature. These results are in good agreement with Navarro et al (2013) where the FAE of *Mytilus chilensis* fed *Isochrysis galbana* ranged from 50 to 60% at 24 °C. A high FAE (83.3%) was also reported in *Argopecten irradians-concentricus* where the animals were fed with *Dunaliella tertiolecta* at 20 °C (Peirson, 1983). In contrast, the FAE of *M. coruscus* fed with *Chlorella* spp. was relatively low at a high temperature (30 °C), ranging from 25 to 45%, probably due to high faecal production (Anestis et al., 2010). These authors also claimed that the *Chlorella* spp. can be less efficiently digested due to the touch and indigestible cellulose cell wall. In contrast, a low food assimilation efficiency (17.4%) was found in oysters fed on the same species of microalgae (Dickinson et al., 2012), but a high efficiency (40 - 50%) was found in mussels with the same microalgae (Wang et al., 2005).

Therefore, the high FAE is not necessarily indicative of fast growth or a better nutritional value of microalgae (Han et al., 2008). The effect of temperature on FAE in bivalve species is, however, inconsistent. For instance, (Anestis et al., 2010)

reported that the difference of FAE in *M. coruscus* was not significant when exposed to different temperatures (25 - 30 ^{o}C). This claim is supported by other studies that show FAE is relatively independent of temperature (Laing et al., 1987; Albentisa et al., 1994), indicating that the temperature-dependent FAE may be regulated by other factors besides food type (Griffiths and Griffiths, 1987; Widdow, 1978). Similarly, in pearl oysters, the FAE of *Pinctada margaritifera* is not influenced by temperature, while *Pinctada maxima* show a significant increase in FAE from 19 to 32 °C (Yukihira et al., 2000). According to Newell and Jordan, (1983) FAE depends on the length of time that the food remains in the digestive tract and the rate of intake, suggesting the difference between CR and FAE could be related to gut morphology and the body size of animals.

Respiration rate or oxygen consumption rate (OCR) in most molluscan species is a temperature-dependent process though the period of adaptation may govern the rate of oxygen consumption [Sara et al, 2008; Bayne et la., 1976). In the present study, irrespective of species, the OCR increased with temperature after the experiment lasted 30 days. At high temperatures where the maximum respiration rate was passed, there was a marked decline in oxygen consumption. This phenomenon is partially due to oxygen limitation and reduction in ventilation rate, and a rapid decline in respiration rate is usually related to the dominance of anaerobic metabolic pathways (Portner and Farrell, 2008; Tang et al., 2005; Jansen et al., 2007). The OCR in the present study increases linearly with the temperature increase up to 25 ^{o}C, suggesting that the thermal tolerance limits are likely to be close to 25 °C. This pattern is also common in *M. coruscus* (Wang et al., 2005), *Donax vittatus* (Ansell, 1973) and *Mytilus edulis* (Sukhotin et al., 2003).

The excretion rate (ER) is commonly used as an indicator of stress in marine organisms (Fernandez-Reiriz et al., 2011). In bivalves, most ammonia excreted is a product of the catabolism of amino acids from food (Saucedo et al., 2004; Mao et al., 2006), which can vary with the nutritional status of the species (Griffiths and Griffiths, 1987). In the present study, ER increases with temperature due to the demand of metabolic energy in bivalves. The positive relationship between temperature and on bivalve excretion rates have also been reported in *Venerupis pullastra* (Albentosa et al 1994), *M. edulis* (Anestis et al., 2010), and *Crassostrea corteziensis* (Guzman-Aguero et al., 2013). In contrast, Wang et al. (2005) found that ammonia excretion of mussels is not significantly different between 25 ºC and 30 ºC in *M. coruscus*, indicating that the animal may be able to regulate the rate of amino acid catabolism under a thermal stress.

2.5.2. Effects of temperature on metabolic enzyme activities

The physiological adjustment occurred in these bivalve species when they were exposed to 25 ℃ for 30 days as evidenced by the corresponding change in enzyme activity. Pyruvate kinase (PK) is an important regulatory enzyme in the glycolytic pathway, particularly, in the regulation of the transition from aerobic to anaerobic energy metabolism (Anestis et al., 2010; Doucet-Beaupre et al., 2010). In the present study, the increases of PK and PEPCK activities at high temperature in both muscle and mantle tissues indicate a high glycolytic rate as well as a high rate of pyruvate supply to aerobic metabolism at 25 °C. Furthermore, the decrease of the ratio PKA/PEPCK implies an activation of anaerobic component of metabolism in the organism. In contrast, Jansen et al. (2007) reported that when the summer starts, and the peak of ambient temperature occurs at 24 ℃, but the respiratory response of *Mytilus* spp declines at high temperatures. It has been proposed that the modulation

of metabolic sensitivity at high temperature is an adaptative mechanism that prevents excessive metabolic rates to occur at high temperature. As pointed out by other researchers, metabolic depression could be an adaptation to avoid a further rise in energy demand at high temperature at the expense of reduction in the scope of aerobic activity (Anestis et al, 2010; Portner, 2002a). The energy saving strategy may thus contribute to passive alleviation from thermal stress. In addition, Bayne et al. (1976) suggested that the exposure to temperatures over 22 ℃ might result in a cumulative stress in mussels by imposing a metabolic deficit which could be recovered during the period of a subsequent high tide.

2.5.3. Effect of temperature on growth

Temperature is the driving force to influence the energy budget and growth in bivalves (Ezgeta-Balic et al., 2011). The SFG is a useful index to estimate the effect of environmental stressors on the overall performance of bivalves under thermal stress (Navarro et al., 2013; Brown et al., 2004). In the present study, the SFG values were higher at 15 °C during the whole period of experiment, with the highest SFG in oysters followed by mussels and cockles. Significant reductions of SFG were observed at 20 °C in all species. The increase of temperature from 20 °C to 25 °C resulted in negative SFG values in all three species, which implies that animals are utilizing energy reserves rather than accumulating energy from food at thermal stress. The poor growth at 25 °C seems to be due to the temperature induced reduction in clearance rate, as demonstrated in other studies (Sobral and Widdows, 1997; Smaal and Widdows, 1994) that the CR or food acquisition is the most sensitive parameter reflecting the change of SFG. Besides, higher temperature significantly increased the OCR and metabolic activities which suppressed the growth rate. These results were similar to the finding of Anestis et al. (2010) who studied the response of *M. galloprovincialis* to the increase of seawater temperature.

These authors claimed that the SFG values became negative at temperatures over 24 °C due to significant reduction in clearance rate. Similarly, Bayne et al. (1976) reported that the highest SFG values for *Mytilus californianus* was at 17–22 °C and declined at 26 °C. In addition, the increase of temperature from 20 to 32 °C resulted in a marked reduction of SFG in the clam *Venerupis decussates* (Sobral and Widdows, 1997) and the oyster *C. corteziensis* (Guzman-Aguero et al., 2013). These SFG results suggest that the temperature beyond 25 °C is stressful and does not sustain the growth of any of these three bivalve species used in this study.

In conclusion, this study shows that temperature has a strong effect on physiological and metabolic activities as well as the growth performance of marine bivalves. Oysters and mussels have greater capacity to adapt to the increasing water temperature compared to cockles. The relationships between all parameters and temperature stress allow us to predict the optimal temperature regimes in bivalve culture, and provide a valuable insight into the physiological energetics of commercially and ecologically important bivalve species. The temperature elevation caused by climate change would ultimately result in a catastrophic consequence in marine bivalves as the growth could not be sustained at high temperatures. This study alerts to the design and site selection for bivalve farming and fishery operations to work out plan to cope with high temperature episodes in near future to reduce the possible loss in molluscan farming.

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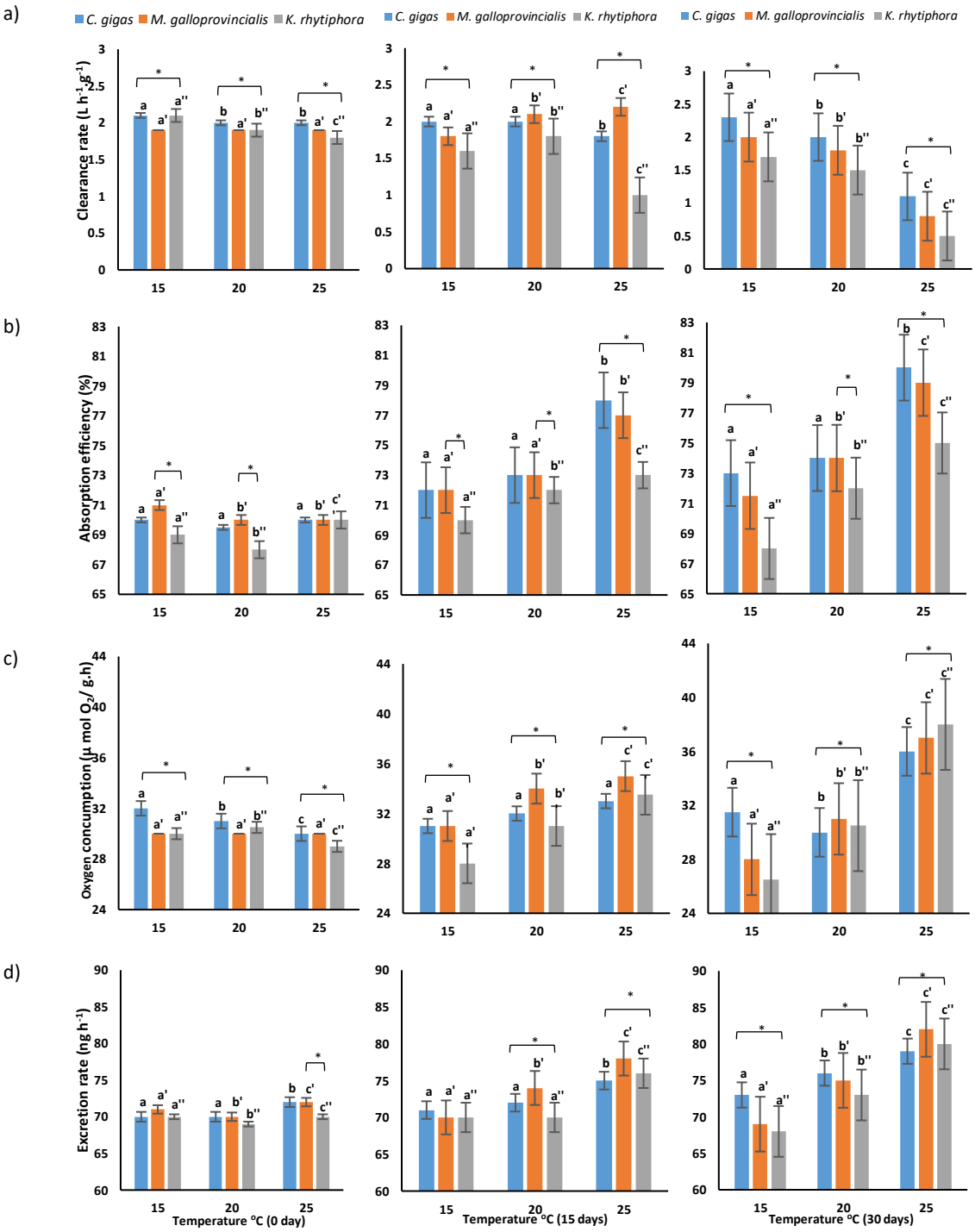

Fig 1 Effects of water temperature on a) clearance rate b) food absorption efficiency, c) oxygen consumption and d) excretion rate by *C. gigas, K. rhytiphora and M. galloprovincialis* during exposure at 0 day (left), 15 days (middle) and 30 days (right). Values are means ± Sd, n = 9. Three sets of letters (a, b and c for oysters; a', b' and c' for mussels and a", b" and c" for cockles) were used at three temperatures, respectively) to differentiate significant difference between temperatures for each species ($P < 0.05$). Bars with * represents significant difference between species at the same temperature. The same letter with different apostrophes (e.g., a, a' and a") is not for comparison between species at a same temperature. Error bars represent standard deviation.

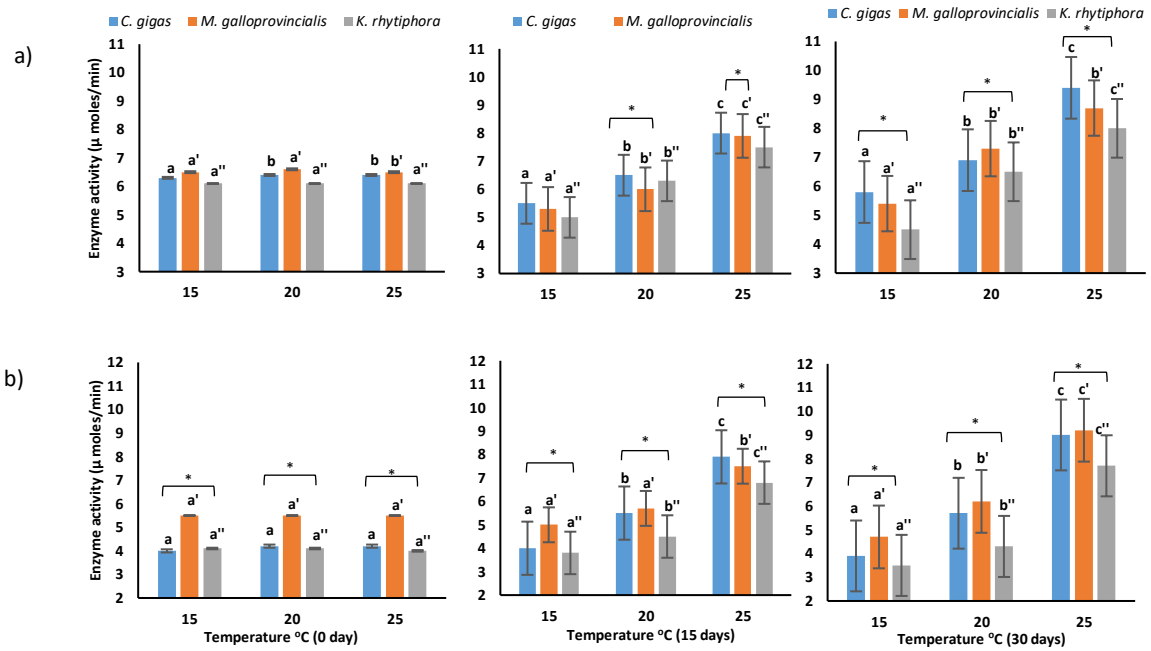

Fig 2 Pyruvate kinase activities (µ mol/min) from a) posterior adductor muscle (PAM) and b) mantle of three marine bivalves during exposure to different water temperatures ($P < 0.05$). Values are means ± Sd, n = 9. *$P <$ 0.05. Different sets of letters (a, b and c for oysters; a', b' and c' for mussels and a", b" and c" for cockles at three temperatures, respectively) indicate significant difference between temperatures within a species. Bars with * represents significant difference between species at the same temperature. The same letter with different apostrophes (e.g., a, a' and a") is not for comparison between species at a same temperature. Error bars represent standard deviation.

a)

b)

