INTRACELLULAR BASIS OF CLIMATE CHANGE RESILIENCE IN OYSTERS

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This thesis is dedicated to my parents, with all my love and gratitude.

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SUMMARY

Climate change is making the world's oceans warmer and more acidic. This environmental disturbance represents a major threat to marine life, particularly to calcifying organisms, such as oysters. Although adaptation to climate change is possible for many marine species, the intracellular basis of such stress-induced modifications is largely unknown. This thesis aims to discover the biological mechanisms that provide heritable protection against the adverse effects of climate change stressors in oysters. I explore the molecular processes underlying the improved performance of selectively bred populations of Sydney rock oysters (*Saccostrea glomerata*) under acidifying conditions. The thesis assesses the intracellular responses of CO_2-resilient and wild type (non-selected) oysters following single or transgenerational exposures to elevated CO_2 using transcriptomics, proteomics and cytology. The molecular profiles of CO_2-resilient oysters were also investigated in response to a combination of CO_2 and thermal stresses. It was found that elevated CO_2 affected different levels of biological complexity, ranging from changes in cellular structures (mitochondria and associated systems) to alterations in protein concentrations and gene expression. The data showed that oysters are highly responsive to ocean acidification, and that discrete populations of oysters differ in their responsiveness. CO_2-resilient oysters exhibited differential regulation of genes and proteins involved in a variety of fundamental cellular processes, including control of the cell cycle, maintenance of cellular homeostasis, energy metabolism and stress responses. Transgenerational exposures to elevated CO_2 further altered the transcriptional profiles of oyster populations, suggesting that they may also be able to undergo rapid acclimation or adaptation to CO_2 at the transcriptional level. However, concurrent exposure to CO_2 and thermal stresses did not produce additive or synergistic effects on the molecular responses of CO_2-resilient oysters. This indicates that the inherent capacity of selectively bred oysters to better cope with CO_2 stress may not extend to ocean warming, or that the addition of thermal stress overwhelms oyster stress responses. Overall, the thesis reveals the molecular processes that may enable marine organisms to survive and thrive as climate change progresses. I conclude by considering the implications of this knowledge to ecosystem dynamics and fisheries production, highlighting potential strategies to minimise the impacts of this imminent threat.

STATEMENT OF CANDIDATE

I certify that the work in this thesis entitled "*Intracellular basis of climate change resilience in oysters*" has not previously been submitted for a degree nor has it been submitted as part or requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Priscila Goncalves

STATEMENT OF THE CONTRIBUTIONS TO THESIS CHAPTERS

I was involved in the conception and experimental design of all studies presented in this thesis. I performed all aquarium and laboratory-based experiments, collection and processing of samples, as well as data management, analysis and interpretation. I was also the lead author of all the chapters of this thesis. My estimated proportion of contribution to each of the chapters of this thesis is outlined below, followed by the specific contribution of the co-authors.

Thesis chapters	**Extent of intellectual input by the candidate (%)**				
	Study concept and design	Acquisition of data	Analysis and interpretation of data	Drafting of manuscript	Critical revision
Chapter 1: *General Introduction*	-	-	-	100%	50%
Experimental chapters:					
Chapter 2	70%	100%	90%	100%	50%
Chapter 3	60%	100%	90%	100%	50%
Chapter 4	70%	100%	90%	100%	50%
Chapter 5	70%	100%	90%	100%	50%
Chapter 6: *Synthesis and Conclusions*	-	-	90%	100%	50%

Thesis chapters	Nature and extent of contribution by co-authors
Chapter 1: *General Introduction*	**Raftos DA** Editing and critical revision
Experimental chapters:	
Chapter 2	**Anderson K, Thompson EL & Raftos DA** Conception and design of the experiments **Anderson K & Thompson EL** Experimental exposure and sampling, manuscript editing **Raftos DA** Editing and critical revision
Chapter 3	**Anderson K, Thompson EL & Raftos DA** Conception and design of the experiments **Anderson K & Thompson EL** Experimental exposure and sampling, manuscript editing **Raftos DA & Melwani A** Assistance with data analysis **Raftos DA** Editing and critical revision
Chapter 4	**Thompson EL & Raftos DA** Conception and design of the experiments **Thompson EL** Experimental exposure and sampling **Jones DB** Assistance with bioinformatics analysis and manuscript editing **Raftos DA** Editing and critical revision
Chapter 5	**Thompson EL & Raftos DA** Conception and design of the experiments **Thompson EL** Experimental exposure and sampling **Raftos DA** Editing and critical revision
Chapter 6: *Synthesis and Conclusions*	**Raftos DA** Editing and critical revision

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CHAPTER 1

GENERAL INTRODUCTION

PREFACE

Shifts in seawater physicochemical variables resulting from climate change represent a major threat to marine life. This thesis explores the inherent capacity of some organisms to cope with rapidly changing oceans. I investigate the intracellular effects of ocean acidification and warming on genetically distinct populations of oysters in order to understand the key subcellular processes involved in their responses to CO_2 and thermal stresses, and to identify factors that could provide resilience against these harmful conditions. This introductory chapter provides an overview of our current understanding of the impacts of decreasing pH and increasing temperature on calcifying marine species, with a particular focus on oysters. It begins by defining the physicochemical changes in seawater driven by global climate change. I then describe the effects of these changes on calcifying marine species, and the mechanisms and strategies that could potentially mitigate such impacts. The chapter also highlights novel approaches for cellular and molecular analyses of organisms responding to environmental stress, and concludes by outlining the specific aims and objectives of the thesis.

1.1 CHANGES IN SEAWATER CHEMISTRY AND PHYSICS RESULTING FROM CLIMATE CHANGE

Increasing concentrations of atmospheric carbon dioxide (CO_2) have been driving substantial changes in the fundamental chemistry and physics of marine ecosystems. The observed increases in atmospheric CO_2 concentrations and temperature ascribed to climate change are derived mainly from anthropogenic activities that have become more intensive and widespread since the industrial revolution (IPCC 2013). Oceans take up a substantial proportion of excess atmospheric heat and CO_2, resulting in increased seawater temperature and decreasing pH (Fig. 1.1) (Pörtner *et al.* 2014; Watson *et al.* 2011). In other words, world's oceans are becoming warmer and more acidic as climate change progresses.

Ocean acidification (OA) is a result of major shifts in seawater carbonate chemistry elicited by rising global CO_2. Once dissolved in seawater, CO_2 increases the partial pressure of CO_2 (pCO_2), as well as the concentration of bicarbonate ions ($[HCO_3^-]$) and dissolved inorganic carbon (C_T). Concurrently, CO_2 reduces the concentration of carbonate ions ($[CO_3^{-2}]$) and the saturation state of calcium carbonate minerals (Fig. 1.1) (Gattuso & Hansson 2011). The higher abundance of protons ($[H^+]$) derived from the imbalance between the concentrations of bicarbonate and carbonate ions ($[HCO_3^-]/[CO_3^{-2}]$) reduces seawater pH. The reduced concentration of carbonate ions ($[CO_3^{-2}]$) further affects the availability of three major calcium carbonate ($CaCO_3$) minerals: aragonite