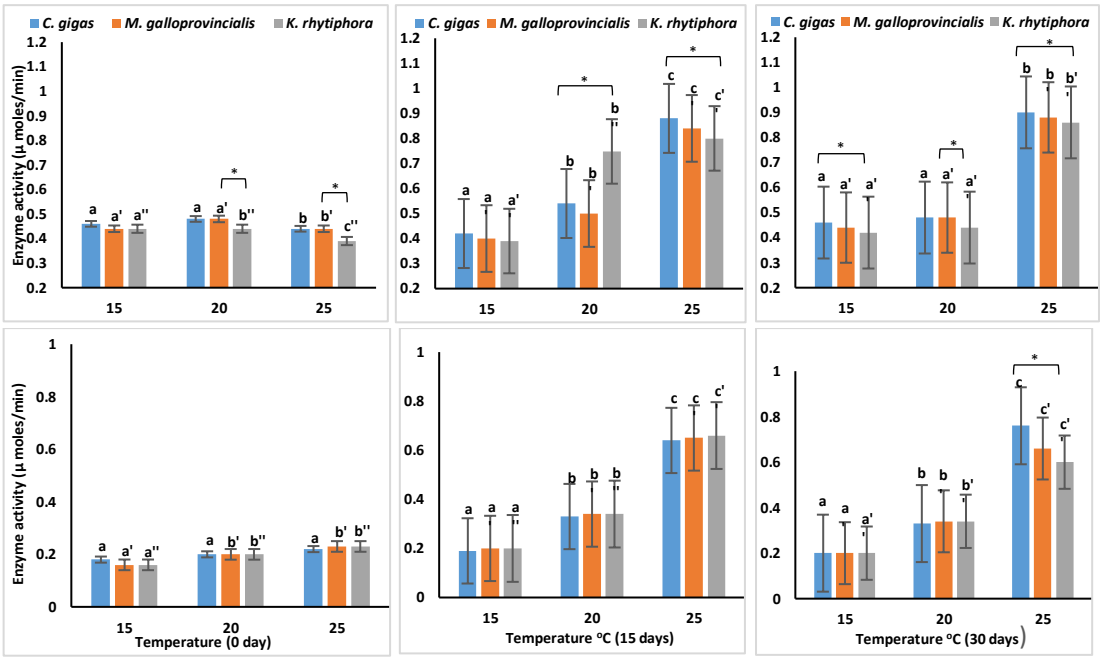

Fig 3 Phosphoenolpyruvate carboxykinase (PEPCK) activities (µ mol/min) from a) posterior adductor muscle (PAM) and b) mantle of three marine bivalves during exposure to different water temperatures ($P < 0.05$). Values are means ± Sd, n = 9. *$P < 0.05$. Different sets of letters (a, b and c for oysters; a', b' and c' for mussels and a", b" and c" for cockles at three temperatures, respectively) indicate significant difference between temperatures within a species. Bars with * represents significant difference between species at the same temperature. The same letter with different apostrophes (e.g., a, a' and a") is not for comparison between species at a same temperature. Error bars represent standard deviation.

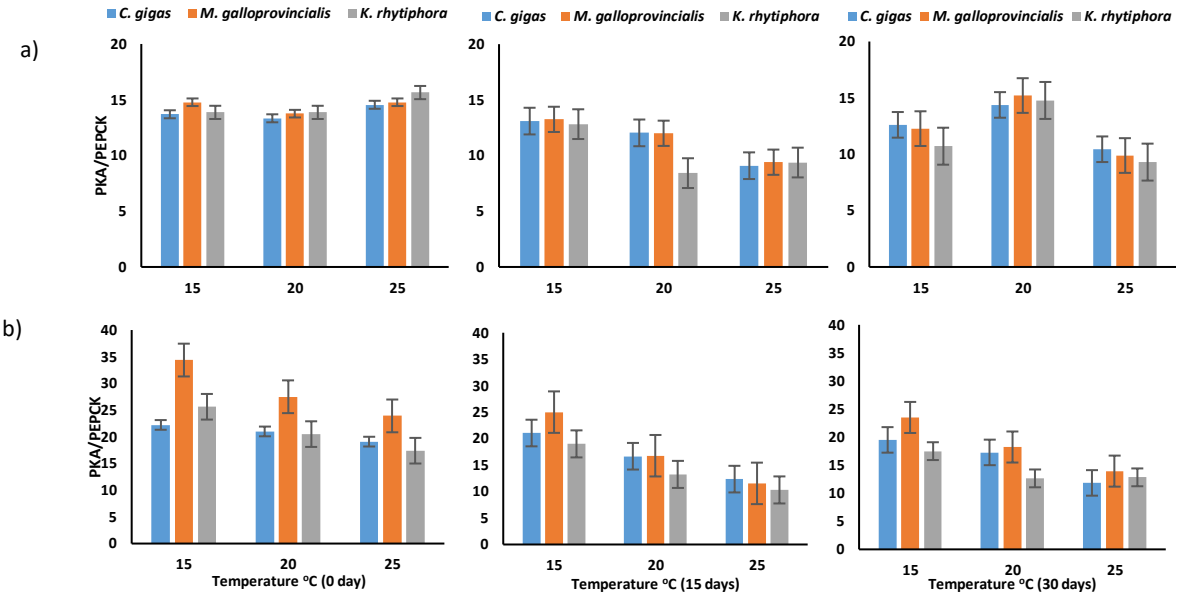

Fig 4 The PKA/PEPCK ratio in the a) posterior adductor muscle (PAM) and b) mantle during exposure to different water temperatures. Values are means ± Sd, n = 9.

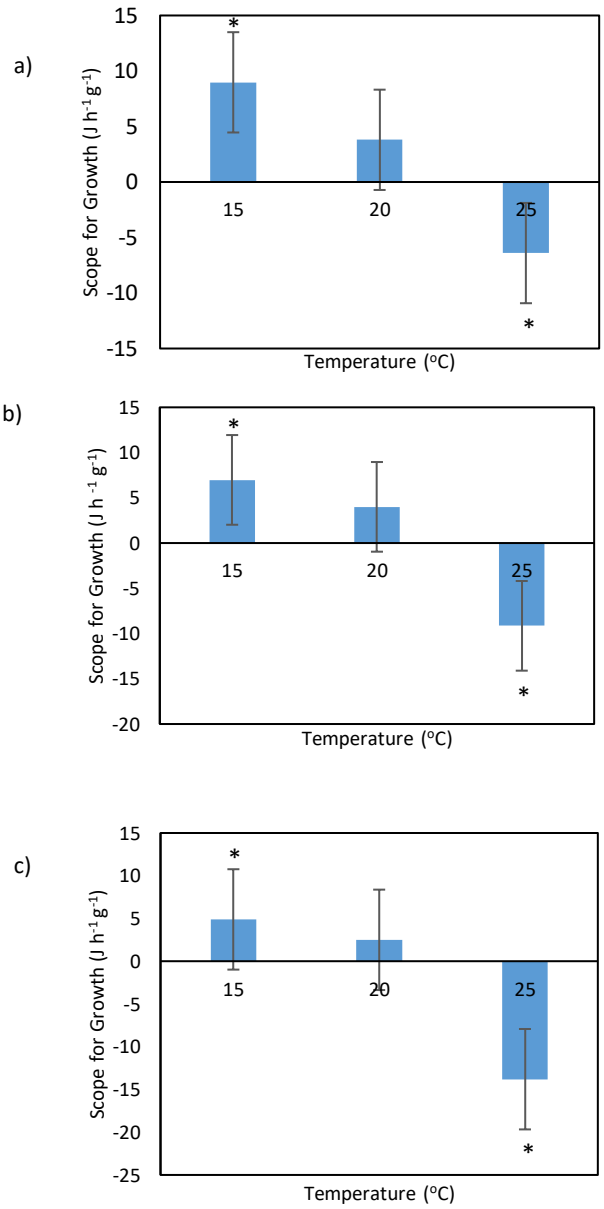

Fig 5 Changes in the scope for growth (SFG) in a) *C. gigas*, b) *M. galloprovincialis, and* c) *K. rhytiphora* after 30 days of exposure. Values are means ± Sd, n = 9. *$P < 0.05$

Table 1 Summary of two-way ANOVA results on effect of temperature (T) and species (S) on clearance rate (CR), food absorption efficiency (FAE), oxygen consumption rate (OCR), excretion rate (ER), pyruvate kinase activity (PKA) in posterior adductor muscle (PAM) and mantle, phosphoenolpyruvate carboxykinase (PEPCK), and scope for growth SFG).

Source	CR			FAE			OCR		
	T	S	T * S	T	S	T * S	T	S	T * S
0 day	0.055	0.007	0.174	< 0.05	< 0.05	0.430	< 0.05	< 0.05	0.060
15 days	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
30 days	< 0.05	< 0.05	0.987	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
	ER			PKA-mantle			PKA-PAM		
	T	S	T * S	T	S	T * S	T	S	T * S
0 day	< 0.05	0.477	0.278	0.812	0.570	0.105	0.680	0.097	0.845
15 days	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.090	< 0.05	< 0.05	0.278
30 days	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.233	< 0.05	< 0.05	0.307
	PEPCK-mantle			PEPCK-PAM			SFG		
	T	S	T * S	T	S	T * S	T	S	T * S
0 day	0.734	0.490	0.720	0.650	< 0.05	0.478	< 0.05	< 0.05	0.115
15 days	< 0.05	0.356	0.295	< 0.05	0.084	0.295	< 0.05	< 0.05	0.087
30 days	< 0.05	0.301	0.321	< 0.05	0.063	0.512	< 0.05	< 0.05	< 0.05

Chapter 3

Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*

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3.1. Abstract

Summer mortality of some bivalve species is often associated with the change of environmental temperature. This study compares the response of immunological parameters to temperature change in three marine bivalves: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*. Each species was exposed to three temperatures, 15 ^{o}C, 20 ^{o}C and 25 ^{o}C for 14 days. The total haemocyte count (THC), phagocytosis, reactive oxygen species (ROS) and the activity of antioxidant enzymes; superoxide dismutase (SOD) and catalase (CAT) were used as indicators to measure the response of each species to different temperatures. The highest temperature (25 ^{o}C) significantly increased the THC and phagocysis of haemocytes in all species. The SOD and CAT activities in the haemocytes of *M. galloprovincialis* and *K. rhytiphora* rapidly increased with temperature elevation, concomitantly with the increase of ROS ions. In contrast, the increases of ROS and SOD in *C. gigas* only occurred from 20 ^{o}C to 25 ^{o}C, suggesting that this intertidal species is more adaptive to different temperature levels. This study indicates that the activities of antioxidant enzymes can reflect the immune response of marine bivalves to thermal stress. Intertidal species such as Pacific oysters have a greater tolerance to thermal stress than subtidal species (e.g. Mediterranean mussel) and demersal species buried in sand (e.g. cockle).

Highlights

Antioxidant enzymes in the haemocytes reflect a response of bivalves to thermal stress.

Thermal stress increases haemocyte number and phagocytic activity.

Intertidal species tolerate more thermal stress than subtidal or buried demersal species.

Keywords: bivalves; haemocytes; temperature; immune response; antioxidant enzymes

3.2. Introduction

Shellfish farming is an important aquaculture industry in Australia, with the Pacific oyster *Crassostrea gigas* alone contributing a yearly revenue of >AUD60 million [1]. Mediterranean mussel *Mytilus galloprovincialis* production is currently >1400 tonnes, worth AUD2.9 million [2], and the mud cockle *Katelysia rhytiphora* is an emerging aquaculture species. These bivalves are all filter feeders, inhabiting coastal waters and estuarine areas that are subject to a wide range of stressors including seasonal temperature change, pathogens and pollution [3,4,5]. In bivalve farming, seasonal temperature change has a significant impact on growth, reproduction and mortality [6,7,8]. In many farming regions, summer mortality of molluscs is often associated with temperature increase [9] and reproductive cycles [10]. Summer mortality events are a consequence of complex interactions between pathogens and environmental factors. The increase of water temperature may lead to bacterial proliferation in the water and bacterial accumulation in the tissues, leading to stress [11], disease and mortality [12].

Immune system function in shellfish has been a research focus in the past two decades. Molluscs, such as oysters, mussels and cockles, have evolved mechanisms that rely heavily on their innate immune system to defend against infection from exogenous pathogens [13]. It consists of both humoral and cell-mediated immunity systems to recognise and respond to pathogens in a generic way. Innate immunity is activated by the chemical properties of the antigen and comes into play immediately after antigens infect. When microbial infection occurs, the organism will then use enzymes to trigger immune responses [12,14,15].

The immune capacity can be measured by the response of immunological and pathological parameters [16,17]. In bivalves, the ability to counteract environmental

stress and bacterial infections is mediated by plasma proteins, glycoproteins and circulation of haemocytes [11]. The haemocytes are responsible for recognition, phagocytosis and elimination by enzymatic or oxidative degradation of exogenous organisms. This process is accomplished by phagocytic haemocytes within the blood and haemolymph of the organism [11,18,]. The response of immunocytes in invertebrates includes generation of reactive oxygen species (ROS), which is induced by the stress of environmental factors [19] and exposure to pathogens. This process involves reduction of oxygen to a superoxide anion (O_2^-), which generates various highly reactive oxygen species such as hydrogen peroxide, singlet oxygen or hydroxyl radicals [20]. Excess of these components can damage cellular structure and protein function [21]. However, the effect can be minimised by the physiological antibody defence mechanisms regulated by enzymes such as superoxide dismutase (SOD) and catalase (CAT). These enzymes can catalyse the conversion of hydrogen peroxide into less-reactive gaseous oxygen and water [22] and strengthen the defence mechanism for species relying on the innate immunity system [23]. Extracellular SOD is a group of protein in the plasma of oysters. The purified SOD from the haemolymph in Pacific oysters and has a function in the host defense to reactive oxygen species by binding with lipopolysaccharides in *Escherichia coli* bacteria [44]. Water temperature fluctuation can significantly change immune functions in molluscs [11,13,23]. Despite the adverse effect of thermal stress on molluscan survival on farms, little is known on the physiological and immunological responses of commonly farmed molluscan species to temperature variation. Summer mortality events are more commonly reported on the sedentary Pacific oysters, but our knowledge is limited on other molluscan species for their susceptibility to increasing temperatures.

This study aims to understand the response of three bivalve species, Pacific oysters, Mediterranean mussels and mud cockles, within their respective habitats of the intertidal, sedentary and underground zones, to changes in ambient water temperatures experienced in temperate southern Australia. The effect of temperature on the defence system was examined by measuring immunological parameters and antioxidant enzymes (SOD and CAT) relevant to stress response in aquatic animals. The results of this study provide an insight into the understanding of the immune response of different molluscan species to temperature variation and identify possible strategies to reduce economic loss due to unexpected mortality of commercially important marine bivalves in southern Australia.

3.3. Materials and Methods

3.3.1 Animal collection and management

Ninety Pacific oysters (*C. gigas*, mean shell length = 64.9 ± 2.5 mm), 90 Mediterranean mussels (*M. galloprovincialis*, mean shell length = 59.1 ± 1.8 mm), and 180 mud cockles (*K. rhythiphora*, mean shell length = 42.9 ± 1.1 mm) were collected from Coffin Bay, South Australia. Animals were dry-transported to the marine laboratory at Flinders University in foam boxes within 48 hours of collection. The animals were acclimatised in a flow-through system for 2 weeks at 20-21 ^{o}C. During the acclimatisation and experimental periods, animals were fed daily *ad libitum* with mixed species of microalgae (*Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros muelleri*). Dead animals were removed from the tanks and replaced with similar sized animals and 50% of seawater in the tank was replaced once every 24 h through continuous water flow. The experiment was conducted in 25-L aquaria with aeration in triplicate. Dissolved oxygen (DO), pH, and salinity were monitored daily and ranged from 7.8-8.3 mg/L, 8.0-8.4 and 35.5-37.5‰, respectively. The

nitrate, nitrite and ammonia levels were measured every second day using the Aquaspex test kit (Aquaspex Water Testing Product, Australia) and maintained at <0.05 mg L^{-1} during the experimental period.

3.3.2. Experimental temperatures

Prior to the temperature trial, all animals were acclimatised to the experimental conditions by increasing or decreasing the water temperature from 20 ± 0.35 ^{o}C progressively (2 ^{o}C/day) to achieve a low (15 ± 0.77 ^{o}C) and a high (25 ± 0.59 ^{o}C) temperature. The water temperature in the experimental tanks was monitored every day and maintained using a thermostat device for temperature consistency throughout the experimental period. All species were analysed after being maintained at their respective temperatures for 14 days in triplicate. Each tank contained 10 animals of each species.

3.3.3. Haemolymph collection

Haemolymph (500 µl per individual) was collected from the posterior adductor muscle using a 16-gauge needle with 3 ml disposable syringe. The volume of haemolymph from five individuals was pooled as one sample for each replicate, centrifuged at 1000 × g for 10 min, and stored in liquid nitrogen. Fresh haemolymph (200 µl) of pooled haemolymph from five individuals was used to determine total haemocyte count (THC) and phagocytic rate. For each enzyme assay, 500 µl of haemolymph was pipetted off, transferred to a new tube, immediately on ice and kept at -80 ^{o}C until analysis.

3.3.4. Total haemocyte count (THC)

The THC was determined according to the method described by Chen et al [24] with a slight modification of mixing 50 µl of haemolymph with 100 µl of 6% formalin (35 ppt saline solution). A drop of mixture was placed on a haemocytometer and the

number of haemocytes was counted on a phase contrast microscope (CK40, Olympus).

3.3.5. Phagocytic activity

The phagocytic rate was measured using a modified method [25]. Briefly, a yeast solution for phagocytosis assay was prepared by autoclaving 2.5% baker's yeast *Saccharomyces cerevisiae* (Tandaco, Cerebos Foods, Seven Hills, NSW, Australia) in 4% Congo red (Sigma) in filtered seawater (FSW). The stained yeast cells were centrifuged at 1500 × g for 10 min, washed three times with FSW and re-suspended in FSW (0.2 µl) at 1×10^7 cells mL^{-1}. Fresh haemolymph (150 µl) was added to a 40 µl yeast suspension at room temperature, lightly vortexed and then settled for 20 min in the dark. At the end, tubes were vortexed and two drops (~50 µl) were placed onto a glass slide with a coverslip. Phagocytic rate was determined in triplicate as percentage of phagocytic haemocytes in 100 haemocytes under a microscope at 400× magnification.

3.3.6. Quantification of reactive oxygen species (ROS)

ROS values were determined using an OxiSelect^{TM} in vitro ROS/RNS assay kit (Green Fluorescence, STA-347; Cell Biolabs, Inc., San Diego, USA). The ROS free radical content in an unknown sample was measured fluorometrically by comparing with the hydrogen peroxide standard curve. A 50-µl plasma sample was transferred to a 96-well plate for fluorescence measurement. Next, 50 µl of catalyst was added to all wells and incubated for 5 min at room temperature. Subsequently, 100 µl of 2,7-dichlorodihydrofluorescein solution was added to each well and incubated at room temperature for further 15–45 min in dark. The plate reaction was then read at 480-nm excitation/530-nm emission on a FLUOstar Omega microplate reader (BMG Labtech, German).

3.3.7. Superoxide dismutase (SOD) activity assay

SOD activity was determined with a superoxide dismutase assay kit (706002; Cayman Chemical, USA) to evaluate the ability of the xanthine/xanthine oxidase system for generating superoxide anions. In brief, 200 µl of the diluted radical detector and 10 µl of plasma were mixed and added on a 96-well microtiter plate. Next, 20 µl pre-diluted xanthine oxidase solution was added into each well and shaken for a few seconds to mix and cover the plate. The plate was then incubated for 30 min at room temperature. The absorbance was read at 440-460 nm on a FLUOstar Omega microplate reader (BMG Labtech, German).

3.3.8. Catalase (CAT) activity assay

Catalase activity was determined using a catalase assay kit (707002; Cayman Chemical, USA). In the current study, 30 µl methanol, 20 µl sample, and 20 µl diluted hydrogen peroxide were mixed thoroughly on a 96-well microtiter plate and incubated on a shaker for 20 min at room temperature. Next, 30 µl of the potassium hydroxide was added into each well and mixed thoroughly to terminate the reaction and then followed by 30 µl catalase purpald (Chromogen). The plate was covered and incubated for 10 min at room temperature on a shaker. Catalase potassium periodate (10 µl) was then added to each well, covered and incubated for 5 minutes at room temperature on a shaker. The plate was read at 540 nm absorbance on the CLARIOstar Omega plate reader (BMG Labtech, German). The catalase activity was measured using the catalase activity assay standard curve.

3.3.9. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Data were tested for normality and homogeneity using Kolmogorov-Smirnov and Levene's test. The normality distributed data were analysed using two-way analysis of variance (two-

way ANOVA) to test the effect of temperature and molluscan species on all dependent variables. When significant interactions between main factors were observed, pairwise comparisons were used to determine significant differences between treatment combinations. If the interaction between the temperature and species was not significant, the main effect was considered and the post-hoc Tukey's HSD was used for multiple comparisons. The level of significant difference was set at $P < 0.05$. All data were analysed using the statistical package IBM SPSS Statistics 22.

3.4. Results

3.4.1. Survival of each bivalve species

The survival of *C. gigas, M. galloprovincialis and K. rhytiphora* was 97.78%, 85.56%, and 78.34% respectively at the end of experiment across all temperature treatments. There was no mortality in *C. gigas* at 15 ^{o}C and 20 ^{o}C and in *M. galloprovincialis* at 15 ^{o}C. The lowest survival (51.66%) was observed in *K. rhytiphora* at 25 ^{o}C. The average survival for *K. rhytiphora* at 15 ^{o}C and 20 ^{o}C was 88% and 95% respectively. In *C. gigas* mortality only occurred at 25 ^{o}C, with two dead animals during the experimental period. The average survival of *M. galloprovincialis* at 20 ^{o}C and 25 ^{o}C was 87% and 80% respectively (Fig. 1).

3.4.2. Total haemocyte count (THC)

An increase in THC was observed as water temperature increased (Fig. 2a). At the end of the experiment, the THC was significantly affected by temperature (2-way 3×3 ANOVA; $P < 0.001$). The animals of all species held at 15 ^{o}C had significantly lower THC than those at 20 ^{o}C and 25 ^{o}C ($P < 0.001$). Furthermore, the highest THC was observed in the animals at 25 ^{o}C in all species. Post hoc comparisons indicated that there were significantly different THC levels between *M. galloprovincialis* and

K. rhytiphora at 20 °C (P = 0.044). There was no interactive effect between species and temperature on THC (P = 0.466).

3.4.3. Phagocytic activity

The phagocytic activity in haemocytes (Fig. 2b) significantly varied among temperature treatments regardless of species (P < 0.001) and was also significantly different between species regardless of temperature (P < 0.001). There was no interaction between temperature and species in phagocytic activity (P = 0.179). Post hoc comparison detected that phagocytic activity significantly increased when temperature increased from 15 °C to 20 °C to 25 °C in all three species, respectively (P < 0.001). At all temperatures tested, phagocytic activity in *C. gigas* was significantly higher than in *M. galloprovincialis* and *K. rhytiphora*, but no significant difference between *M. galloprovincialis* and *K. rhytiphora* (P = 0.057) was observed.

3.4.4. Reactive oxygen species (ROS)

As an index of oxidative stress, the ROS activity in haemocytes (Fig. 3a) significantly increased with temperature escalation (P < 0.001) and varied among species (P = 0.006). However, there was no significant interaction between temperature and species on ROS production (P = 0.337). Post hoc comparisons indicated when temperature increased from 15 °C to 20 °C and further to 25 °C, the ROS activity significantly increased by each temperature increment in all species (P < 0.001), except *C. gigas* between 15 °C and 20 °C (P = 0.123). The ROS activity in *C. gigas* and *M. galloprovincialis* was significantly higher than in *K. rhytiphora* regardless of temperature (P < 0.05), but there was no significant difference between *C. gigas* and *M. galloprovincialis* (P < 0.05) at all temperatures.

3.4.5. Superoxide dismutase (SOD) activity

In haemolymph fluid, the production of SOD enzyme showed a significant increase with the increase of temperature from 15 ºC to 20 ºC to 25 ºC regardless of species ($P < 0.05$, Fig 2b), with no significant difference between species ($P = 0.134$). No significant interaction was found between temperature and species on the SOD activity (P = 0.995). A post hoc comparison revealed that the SOD activities between 15 ºC to 20 ºC did not significantly differ ($P = 0.45$) in *C. gigas,* which was congruent with a lesser generation of ROS in *C. gigas* from 15 ºC to 20 ºC.

3.4.6. Catalase (CAT) activity

The CAT activity significantly increased with increasing temperature in all species (two-way ANOVA, $P < 0.001$) (Fig. 3c) and was significantly different between species ($P < 0.001$). There was no significant interactive effect between temperature and species ($P = 0.133$). Post hoc comparison revealed that catalase activity significantly increased from 15 ºC to 20 ºC ($P < 0.001$) and from 20 ºC to 25 ºC ($P < 0.001$) regardless of species. Conversely, there was no significant difference of catalase activity between 20 ºC and 25 ºC in *C. gigas* ($P = 0.075$). The catalase activity in *C. gigas* and *M. galloprovincialis* was significantly higher than that in *K. rhytiphora* ($P < 0.001$) at all temperature levels. However, there was no significant difference between *C. gigas* and *M. galloprovincialis* at any temperature ($P > 0.05$).

3.5. Discussion

Mortality in epidemic outbreaks is the most important factor leading to low production in shellfish aquaculture. In marine environments, temperature can have a strong effect on the function of immune defence systems in molluscs [11,13,23]. Indeed, temperature also alters the rate of biological, chemical and enzymatic reactions [26]. In the present study, temperature affected the survival of all

molluscan species with highest mortality occurrence at the highest temperature of 25 ^{o}C. The *K. rhytiphora* is the most affected species by the elevation of temperature. Over the last decade, there has been growing awareness that temperature is related to disease incidence or mass mortality in marine bivalves due to immunosuppression [23,27,28]. This comparative immune study of marine bivalves from different thermal conditions has illustrated that water temperature has a significant impact on immune systems, both at cellular and antioxidant levels. However, the nature of each immune parameter differed among the three species, suggesting a considerable degree of inter-species variation in the relative importance of individual parameters to the overall immune response. Moreover, the induction of oxidative stress in all species tested were minimised by active anti-oxidative enzymes (SOD and CAT) in response to temperature elevation during a short-term exposure.

3.5.1. Cellular response to different temperatures

In this study, the temperature change significantly increased the value of total haemocyte count (THC) in all species. The THC in *C. gigas*, *M. galloprovincialis* or *K. rhytiphora* was sensitive to temperature stress and increased from 15 ^{o}C to 20 ^{o}C or from 20 ^{o}C to 25 ^{o}C after a period of 14 days exposure, indicating that variations of temperature could affect the functional responses of mollusc haemocytes. The results were also in agreement with other studies where haemocyte counts in *C. gigas, M. galloprovincialis,* and *Ruditapes phillipinarum* are positively correlated with increasing water temperature [29, 30, 31]. It is suggested that the increased THC in organisms at the different temperature levels could be a result of cell mobilisation or cell proliferation from tissues into the haemolymph circulation [23,32]. In any event, haemocytes are important and known to be involved in wound healing to avoid loss of haemolymph and interference of microorganisms upon

injury. In addition, haemocytes also secrete antimicrobial metabolites for bacterial recognition and killing [11]. It is clear that temperature is a crucial factor to regulate THC levels. This coincides with previous studies that temperature can influence haemocyte activities of mollusc species [13,18,28,32].

Temperature change affects other important immune functions, such as inhibition of phagocytic activity [28,32,33,34]. In the current study, the phagocytic activity of haemocytes increased significantly over the thermal stress application, which shows a similar pattern to the changes of THC in *C. gigas*, *M. galloprovincialis*, and *K. rhytiphora*. Similar observations were reported previously in *M. galloprovincialis* where the capability of haemocytes to engulf foreign particles is lower at 10 ^{o}C than at 20 ^{o}C and 30 ^{o}C [31]. Likewise, Monari [23] has reported that the clam *Chamelea gallina,* exposed to 30 ^{o}C water temperature, suffered a significant inhibition to phagocytic activity and Hegaret et al. [18] demonstrated a significant decrease in phagocytic activity in *Crassostrea virginica* kept at 28 ^{o}C for 7 days. In a similar experiment, Chu and La Peyre [34] indicate that phagocytosis reduces in oysters at 25 ^{o}C compared to oysters held at 10 ^{o}C and 20 ^{o}C for 68 days. Above a certain threshold, the temperature-induced reduction in enzymatic processes for phagocytic activities leads to increased cells damage [27,29].

Thermal stress induces the formation of ROS in haemocytes, and other small toxic molecules including superoxide radical (O_2^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$), which are involved in internal defence to eliminate non-self-particles [19]. The production of ROS was previously reported in the haemocytes of bivalve molluscs, including oysters [20,35], mussels [36], scallops [32], and clams [28]. All of these studies demonstrate that higher temperatures strongly influence several metabolic, physiological and immune parameters. In the

present study, the increased level of ROS varied among species. The *M. galloprovincialis* and *K. rhytiphora* were significantly stressed by the increasing or decreasing temperature. Despite an increase of ROS level in *C. gigas* throughout temperature treatments from 20 °C to 25 °C, no mortality was observed, suggesting a greater resilience to temperature stress. Gagnaire et al. [29] also demonstrate that only extreme temperature conditions (40 °C, 50 °C, and 60 °C) significantly affect the function of *C. gigas* haemocyte activities. Additionally, this phenomenon has also been observed in *C. gigas* under heavy metal stress, where only high concentrations of mercury chloride are able to affect and kill haemocyte cells after 4 h of in vitro exposure [37].

3.5.2. Response of antioxidant enzymes to different temperatures

Antioxidant defences are composed of three general groups including fat-soluble vitamins, water-soluble reducing agents, and enzymes including superoxide dismutase and catalase [38]. One of the unique characteristic of these enzymes is their inducibility as an adaptation to the environmental change when marine invertebrates are under oxidative stress [38,39]. In this study, temperature increases enhanced ROS production, thereby increasing the risk of oxidative damage. The radical formation and damage to tissues is balanced by an array of main cellular antioxidants, superoxide dismutase ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) and catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$) to neutralise ROS before starting the radical reaction chains. The SOD activity in these marine bivalves increased significantly from a low temperature at 15 °C to a high temperature at 25 °C, suggesting that the enzyme has protective responses to catalyze reactive free radicals. We observed a higher SOD activity in *M. galloprovincialis* and *K. rhytiphora* compared to *C. gigas*. The change in environmental conditions of the habitat can lead to a functional response of

antioxidant enzymes [40]. The off-shore and underground species are exposed to a less fluctuation of environmental condition compared to the intertidal species, such as *C. gigas*, that can adapt to elevated temperatures. However, differences in SOD between temperature treatments were not significant between *M. galloprovincialis* and *K. rhytiphora*. This is possibly because SOD activity could instantaneously counteract with the higher concentration of ROS in *M. galloprovincialis* and *K. rhytiphora* and result in no significant variation between these two species. The different of SOD level by *K. rhytiphora* supports the observation by Monari et al. [24] that the SOD activities in both haemocytes and cell-free haemolymph of clams *Chamelea gallina* after 7 days of experiment are higher in animals at 25 ^{o}C and 30^{o}C than those at 20 ^{o}C. Similarly, in the mussel *Perna viridis* the level of SOD activity increases significantly with the increase of body toxicant concentrations when the animals are transferred from relatively clean sites to various polluted sites [38]. These studies demonstrate that marine bivalves could often experience intensified oxidative stress and their mortality in estuarine habitats could partly depend on their ability to activate the antioxidant defence mechanism. In this study, the mortality of each species could not be well explained by the change of immunological parameters measured, suggesting that the cause of mortality is complex and goes beyond the change of sole immunological responses.

When marine bivalves are exposed to H_2O_2 they can activate catalase to counteract stress. However, antioxidant activity may not necessarily increase as a result of environmental stress [23,41]. The activity of CAT showed high variation compared to the SOD responses in all three-molluscan species. The high CAT activity observed in the haemocytes of *M. galloprovincialis* and *K. rhytiphora* under temperature stress, suggests that the oxidative stress is prone to peroxide radicals. This result

agrees with the elevated CAT activities after exposed to stressors in other molluscan species such as *Mytilus edulis*, *M. galloprovincialis* [43,44] and *Crassostrea* sp. [43]. In contrast, the CAT activity in *C. gigas* was not significantly different among temperature treatments. The CAT enzyme level increased from 15 ºC to 20 ºC, but decreased from 20 ºC to 25 ºC. The weak response may be associated with the exposure to stress, which can be explained by the induction of antioxidant mechanism. In this case, *C. gigas* may be able to acclimate better to elevated temperatures as they dominate the region where fluctuating environmental conditions prevail.

In conclusion, the present study demonstrates that water temperature change affects haemocytic function and leads to oxidative stress, reducing immunosurveillance in these three marine bivalves. The major differences in immune dynamics are related to the habitat of these three species in the wild. Animals in the intertidal zone need to cope with more extreme temperatures compared to those in the deeper water. The *K. rhytiphora,* appears to be more sensitive to temperature changes than other species as indicated by haemocyte activities. However, further investigation is needed to understand the relationship between immune response and mortality of bivalves to pathogen infection under thermal stress. Conversely, the intertidal species *C. gigas* can tolerate extreme thermal stress allowing it to densely dominate the intertidal and subtidal zones. This study improves our knowledge on temperature-induced immune modulation of marine bivalves in the scenario of possible global warming in future.

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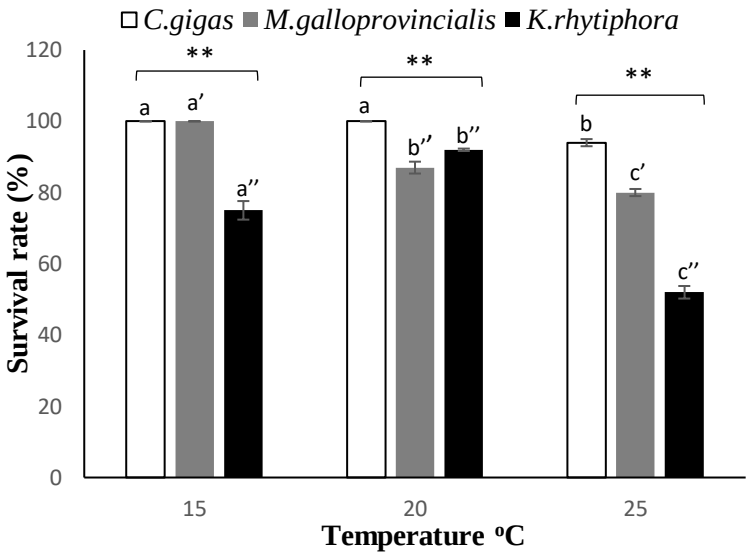
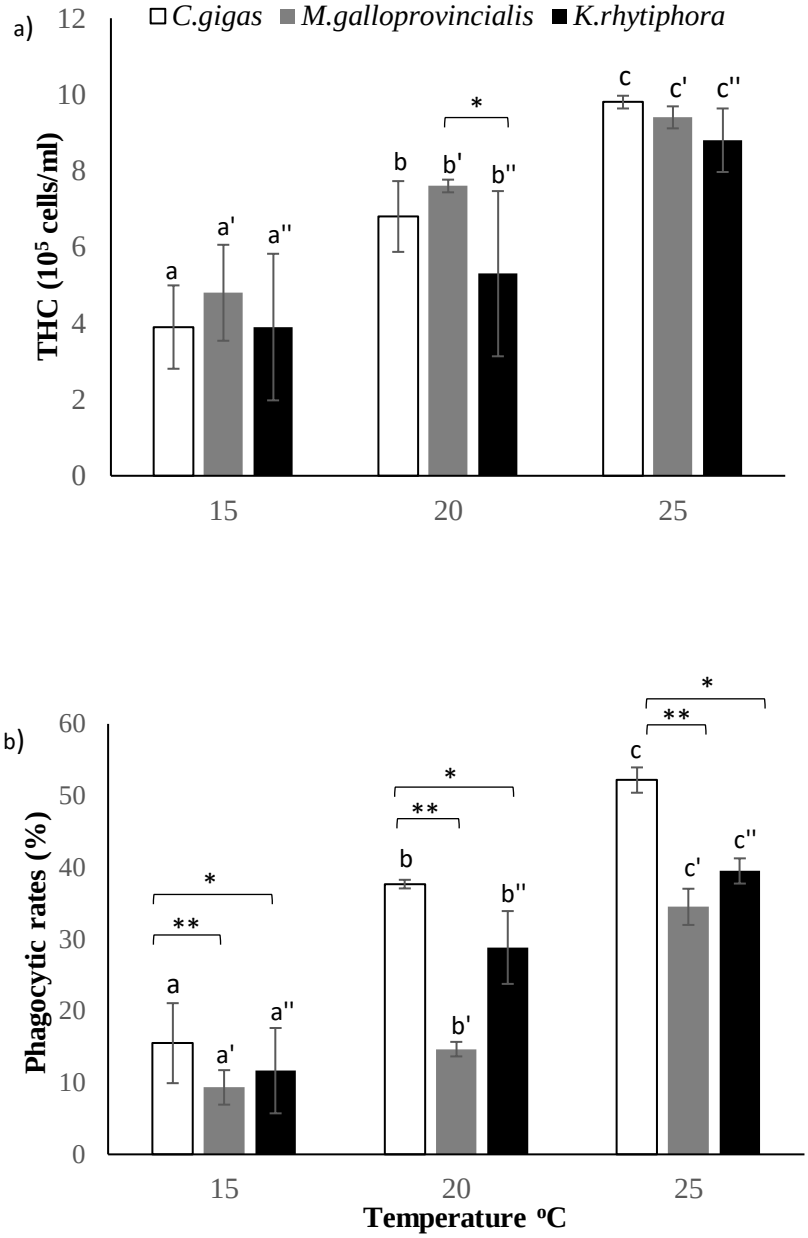


Treatment	*P*
Temperature	<0.001
Species	<0.001
Temp x Species	<0.001

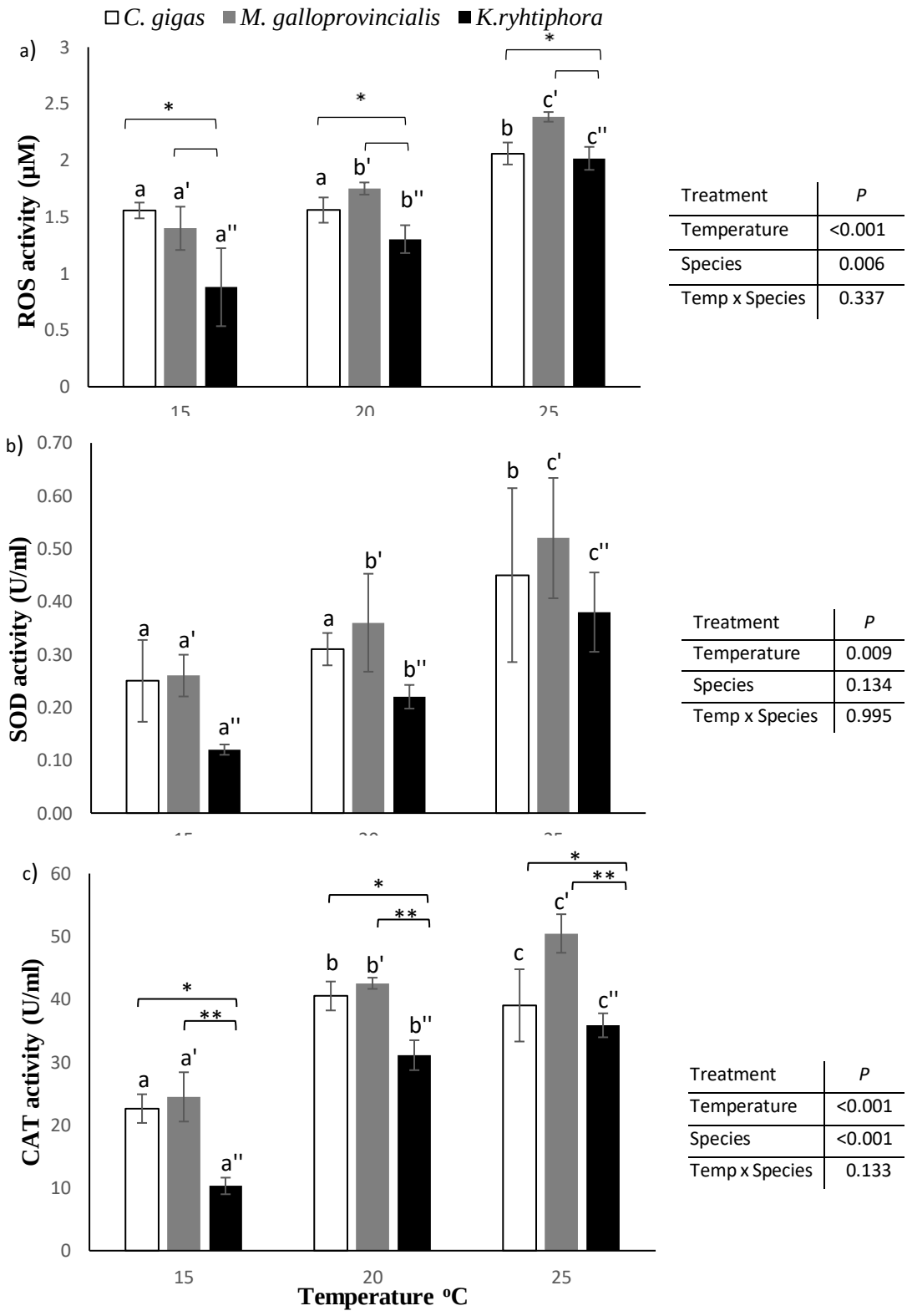
Fig. 1. Survival of animals (*Crassostrea gigas, Mytilus galloprovincialis* and *Katelysia rhytiphora*) after 14 days exposed to temperature treatments (15 °C, 20 °C or 25 °C). Different sets of letters (a, b and c for oysters; a', b' and c' for mussels and a", b" and c" for cockles at three temperatures, respectively) indicate significant difference between temperatures within a species. The hatching bracket between two bars represents significant difference between species at the same temperature. The double asterisks (**) are for a difference at $P < 0.001$. The same letter with different apostrophes (e.g., a, a' and a") is not for comparison between species at a same temperature. Error bars represent standard deviation.



Treatment	*P*
Temperature	<0.001
Species	0.051
Temp x Species	0.466

Treatment	*P*
Temperature	<0.001
Species	<0.001
Temp x Species	0.179

Fig. 2. Water temperature changes affect a) total haemocyte counts (THC); b) phagocytic rates for three molluscans after 14 days exposed to temperature treatments (15 ºC, 20 ºC or 25 ºC). Symbols and signs refer to Fig. 1.



Treatment	*P*
Temperature	<0.001
Species	0.006
Temp x Species	0.337

Treatment	*P*
Temperature	0.009
Species	0.134
Temp x Species	0.995

Treatment	*P*
Temperature	<0.001
Species	<0.001
Temp x Species	0.133

Fig. 3 Average of a) reactive oxygen species (ROS); b) superoxide dismutase (SOD) and c) catalase (CAT) activities in haemocytes for three molluscan species after 14 days exposed to temperature treatments (15 ºC, 20 ºC or 25 ºC). Symbols and signs refer to Fig. 1.

Chapter 4

Analysis of the seasonal impact of three marine bivalves on seston particles in water column

Published as

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4.1. Abstract

Suspension-feeding bivalves are the keystone species that affect the abundance and composition of phytoplankton communities. This study compares the food selectivity of oysters *Crassostrea gigas* (Thunberg, 1973), mussels *Mytilus galloprovincialis* (Lamarck, 1819), and cockles *Katelysia rhytiphora* (Lamy, 197) on natural particle assemblages in the laboratory using water collected from Coffin Bay, South Australia, in spring, summer, autumn and winter. The phytoplankton community was dominated by *Bacillariophyceae* (diatoms), *Dinophyceae* (dinoflagellates), *Synechococcus* (cyanobacteria), and picophytoplankton (2-5 µm) from September 2016 to August 2017. Diatoms and picoplankton, including cyanobacteria, were dominant in spring and summer, while dinoflagellates were dominant in autumn and winter. The present study showed significant differences in filtration capacity among oysters, mussels and cockles between seasons. Oysters and mussels selectively fed on large food particles (e.g., diatom, dinoflagellate and large picoplankton >2 µm) regardless of season, but mussels could access a wider size spectrum of food particles compared to oysters. In contrast, cockles selected for both large and small food particles (e.g., *Synechococcus* and small picoeukaryotes <2 µm) and fed more efficiently on small particles than both oysters and mussels. Bacteria were more abundant in warmer seasons, but mussels and cockles selectively reduced bacterial abundance. The abundance of virus-like particles was not affected by season nor by filtration of any molluscan species. This study demonstrates that oysters and mussels utilise similar food sources but varies in term of size spectrum of food particles, whereas cockles consume a broad range of smaller food particles. Additionally, these three molluscan species do not necessarily compete for food; hence, stocking multiple filter-feeding species in an ecosystem could exploit a wider spectrum of food particle size and increase system productivity.

Keywords: *Crassostrea gigas*; *Mytilus galloprovincialis*; *Katelysia rhytiphora*; selective feeding; phytoplankton; natural seston

4.2. Introduction

The South Australian coast, experiences extreme high temperature and salinity in summer, but is one of the most diverse marine ecosystems in the world and supports significant fishery and aquaculture industries (O'Hara, 2002). The management of the coastal marine ecosystem is paramount to sustain these economic activities (Cranford et al., 2012). Phytoplankton at the bottom of the marine food web supply energy to animals at all upper trophic levels (Uitz et al., 2010). In coastal ecosystems, phytoplankton abundance is primarily governed by seasonal environmental changes such as temperature, light, and nutrient fluctuation caused by upwelling circulation and nutrient inputs from land run-off (Balzano et al., 2015; Racault et al., 2012).

Most marine bivalves are filter-feeders that consume organic particles in the water column, with phytoplankton being the dominant food source (Espinosa et al., 2016; Hawkins et al., 1998, Sonier et al. 2016). Phytoplankton span a broad size range; however, in seawater, the small picophytoplankton (<2 μm) account for a significant proportion of whole biomass and trophic resources, especially in oligotrophic environments (Marañón et al., 2001). Worldwide, the prokaryotic component of picophytoplankton primarily consists of two cyanobacterial genera, *Prochlorococcus* and *Synechococcus* (Partensky et al., 1999). In contrast, the diversity of picoeukaryotes is more complex (Vaulot et al., 2008). The larger fraction of seston, which includes nanoplankton (2–10 μm) and microplankton (>10 μm) are highly diversified, with diatoms and dinoflagellates often being the dominant taxa (Balzano et al., 2015; Leterme et al, 2014). In addition to phytoplankton, recent studies have revealed that bivalves can ingest a wide range of seston particles such as detritus, bacteria, faecal pellets, microzooplankton and mesozooplankton (Ezgeta-

Balić et al., 2012; Peharda et al., 2012; Webb et al., 2013). The size range and density of food particles in an ecosystem underlie the success and sustainability of bivalve farming. Bivalves are filter feeders and have evolved the ability to discriminate food particles through size selection (Espinosa et al., 2016; Ward and Shumway, 2004). The selection mechanism in suspension-feeders is controlled by physical, chemical, and biological factors in the environment and previous studies have shown that changes in size, density, or concentration of particles can affect selection efficiency of bivalves (Espinosa et al., 2008; Ward and Shumway, 2004).

In recent years, studies have endeavoured to understand complex interactions between food supply and environmental factors to develop production and carrying capacity models to estimate bivalve production. With the advancement of analytical technology (e.g., flow cytometry), it has become possible to understand the processes of particle feeding and selection in bivalve species (Cranford et al., 2012; Yahel et al., 2009) and to predict the outcomes of competition between filter feeders in different environmental conditions. Temperature is a factor affecting immunological, physiological and metabolic responses in marine poikilotherms (Rahman et al., 2019; Anestis et al., 2010; Berthelin et al., 2000). With flow cytometry and taxonomic identification, it is possible to understand the mechanism of thermal adaptation and food acquiring capacity of filter feeders and to evaluate the competitive outcome and growth performance in a changing environment.

Temperate southern Australian waters are typified by low nutrient input and low food availability for filter feeders. Despite this, the Coffin Bay, South Australia, sustains a large bivalve aquaculture industry. Coffin Bay is the primary oyster growing region in southern Australia due to its relatively fertile water and is internationally recognised for its clean environment and production of high-quality

seafood. Currently our understanding of the carrying capacity, trophic function and feeding physiology of bivalves in this area is limited. Previous studies show that bivalves, primarily oysters, cockles and mussels in southern Australian waters derive their nutrient from a diverse range of food sources (Rubio, 2008). However, the degree of feeding capacity, food items and resource partitioning among major bivalve species within the Spencer Gulf is unknown. Therefore, it is necessary to understand the seston particles that major bivalve species could consume in the water column at different seasons.

We hypothesise that the food overlap and competition differ among major economically important bivalve species in Coffin Bay. The aim of this study is to compare the seasonal filtering capacity of three bivalve species that naturally live or are farmed in this area. The novelty of this study resides in the simultaneous comparisons on the feeding capacity of three filter-feeding bivalve species and their impact on seston particles over four seasons in an area with high potential of molluscan farming in South Australia. We combined the approach of microscopic examination and flow-cytometry to estimate the capacity of filtration of three economically important bivalve species on seston particles. This information is important to estimate food resource and food partitioning among major bivalve species (e.g., Pacific oysters, blue mussels and mud cockles) and provide essential data for the construction of carrying capacity models to support holistic ecosystem management and sustainable farming for bivalves.

4.3. Materials and methods

4.3.1. Collection methods

Seawater used for each of the four feeding trials was collected at 1 - 1.2 m from the surface water adjacent to the central oyster farming zone in Coffin Bay

(34°33'27.79"S, 135°21'40.90"E), South Australia, using a diaphragm hand pump to avoid damaging live organisms in the water. The water was stored in plastic containers and transported to the laboratory at the Lincoln Marine Science Centre, Port Lincoln, South Australia. Thirty Pacific oysters (*Crassostrea gigas*, Thunberg, 1973) were collected from the farm, and 30 mussels (*Mytilus galloprovincialis*, Lamarck, 1819) and 60 mud cockles (*Katelysia rhytiphora*, Lamy, 1937) were collected from the seabed adjacent from the farm each season. The animals collected had shell lengths ranging 50 – 60 mm for oysters and mussels, and 30 – 40 mm for cockles. Four feeding trials were conducted with each representing a different season in November 2016, February 2017, May 2017 and August 2017, respectively, and each feeding trial lasted 10 h. Upon arrival of the water from the collection site, the water in different plastic containers was thoroughly mixed in a tank prior to the experiment. All animals were cleaned to remove barnacles and other attached organisms and acclimated in the laboratory for 4 h in the experimental water for each trial.

4.3.2. Experimental design and system

Ten individuals of each species (20 individuals for cockles) were separately stocked into a 20 L container in three replicates with an additional container without animals as a control in three replicates (n = 3). Each experimental unit consisted of 10 individuals placed on a plastic frame that was held off the bottom in the approximate middle of the water column (Fig. 1). All units were temperature controlled to maintain a similar temperature to that of the sampling site for the duration of the experiment. Water collected from Coffin Bay with its natural seston was supplied to each experimental unit for a period of 10 h. Water samples were

collected at the beginning and end of the feeding period for flow cytometry analysis and phytoplankton enumeration.

4.3.3. Flow cytometry analysis

Flow cytometry was used to determine the abundance of picoplankton including bacteria and viruses. Smaller size of photosynthetic picophytoplankton (i.e. *Prochlorococcus*, S*ynechococcus* and picoeukaryotes; collectively termed as picophytoplankton herein), bacterioplankton (inclusive of bacteria and archaea) and virioplankton were enumerated by flow cytometry. Seawater samples (1.0 ml) in triplicate were fixed in 25% glutaraldehyde (0.5% final concentration) in the dark for 15 min and stored in liquid nitrogen until analysis (Patten et al., 2011). Picophytoplankton samples were thawed at 37 ºC, and 1 µm fluorescent beads (molecular probes) were added as an internal standard. Samples were then analysed using a flow cytometer (FACSCanto II, Becton– Dickinson) fitted with a 488 nm laser on high throughput mode at a flow rate of 60 ml min^{-1} for 2 min. Prochlorococcus, Synechococcus and picoeukaryotes were discriminated on the basis of red and orange autofluorescence of chlorophyll and the accessory pigment phycoerythrin (Patten et al., 2011). Bacterioplankton and virioplankton samples were thawed as above, diluted fivefold in 0.02 µm filtered Tris EDTA buffer (pH 8, Sigma–Aldrich), stained with SYBR I Green (0.5×10^{-4} final concentration) in the dark at 80 ºC. The 0.75 µm fluorescent beads (molecular probes) were then added as an internal standard (Brussaard, 2004). Bacterioplankton and virioplankton were analysed using the same flow cytometer, but at a flow rate of 30 ml min^{-1} for 2 min and were discriminated based on side scatter and green (SYBR I) fluorescence. The flow cytometry method differentiated the particle size of seston based on the maximum dimension of the particle diameter.

4.3.4. Phytoplankton composition of water samples

Phytoplankton composition was quantitatively analysed following the filtration method outlined in Wilkinson (2015). In brief, approximately 200 ml water was collected from each tub at the beginning and after the 10-h feeding period. Water samples were filtered through polycarbonate membrane filters with 5.0-µm pore size. Once the meniscus of the sample reached the bottom lip of the funnel, approximately 5 ml of the remaining sample was transferred into a 10-mL measuring cylinder. All samples for larger phytoplankton identification and enumeration were fixed in 20-µl Lugol's iodine prior to analysis.

Algal cell abundance was quantified on a Sedgewick-Rafter counting chamber using phase contrast microscopy under 400x magnification on an Olympus BX-40. One complete row around the middle of the chamber was viewed and all species were identified and counted. Detailed observations sometimes required higher magnification. Phytoplankton abundance (cell L^{-1}) was calculated using the following equation (Wilkinson, 2015).

Abundance = unit count x [1/ (filtered volume/final volume)] x [No. of rows (20)/rows counted] x 1000 (cell/L)

4.3.5. Statistical analysis

Data were expressed as mean ± standard deviation and analysed using two-way analysis of variance (two-way ANOVA: four seasons vs four grazing treatments, i.e., three grazers and a control) to test the impact of molluscan species, seasons and the species/season interaction. All data were checked for normality and homoscedasticity before analysis. For those did not pass the test, we used logarithm transformation or non-parametric analysis (Kruskal–Wallis test). When significant interactions between main factors were observed, pairwise comparisons were used to

determine significant differences between treatment combinations. If the interaction between the season and species was not significant, the main effect was considered and the post-hoc Tukey's HSD was used for multiple comparisons within four levels of the main effect (i.e., between four seasons, or between four feeding treatments). The level of significant difference was set at $P < 0.05$. All data were analysed using the statistical package IBM SPSS Statistics 20.

4.4. Results

During the experimental period, water temperature averaged 18.4 ± 2.7 ^{o}C from the end of October 2016, and gradually increased to the maximum of 25.3 ^{o}C in mid-December (Fig. 2). The temperature declined to a minimum of 11.5 ^{o}C in mid July 2017. The average dissolved oxygen (DO) was 7.32 ± 1.0 mg/l with the maximum (8.11 mg/l) in July 2017 and minimum (6.41 mg/l) in mid-December 2016. Salinity ranged from 35.8 to 39.4 ppt throughout the year and averaged 37.6 ± 0.5 ppt.

4.4.1. Diatoms (5-300 µm)

Within Coffin Bay's central oyster farming zone, the abundance of seston (mainly diatoms) ranged from 1.7×10^4 to 7.0×10^5 cells ml^{-1} and was particularly higher in spring and summer than any other seasons ($P < 0.001$). The dominant diatom species included *Chaetoceros* sp., *Coscinadiscus* sp., *Licmophora* sp., *Navicula* sp., *Nitzschia* sp., *Leptocylindrus* sp., and *Pseudo-nitzschia* sp. The reduction of diatoms was significantly affected by season and grazer species (Fig. 3a). In spring, there were significant differences in diatom reduction between the control and three molluscan species. Similarly, in summer, both oysters and mussels removed significantly more diatoms than cockles. Oysters and mussels removed 92-94% and 76-95% of diatoms respectively in spring and summer, while cockles

removed 35% in spring and 77% in summer. In autumn and winter, the abundance of diatoms was low, but oysters and mussels removed more diatoms than cockles. Regardless of season, the reduction of diatoms by oysters, mussels and cockles was significant compared with the control.

4.4.2. Dinoflagellates (5-100 µm)

The abundances of dinoflagellates in autumn and winter were significantly higher than in spring and summer, but there was no significant difference between spring and summer or between autumn and winter. The abundance of dinoflagellates was significantly affected by season and by grazer (Fig. 3b). In all seasons, dinoflagellates were significantly reduced by all three molluscan species compared with the control. Regardless of season, oysters and mussels reduced significantly more dinoflagellates than the cockles.

4.4.3. Large pico-eukaryotes (2-5 µm)

The abundance of large pico-eukaryotes (1.9×10^3 to 7.1×10^3 cells mL^{-1}) was highest in summer, followed by spring, both of which were significantly higher than in autumn and winter. The abundance of large pico-eukaryotes was affected by season and grazer species (Fig. 4a). Large pico-eukaryotes were reduced significantly in each season by molluscan grazing. In spring, oysters and cockles reduced more large pico-eukaryotes than mussels. In summer, autumn and winter, oysters and mussels suppressed > 80 % large pico-eukaryotes except for cockles which reduced 66 -77 %.

4.4.4. Small pico-eukaryotes (<2 µm)

Small pico-eukaryotes ranged from 2.0×10^4 to 5.5×10^4 cells mL^{-1} and were significantly affected by both season and grazer species (Fig. 4b). Small pico-eukaryotes were more abundant in spring than in other seasons, but there was no

significant difference among summer, autumn and winter. In all seasons, cockles reduced small pico-eukaryotes by 90 - 98%, more than oysters and mussels. Mussels reduced small pico-eukaryotes by 41- 67% in all seasons except winter. Pacific oysters only reduced 4 -15% of small pico-eukaryotes in all seasons, expect in summer where the reduction was much higher (46%).

4.4.5. Synechococcus sp. (0.7-2 µm)

The abundance of *Synechococcus* was significantly affected by both season and grazers (Fig. 4c). The highest Synechococcus abundance occurred in spring and summer, but there was no significant difference between autumn and winter. Pacific oysters did not significantly reduce Synechococcus. Mussels reduced 9 -13% of Synechococcus in spring and summer, and 32% in winter. In all seasons, cockles reduced more Synechococcus than oysters and mussels, totalling of 81- 91 % in spring and summer and 64-73% in autumn and winter.

4.4.6. Bacteria (0.2-2 µm)

Total bacteria varied in the order of magnitude from 2.0×10^5 to 1.2×10^6 over the study period. The bacterial abundance was affected by both grazer species and season (Fig. 5a). Bacterial abundance in spring was higher than in autumn, and bacteria in summer were more than those in autumn and winter. However, there was no significant difference in bacterial abundance between spring and summer. In all seasons, a similar pattern was observed, i.e., cockles reduced bacteria between 32 and 67 %, but oysters hardly removed any bacteria compared with the control. Mussels significantly decreased the abundance of bacteria by 10 - 44 % in all seasons except in spring. In autumn, cockles and mussels reduced 100% of bacteria in contrast with oysters that did not significantly reduce bacteria. In summer, both mussels and cockles reduced bacteria from the water column.

4.4.7. Virus-like Particles (VLP's) (0.02-1 µm)

The abundance of smallest particles VLPs was not significantly different between seasons or between species (Fig. 5b). The VLPs in spring and summer were more abundant than in autumn and winter, but there was no significant difference between spring and summer or between autumn and winter. In all seasons, only cockles significantly reduced VLPs.

4.4.8. Abundance of particles before and after grazing

Overall particle abundance differed significantly between seasons and species, though the impact varied between particle sizes (Fig. 6). In spring, cockles significantly reduced 2-µm particles, but oysters and mussels removed >10 µm particles. However, the abundance of 2-µm particles was increased by the presence of mussels. The abundance of 4 - 8 µm particles in the water was very low across all seasons. Summer showed a similar pattern to spring, with the exception of a significant increase in 2-µm particles, which mussels significantly suppressed and the loss of >10 µm particles in the control. In autumn, oysters suppressed >10 µm particles, and mussels and cockles removed 2-µm particles from the water. In winter, a similar pattern was observed in autumn except that mussels did not significantly suppress 2-µm particles.

4.4.9. Filtration rate

Visual observations found that the shells of all animals were open and appeared to be feeding normally during the period of experiments. There were less than 5% changes in algal concentrations between at the beginning and at the end of experiment in the control group compared to the treatments with the presence of animals. Overall, the filtration rate of oysters and mussels on >8 µm particles was significantly higher than that of cockles irrespective of seasons (Fig. 7). In contrast,

cockles and mussels significantly filtered more small particles (<5 µm) than oysters in autumn and winter. Mussels showed negative filtration activity on particles < 5 µm in spring, but oysters showed negative filtration on small particles in winter. In summer, mussels showed the highest filtration capacity on both small and large particles compared to oysters and cockles. Mussels and cockles had significantly higher filtration rate on small particles than oysters in autumn.

4.5. Discussion

4.5.1. Food selection by molluscan species

The present study reveals significant differences for particle selection among oysters, mussels and cockles and their food selectivity varies between seasons. The degree and particle selection efficiency varies among bivalve species due to the structural variation of filtering apparatus and feeding behaviour (Hawkins et al., 1998; Ward et al., 1998). Mohlenberg and Riisgard (1978) found that the retention efficiency of 13 species of bivalves, fed mixtures of natural particles, decreases with particle size. The capacity of filter feeders differ between species and each species has developed adaptations to reduce inter-specific food competition. Food particle size and shape are also important variables to predict the capacity of food particle selection and ingestion in oysters (Defossez and Hawkins, 1997), although there is still a debate on whether size alone can determine the results of food selection (Ward and Shumway 2004). Other studies on food selection of four marine bivalves species *Cardium edule, Chlamys opercularis, Chlamys islandica, and Mytilus edulis* show that food selection efficiency increases with particle size, but capture efficiency on small particles (<7 µm) is species-specific (Vahl, 1972).

Oysters

Individual oysters can filter a large volume of water each day, and food ingestion rate is related to the gill structure and the volume of water cleared per unit of time (Honkoop et al., 2003). The oyster has been considered an ecosystem engineer due to its feeding efficiency and food selection, which regulates energy transfer between trophic levels. The clearance rate of an oyster also depends on environmental conditions such as temperature and food size (Honkoop et al., 2003). In the present study, oysters removed large particles (> 5 µm) such as diatoms and dinoflagellates more effectively than small particles (<2 µm) in spring and summer. This is similar to the report by Heral (1983) who found that *C. gigas* retains less than 10% of 1 µm particles, more than 50% of 3 µm and 100% of 7 µm particles. Interestingly, picophytoplankton abundance (particles <2 µm) was similar between the control and the oyster grazing treatment, suggesting that oyster gills are not able to retain these small particles. These results are in an agreement with Moore (1982), who used a golden alga *Isochrysis galbana* (3 - 7 µm) in a feeding trial and observed that Pacific oysters selectively removed algal cells > 6 µm. Moreover, Vaquer et al. (2000) demonstrated that *C. gigas* have a high clearance rate for > 5 µm particles, including flagellates, microphytoplankton and ciliates, in a natural planktonic community. These results suggest that oysters have a poor ability to retain small flagellates and picophytoplankton (<5 µm) despite the high availability of these small particles in the environment.

Food quality and quantity influence ingestion rate in filter feeders and Pacific oysters show high absorption efficiency on food particles containing 5% of organic contents (Ren, 2009). Furthermore, oysters can select food during various stages of food collection and can expel unwanted food as pseudo-faeces (Bayne, 2002). The process of food selection in oysters has fascinated scientists for decades, especially

when the availability of food sources fluctuates over seasons (Balzano et al., 2019; Espinosa et al., 2008). Our data show that irrespective of seasonal change, oysters ultimately retained large food particles as the main food source. This is supported by a study in oligotrophic waters where diatoms and dinoflagellates are highly retained as the major food sources in oysters (Vaquer et al., 2000). It can be concluded that picophytoplankton has a limited contribution to the food supply of oysters due to an incapability of this species retaining smaller particles.

Mussels

The particle size retained by the mussels *M. galloprovincialis* was intermediate between oysters and cockles. In the present study, mussels ingested a large proportion of larger size particles (> 5 µm) comprised of diatoms and dinoflagellates species, and also filtered a small portion of particles of 2-5 µm, including large picophytoplankton. Like oysters, mussels are unable to efficiently collect picophytoplankton (<2 µm) regardless of season. These findings are consistent with other studies (Kiorboe and Mohlenberg, 1981; Safi and Hayden, 2010) where mussels preferentially consumed a large fraction of phytoplankton rather than consuming smaller picoplankton and bacteria (Trottet et al. 2008).
In a previous study by Shumway et al (1985), showed that bivalves can only effectively consume particles greater than the size of a bivalve's gill sieve. For example, *Mytilus edulis* can retain food particles > 2 µm at an efficiency close to 100% as the distances between gill cilia range from 1 to 1.7 µm (Jørgensen, 1974). This adds to the argument on how food size affects the clearance capacity of bivalves and whether filtering efficiency is proportional to food particle size. In New Zealand mussels *Perna canaliculus*, Safi and Hayden (2010) reported that organisms of 5 µm and larger, including zooplankton, are preferred.

In the present study, a significant difference in feeding behaviour was observed between oysters and mussels. Both species can effectively remove diatoms, dinoflagellates and large picoplankton, but only mussels can further access smaller picoplankton as food sources. These results suggest that oysters and mussels are close competitors for the same natural assemblages of algae as reported in Deslous-Paoli et al (1987). Moreover, a study by Bougrier et al (1997) indicates that despite the similarity of food selection between *C. gigas* and *M. edulis*, mussels can further exploit food particles at the lower spectrum of size distribution that oysters are unable to access.

Cockles

Unlike sessile bivalve species such as oysters and mussels, cockles are a sediment dweller living in loose sand in the littoral zone (Kiorboe and Mohlenberg, 1981). In the present study, cockles could ingest smaller particles than oysters and mussels across all seasons. Small picoeukaryotes and bacteria were significantly removed from the water column. Similarly, Iglesias et al (1992) reported that cockles *Cerastoderma edule* can effectively remove 17 - 53% of small particles while feeding on a mixture of suspension microalgae. It is important to consider the characteristics of food particles when evaluating the differences in particle selection between species. Feeding capacity needs to be tested using a food source that closely resembles natural particle composition (Hawkins et al., 1998; Iglesias et al., 1992). Diatom cells are protected by rigid siliceous frustules, while flagellates are enclosed by a more flexible cell wall (Rouillon and Navarro, 2003), which may defer cockle's preference on these food particles. This suggests that cell morphology, structure and texture may also affect food selection in bivalves.

Size is an important criterion to discriminate among particles, but simply selecting for smaller food size may not be advantageous in all conditions. In the current study, cockles could fed on both small and large food particles, though its ability to remove large particles was not as efficient as oysters and mussels. Ward and Shumway (2004) reported that the benthic particle feeder *C. edule* was able to discriminate and retain significantly smaller size particles when confronted with a wide range of sediment particulates, including detritus, protozoa and bacteria. In addition, previous studies also reveal that cockles are less selective for food particles by size (Iglesias et al., 1992; Urrutia et al., 2001). In the present study, cockle feeding did not affect virus-like particles (0.02 - 1 μm) suggesting that these small particles could not contribute as a food source for this species.

4.5.2. Filtration capacity of molluscan species

The filtration rate is physiologically plastic as some marine bivalves are able to adjust the amount of water filtrated through in response to the environmental change (Rosa et al., 2018; Cranford et al., 2016). Seston composition, food particle density and temperature are common factors regulating the rate of bivalve filtration (Joyce et al., 2019; Specht and Fuchs 2018; Rosa et al., 2015). The present study showed significant differences in filtration capacity among oysters, mussels and cockles between seasons. Across seasons, oysters and mussels effectively filtered larger particles (> 8 μm) while cockles consumed largely on particles <5 μm. The ability to adjust filtration capacity allows bivalves to optimise particle intake according to the seston load in the environment (Hawkins et al. 1999). Strohmeier et al. (2012) showed that blue mussels *M. edulis* in a Norwegian region exhibited seasonal variation of particle size retention with a gradual increase in filtration rate from particles of 7 μm to 35 μm. Changes in the seston particle size usually coincide

with the change of the ambient particle size distribution. Similar results have been reported in blue mussel *M. edulis* (Rosa et al. 2015), rock-pool bivalve *Venerupis corrugatus* (Stenton-Dozey and Brown 1992) and Japanese oyster *Crassostrea gigas* (Barille et al. 1993). In addition, seawater temperature can affect filtration rate of particles (Richoux and Thompson 2001, Specht and Fuchs 2018). Kittner and Riisgard (2005) studied the effects of temperature on filtration rates of *M. edulis* and found a linear relationship between temperature and filtration rate. Furthermore, a study by Riisgard and Larsen (2007) on blue mussels suggests that the warmer water temperature itself does not influence ciliary beat by affecting physiological processes, but rather temperature alters the viscosity of water and affects fluid mechanics. In particular, increasing temperature decreases water viscosity and reduces drag on the cilia which results in higher rates of filtration activity. In the present study, mussels showed negative filtration on small seston particles (<5 μm in spring and oyster showed negative filtration on these sizes of particle in winter. The possible reason is that these animals defecated small seston to the ambient water, but had limited ability to retake these small particles in these environmental conditions.

4.5.3. Implication of food selection in marine ecology and molluscan farming

Understanding particle selection in suspension-feeding bivalves is essential to improve the growth and productivity of the existing production models, as well as to maintain the sustainability of the ecosystems (Grangeré et al., 2009). Bivalves, due to their filter feeding capacity, are often considered a keystone species in an ecosystem with the ability to affect the function and energy transfer in the surrounding environment (Espinosa et al., 2016; Gallardi, 2014). At the ecosystem level, particle selection by bivalve species can impact the material flux into the benthos (Chauvaud et al., 2000). Depending on species, bivalves can remove suspended matter with a

particle size of 1- 7 µm, and return large faecal pellets (500 - 3000 µm) to the environment. The pellets rapidly settle to the seabed to impact the organic load in the system (Gallardi, 2014). Moreover, through the ability to sort and select foods, bivalves can reduce phytoplankton abundance and change species composition in the water (Smaal and Prins, 1993; Prins et al., 1991).

Depending on the farming model, cultured bivalves affect the planktonic and benthic food web by modifying, repacking and increasing the sedimentation rate of fine suspended particles, ultimately altering the availability of food resources to other species. The bivalve aquaculture industry in Australia is widely spread throughout Tasmania, South Australia and New South Wales. Currently, most of bivalve aquaculture species including mussels, Pacific oysters and Sydney rock oysters, are farmed as a single species in Australia. This practice is purposely to avoid food competition that will slow down the growth and productivity of the target species. By knowing food preferences of the different species, bivalve growers can gain some advantage in diversifying the number of species cultured on farms without affecting the productivity of the main species. This study demonstrates that oysters, mussel and cockles have their own ecological niche and are not necessarily competing for food. Oysters and mussels tend to select larger food particles, with mussels exploring a similar range of food but at the lower end of size spectrum. Cockles, on the other hand, could explore a large spectrum of food particles, with a particular feeding mode to select for smaller particles. Oyster farms are usually located near shore, whereas mussels are farmed in relative deep water. Cockles have not been farmed in any well-established farming model in this region, but have attracted strong interest in its aquaculture recently.

The selection mechanisms used by suspension-feeders to capture and ingest food from a mixture of particles with various sizes and nutrient composition have been the central research topic among bivalve ecophysiologists for several decades (Espinosa et al., 2016). More knowledge is needed in terms of the ecological management of marine resources. This study contributes to our understanding of the particle selection of molluscan species in different seasons to promote growth and maximise productivity. There is a need to develop the molluscan industry in a way that pursues sustainability and that protects and maintains the supporting environment by operating within the ecological carrying capacity. This study further shows that despite the seasonal change of food availability, the nature of selection between bivalve species does not vary substantially with temperature. Importantly, our results provide a scientific base to practice ecosystem based management of multi-species aquaculture in a marine zone and effectively use marine resources for the optimisation of productivity.

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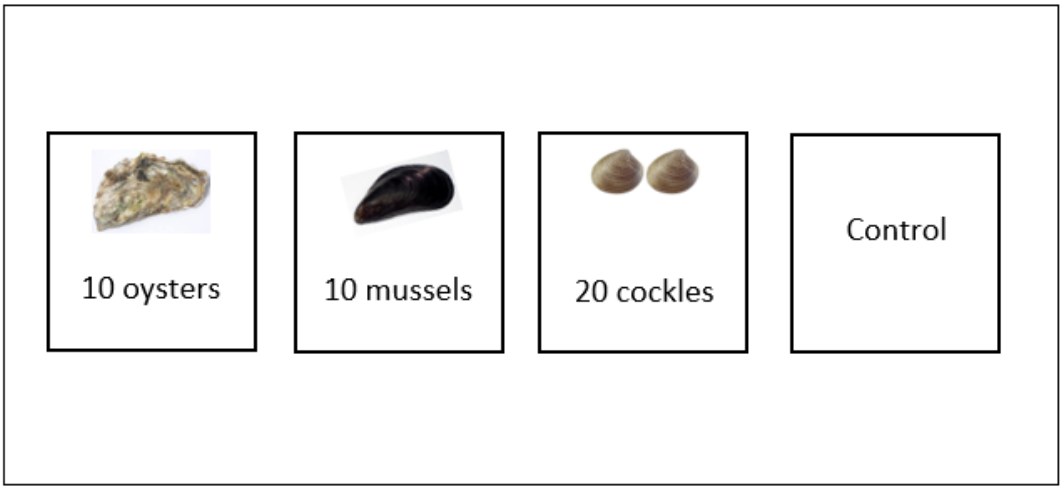

Fig. 1 Design of the feeding experiment with four treatments: oysters (10), mussels (10), cockles (20) and control in 20 L containers (n = 3) in each season.

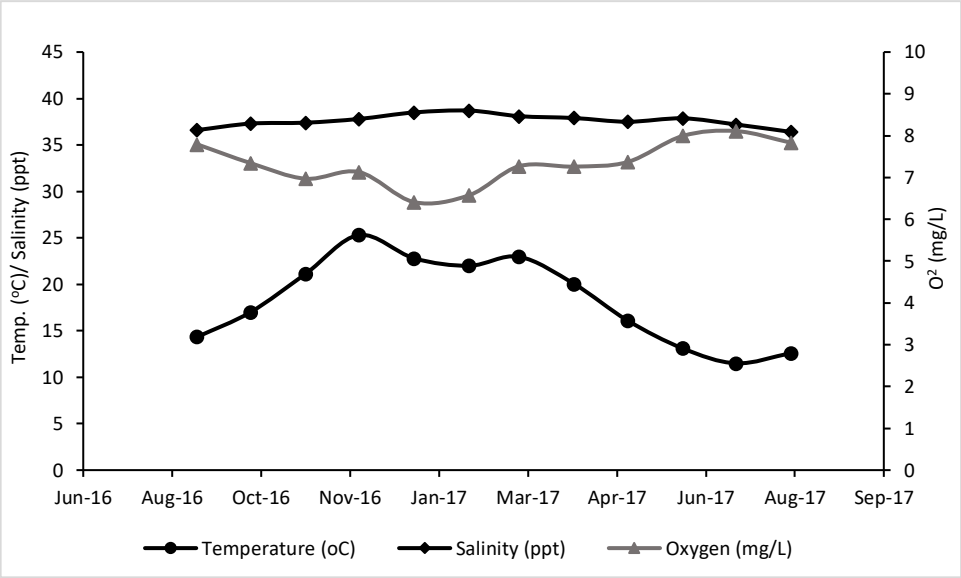

Fig. 2 Fluctuations of temperature, dissolve oxygen (DO) and salinity from September 2016 to August 2017 in the site where water samples were collected.

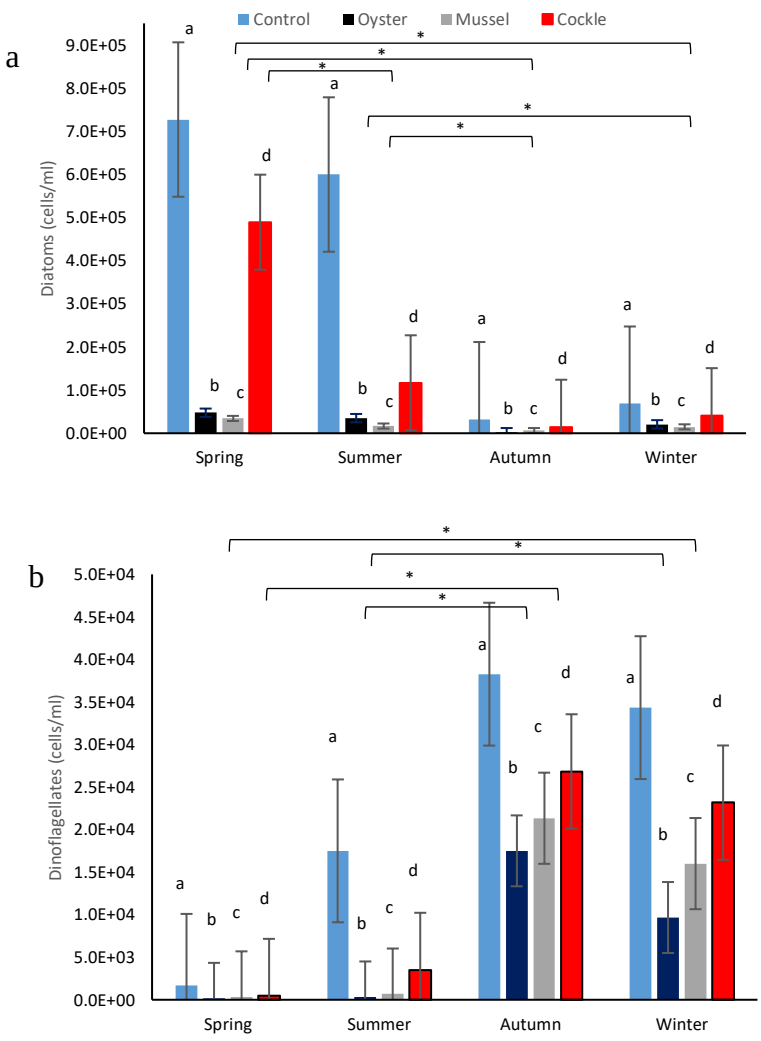

Fig.3 a) Diatoms and b) dinoflagellates reduced after a 10-h feeding trial by oysters, mussels and cockles in four seasons. Error bars represent standard deviation. Different letters indicate significant difference ($P < 0.001$) between species in each season. Bars with an asterisk represent significant difference ($P < 0.001$) between seasons.

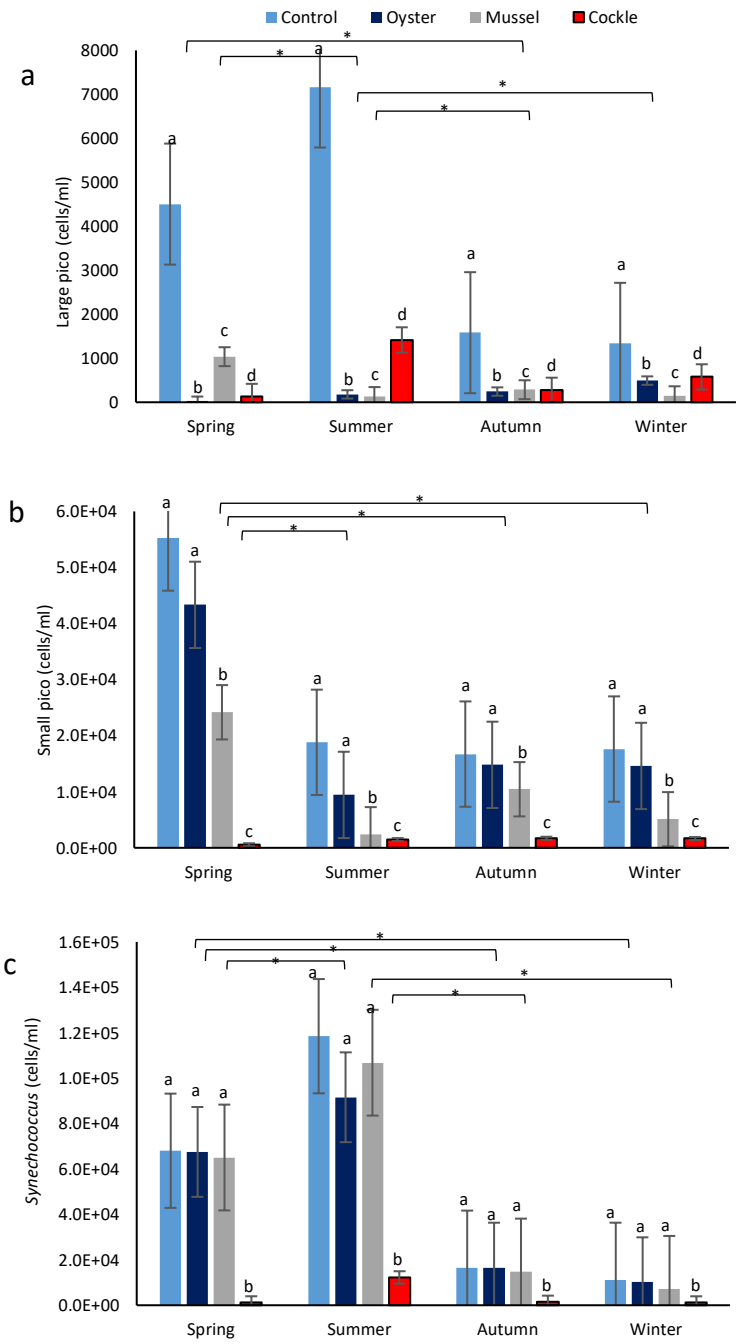

Fig. 4 a) Large picoeukaryotes (2 -5 µm), b) small picoeukaryotes (<2 µm) and c) *Synechococcus* sp. reduced after 10 h feeding by all species in four seasons. Error bars represent standard deviation. Different letters indicate significant difference ($P < 0.001$) between species in each season. Bars with asterisk represent significant difference ($P < 0.001$) between seasons.

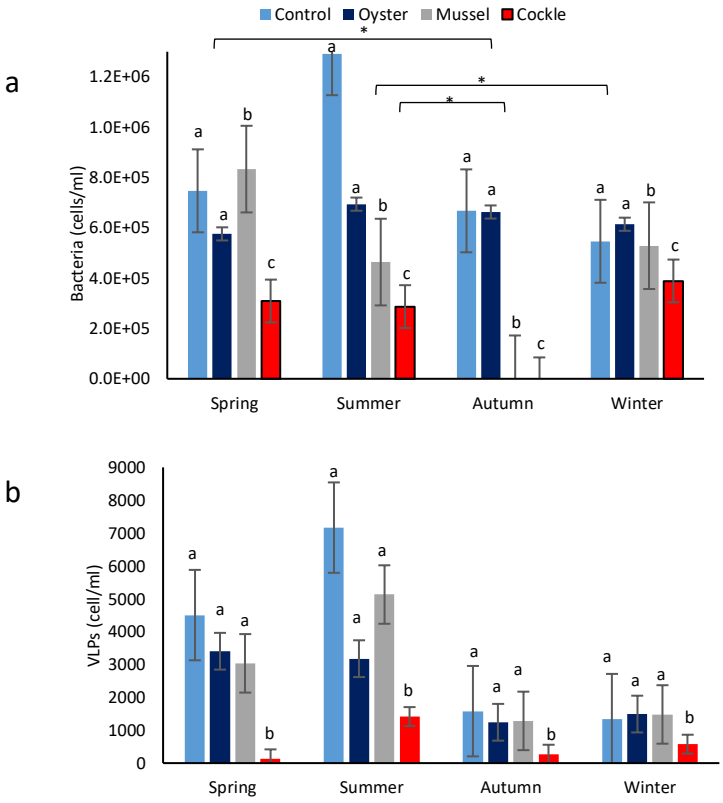

Fig.5 a) Bacteria, b) virus like-particles reduced after 10 hours feeding period by all species in four seasons. Error bars represent standard deviation. Different letters indicate significant difference ($P < 0.001$) between species in each season. Bars with an asterisk represent significant difference ($P < 0.001$) between seasons

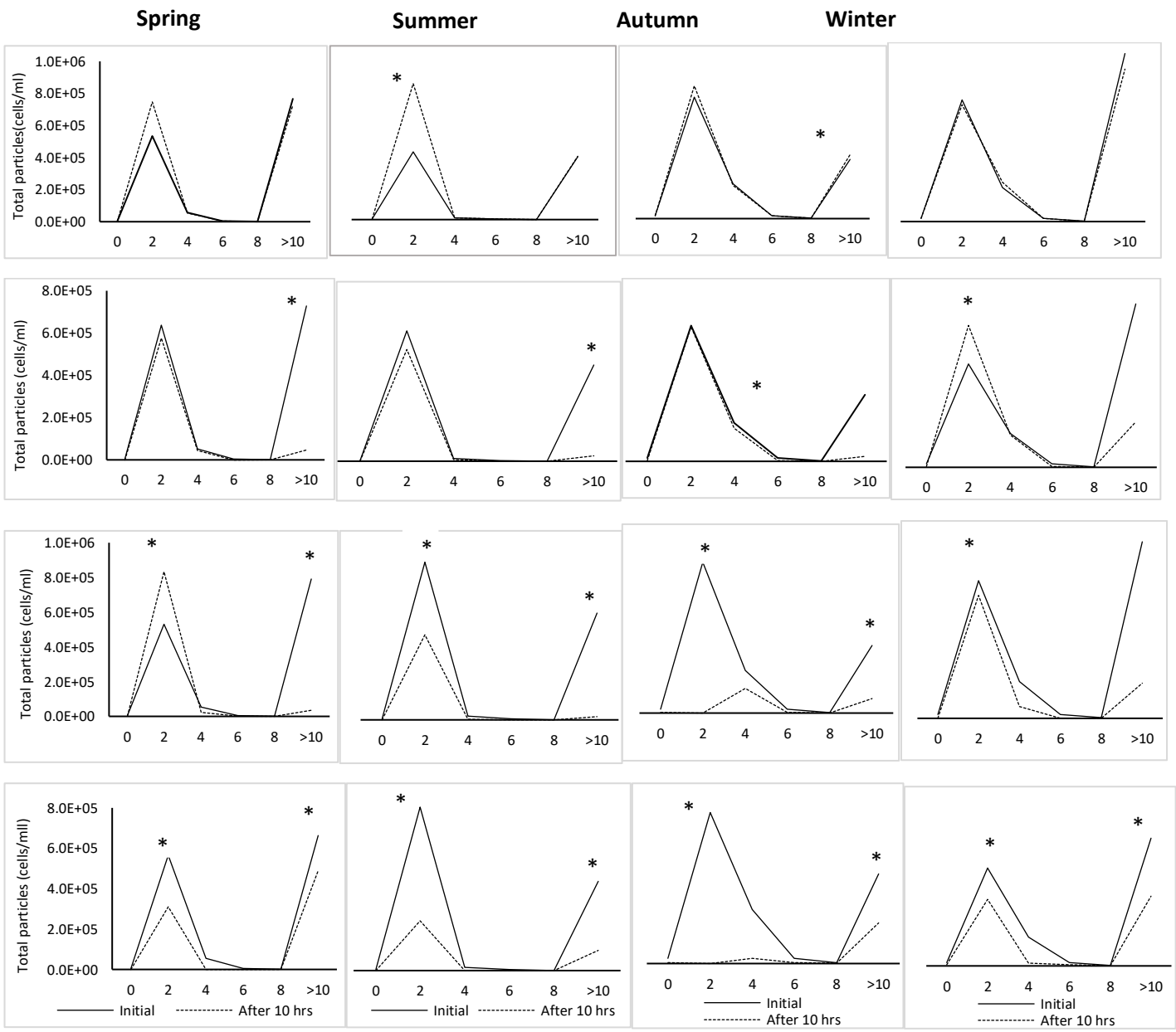
Spring
Summer
Autumn
Winter
a
b
c
d
Total particles(cells/ml)
Total particles (cells/ml)
Total particles (cells/mll)
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
0 2 4 6 8 >10
Initial
After 10 hrs
Particle size (µm)

Fig. 6 Seasonal comparison of initial (dashed line) and after 10 h feeding (solid line) particle size selection by species; a) control, b) oysters c) mussels and d) cockles. The asterisk represents significant difference between the initial and final abundance of particles ($P < 0.001$).

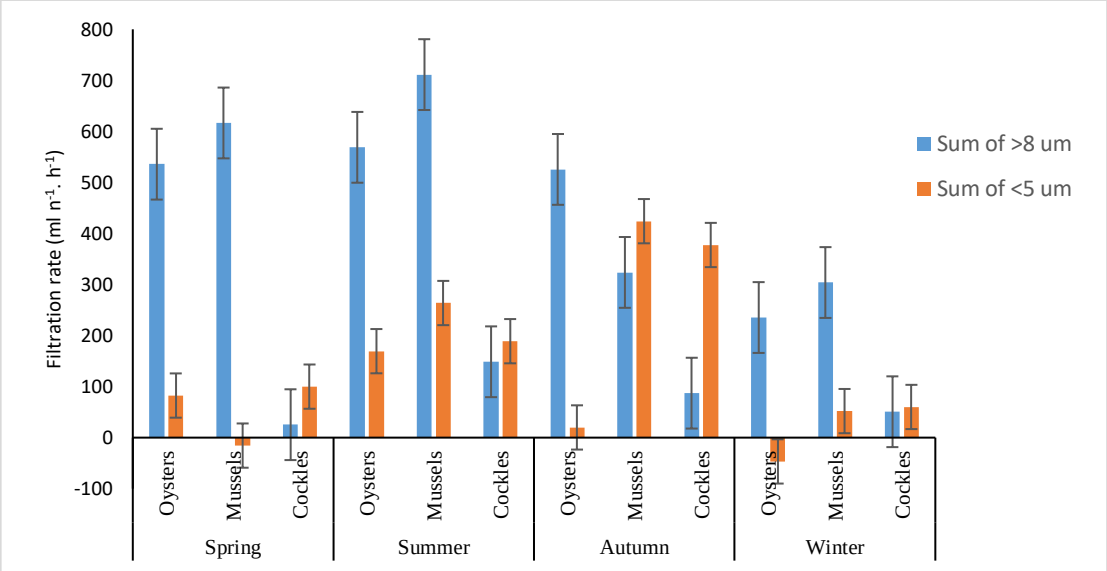

Fig. 7 Seasonal comparisons on the filtration rate of particles < 5µm and particles > 8µm by marine bivalves after 10 h feeding. Values are means ±Sd, n =10 (cockles, n = 20).

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSION

Shellfish aquaculture is a growing industry worldwide and has been thought as an effective means to meet the increasing demand for seafood supply as these animals can effectively utilise primary productivity from the basal food chain. Efforts have been made to sustain the industry shellfish aquaculture, including species diversification, resource management and technical innovation in husbandry. To achieve sustainability of shellfish aquaculture, an important consideration in aquaculture operation is to understand the potential interaction between shellfish farming and the environment. Food availability, feeding physiology and metabolism of bivalve shellfish are closely related to environmental variables such as temperature and salinity that are both spatially and temporally variable in near-shore waters (Hawkins and Bayne, 1992). Temperature is a far more important factor than others in affecting the physiology of marine organisms, and previous studies have demonstrated the impact of temperature on basic physiological and metabolic functions in bivalves such as clearance rate, respiration and heart beating rate (Feng, 1965; Han et al., 2008; Haure et al., 1998; Pandolfo et al., 2009). Changes in temperature also modulate bivalve defence functions both *in vivo* and *in vitro* (Carballal et al., 1998; Yu et al., 2009). Recently, bivalve culture has suffered from summer mortality where catastrophic mortality events exceed more than 20% stock losses in summer (Malham 2009; Berthelin et al., 2000). High temperature has been suggested as the most influential factor associated with the oyster summer mortality syndrome (Cheyney et al., 1998; Samain et al., 2007; Soletchnik et al., 2007).

The overall objective of this thesis research was to improve the knowledge on the impact of temperature on the biology and growth in three important commercial bivalve species, i.e., the Pacific oyster, blue mussel and mud cockle. This thesis provides comparative data on how food availability in the water column could affect

the biological performance of three bivalve species that naturally live in different habitats in four seasons. This research is crucial to develop relevant management strategies for optimising production and limiting the impact of environmental changes caused by climate change. The major findings of this thesis research are summarised below.

5.1. Summary of major findings

1. The increase of seawater temperature has significantly affected suspension feeding efficiency, physiology, metabolism and growth of three bivalve species. The response of these three bivalves to temperature elevation was more pronounced with the elapse of time especially after 30-day exposure to different temperatures. The best growth occurred at 15 ^{o}C whereas the growth rate became negative at 25 ^{o}C in all species. The normal growth of somatic tissues cannot be sustained at 25 ^{o}C in all three bivalve species due to low clearance rate, high metabolism and high faecal excretion rate. Cockles had the lowest scope for growth value ranging from -8.4 to -15.7 J $g^{1}h^{-1}$ compared to oysters and mussels, suggesting that cockles have the poorest adaptability to thermal stress.

2. The temperature changes affected haemocyte functions and led to oxidative stress, reducing immunosurveillance in all these three bivalve species. The temperature of 25 ℃ significantly increased the THC and phagocytosis of haemocytes in all species. The SOD and CAT activities in the haemocytes of mussels and cockles rapidly increased with temperature elevation compared to those in oysters that only showed the change in the temperature change from 20 to 25 ℃. Oysters and mussels have a greater tolerance to extreme thermal stress allowing their dominance in the intertidal and subtidal zones, whereas cockles appeared to be more sensitive to temperature elevation as indicated by the change of haemocyte activities.

The survival of cockles was lowest (51.66%) at 25 °C compared with other two bivalve species.

3. The phytoplankton community in the central zone of the Coffin Bay was dominated by *Bacillariophyceae* (diatoms), *Dinophyceae* (dinoflagellates), *Synechococcus* (cyanobacteria), and picophytoplankton (2-5 µm) from September 2016 to August 2017. Diatoms, picoplankton and cyanobacteria were dominant in spring and summer, while dinoflagellates were dominant in autumn and winter. Selectivity for seston particles by oysters, mussels and cockles and their food availability vary between seasons. Oysters and mussels selectively fed on large food particles (>2 µm) regardless of season, but mussels could access a wider size spectrum of seston particles compared to oysters. Cockles selected for large and small food particles (e.g., *Synechococcus* and small picoeukaryotes <2 µm) and fed more efficiently on small particles than either oysters or mussels.

5.2. Overall discussion on the influence of temperature on marine bivalves

5.2.1. Physiological and metabolic enzyme activities as indices of thermal stress on bivalves

Assessments of the physiological and metabolic conditions of ecologically important marine bivalves are essential to understand how the environmental change can affect their growth performance and survival. Temperature can control the rate of fundamental biochemical processes in marine organisms, and consequently the change of environmental temperature can influence the performance and function at individual, population, and community levels. As the most influential factor in the environment, water temperature can dramatically affect the biological activity and metabolic rates of aquatic organisms (Roessig et al., 2004; Danise et al., 2013) and the thermal stress has been observed in all three bivalve species in this study, though the degree of influence was species-specific.

The mechanism of physiological responses in bivalves exposed to a stressful temperature is complex. Temperature can change the growth performance by influencing the filtration rate, absorption and utilization of available food (Zippay and Helmuth, 2012; Jansen et al., 2007). Each of these processes differs between species and populations according to animal size, physiological status, nutrition, thermal history and other factors. Responses also vary according to the rate and amplitude of temperature changes. In most of aquatic organisms, a 10 °C increase in water temperature will approximately double the rate of physiological function (Bennett and Di Santo, 2011). Interrelations between many of these variables have been widely studied (Gazeau e al., 2014; Matoo et al., 2013; Bougrier et al., 1995). The results of the current study support the findings of previous studies that elevated levels of temperature significantly impact both physiological and metabolic of

bivalves. Cockles were more sensitive to elevated levels of temperature than oysters and mussels. The high temperature (25 ^{o}C) treatment resulted in negative growth rates and the lowest clearance rate for all three bivalve species. The temperature effect became worse once the environmental temperature exceeded the range of thermal tolerance. Furthermore, the temperature of 25 ^{o}C is considered critical as the positive growth rate could not be sustained in all three bivalve species. This study indicates that relationships between all parameters and temperature stress allow us to predict the optimal temperature regimes in bivalve culture and provide a valuable insight into the physiological energetics of commercially and ecologically important bivalve species.

In this study, there was a direct relationship between metabolic rate and water temperature as indicated by the increase of cellular enzymes at high temperatures. The rate of metabolism in ectothermic organisms usually increases as the environmental temperature increases (Ivanina et al., 2013; Brierley and Kingsford, 2009). This study shows that the rise of metabolism is a result of high activity of cellular enzymes in concomitance of temperature increases. The adaptability to the increase of metabolic activities varies among species. In this study, oysters and mussels showed greater capacity in adaptative mechanism compared to cockles. Metabolic stress could be an adaptation to avoid a further rise in energy demand at high temperature that may affect the growth and productivity of animals. Collectively, these results provide critical information on the impact of temperature on the growth of bivalves in coastal ecosystems. Therefore, the identification of mechanisms of thermal limitation and adaptation of bivalves is important, particularly in the light of future shifts in physiological performance of marine organisms due to climate change.

5.2.2. Immune response as indicators of thermal stress

The invertebrate immune response is through a non-adaptive system involving both cellular and humoral components (Schmid-Hempel, 2003). A rise in temperature can induce changes in the extracellular acid–base balance that can cause immune disturbance to adversely affect immune processes such as phagocytosis, cytotoxic and the first line group of protein defence (Matozzo et al., 2012; Ellis et al., 2011). A growing body of evidence indicates that temperature can impact immune response in many bivalve species such as Pacific oysters, mussels, and mud cockles (Rahman et al., 2019), clam (Munari et al., 2011), and surf clam (Yu et al., 2009). Considering the integral role of innate immune defence, it is not surprising that phagocytosis is the measure that has received the greatest amount of investigation to assess the thermal stress on the immune response of marine bivalves. Typically, the measure of phagocytosis investigates the proportion of haemocytes that are phagocytically active in haemocytes (Gagnaire et al., 2006) or the phagocytic index to estimate the number of bacteria engulfed by haemocytes (Duchemin et al., 2007). These findings are consistent with Wootton et al. (2003) who reported that phagocytosis activity in haemocytes of intertidal species *Mytilus edulis* were most active compared to infaunal species like common cockle *Cerastoderma edule* and pod razor *Ensis silique*. This study suggests that oysters and mussels are able to adapt better to elevated temperatures than mud cockles as indicated by a high phagocytosis activity. In addition to assessing the phagocytic activity, the present study also used the total haemocyte counts (THC) to estimate a possible change in the number of haemocytes in the context with the change of other cellular immunological features. This study suggests that the increase of THC in bivalves could be a result of cell mobilisation or cell proliferation in haemolymph circulation which directly will

influence the phagocytosis activity. Furthermore, the changes in the abundance of haemocytes at different levels of temperature will allow a better understanding of the process that is involved in phagocytosis due to the change of environmental stressors (Zhang et al., 2014; Husmann et al., 2011; Gagnaire et al., 2003).

Thermal stress is also accompanied by oxidative stress in various marine mollusc species (Husmann et al., 2011; Weihe et al., 2010) based on indirect measurements of oxidative stress, or changes in antioxidant enzyme activities upon temperature elevation (Perrigault et al., 2011; Yu et al., 2009; Abele et al., 2001). Reactive oxygen species (ROS) are normal by-products of cellular respiration, and the increase of ROS in bivalves is a measure of the ability to counteract exogenous invaders (Marin et al., 2007). Bivalves under the stress of temperature change can produce ROS and activate anti-oxidative enzymes (Abele et al., 2002), and the SOD is an important defence indicator in the antioxidant system (Marin et al., 2007; Yang et al., 2007). The current study suggests that high temperature can lead to alteration of SOD activity in the haemocyte lysate or cell-free haemolymph. However, the adverse effects are not necessarily universal as different species vary in immune sensitivities to temperature stress (Ellis et al., 2011; Wootton et al, 2003). The present study showed that immune cells and functions differed extensively among these three species. The oysters showed a much higher level of immunological vigour that may be linked to its considerable resilience to adverse environmental conditions in the intertidal zone. An understanding of exactly how the invertebrate immune system responds to temperature change in the environment is vital to help further our knowledge on host-pathogen interactions at different temperatures. Furthermore, this knowledge will help us to understand and predict how the changes

in immunocompetence caused by environmental variability may impact marine bivalves at a population or a community level.

5.2.3. Seasonal variation of filtering capacity among molluscan species

Water temperature can have a large influence on phytoplankton composition, distribution and nutrient cycling patterns (Peter and Sommer, 2012; Muren et al., 2005). Phytoplankton communities at the bottom of the marine food web play an important role in changing the growth and survival of species at the upper trophic levels, including bivalves (Huertas et al., 2011). Studies of seasonal fluctuations of the phytoplankton communities have been conducted by many authors worldwide (Balzano et al., 2015; Ajani et al., 2011; Head and Pepin, 2010). Feeding in bivalves generally is understood to be physiologically plastic as organisms respond differently to the changes of environmental fluctuations, seston composition and particle loads (Rosa et al., 2018; Cranford et al., 2016).

This study reveals that the filtering capacity and selectivity among oysters, mussels and cockles varies between seasons. The differences may due to several factors such as variation of filtering apparatus, feeding behaviour and food availability. These results are in a good agreement with the study by Cranford and Hill (1999) who reported variation of seasonal changes in the rates and efficiencies of feeding and absorption in the *M. edulis* and *Placopectan magellanicus*. Both species displayed a different capacity for controlling clearance and absorption rates especially during spring and summer where the abundance of seston is high. The ability to adjust filtration capacity allows bivalves to optimise particle intake according to the seston load in the environment. Furthermore, by knowing dietary sources of the different species, bivalve growers can develop strategies in

diversifying the number of species cultured on farms without affecting the productivity of the principal species.

Differences in food selectivity between bivalves have been reported in other species (Rosa et al. 2013; Espinosa et al. 2010; Beninger et al. 2007). Bivalve species may rely on variables other than physicochemical factors in food collection, retention and filtration. For example, the differences in selection efficiency between mussels and oysters may be due to differences in ctenidial architectures and morphology of filtering apparatus (Ward et al.1998). In the present study, a significant difference in feeding behaviour was observed between oysters and mussels. Both species tend to select larger food particles like diatoms, dinoflagellates and large picoplankton, but only mussels can further access smaller picoplankton as their food preferences. Cockles, on the other hand, can effectively ingest smaller particles than oysters and mussels. Furthermore, the difference in filtration capacity by these molluscan species could be reflected by their growth performance. Investigation of growth rates under various environmental conditions could achieve a better understanding of food competition between these three species to improve aquaculture management. Further defining the mechanisms and determining the role of particle selection by marine bivalves warrant future study.

5.3. Conclusion

This thesis presents findings on the response of three marine bivalves to temperature stress. The outcomes of the research not only contribute to new understanding of the biology of these aquatic organisms but also provides crucial information useful for future improvement of aquaculture management. In summary, the following four conclusions are drawn:

1. Water temperature plays an important role in biological regulations such as feeding, metabolism and immunity, growth and survival of marine bivalves.

2. Oysters and mussels have a greater tolerance to extreme environmental fluctuations than cockles. The different ability to thermal stress may be related to the habitats where these three species live in nature.

3. The cockle is the most sensitive species in response to temperature stress and can effectively remove smaller size particles compared to oysters and mussels.

4. Variability of the thermal responses and food selection among these three marine bivalves contribute to a better understanding to improve the growth and productivity of the existing production models, as well as to maintain the sustainability of the ecosystem.

5.4. Future Research and Recommendations

Overall, the outcomes of this research provide fundamental understanding of the role of temperature in aquatic organisms. Nevertheless, some questions are still prevalent and future research is needed to tackle the following unsolved issues:

1. The present study has demonstrated that elevated temperature has a significant impact on the response of marine bivalves and may constitute a potential threat to the increased susceptibility to diseases that lead to mortality. However, there is a major uncertainty with identification of the causes of mass mortality events in bivalves during summer. Further research should also consider other factors that contribute to this phenomenon such as pathogenic infections, agricultural runoff, and other stressors affecting development in combination with high temperature. A further investigation will improve the understanding of the interaction between biotic and abiotic components in an ecosystem.

2. Temperature does not, however, only interfere with physiological processes through direct impact on catalytic rates but also modulate regulatory processes at a molecular level. Furthermore, comparison with the phylogenetically

distant will allow us to estimate if thermal sensitivities of specific enzymes are conserved or labile in characterisations. More experimental studies in gene expression are required to fully elaborate the background of climate sensitivity and specialisation.

3. Based on the present dataset, the question whether the reduction of growth capacities is due to the increase of water temperature itself, remains unresolved. More long-term studies performed at the proximity of aquaculture sites are required to assess possible adaptation during continuous temperature stress. In addition, the synergistic effects of temperature with other environmental factors such as salinity, ocean acidification and hypoxia also need further investigation. Further research in this area will improve the understanding of the response of marine bivalves to environmental fluctuations as a whole picture.

4. The relationships between temperature and growth, food availability and composition are not well understood. Further research on growth and feeding physiology of bivalves especially in an oligotrophic environment where most southern Australian waters are, is required to understand and predict shellfish distribution, production and survivorship in an oligotrophic environment. The knowledge of feeding physiology will permit enhancing the grow-out and breeding of this species in captivity and optimise site selection for the cultivation of bivalves and maximization of their growth performance.

5. Analysis of particle selection by these three bivalves was done in this study, but the specific diet consumption was not investigated. Identifying the diets of an organism is important for understanding their basic ecology, characterizing trophic interactions, predicting community-level consequences of biotic and abiotic changes as well as their growth performance and productivity. It is recommended to

use fatty acid and stable isotope analysis to reveal the contribution of different food items to their dietary utilisation and possibly to estimate the time required to assimilate nutrients from food into body tissues.

6. Cockle is a potential species for aquaculture as for the capability to access a broad range of food sources in the water column. The knowledge gained in this thesis should be applied to the study on the feasibility and practicality for farming this potential aquaculture species under captivity.

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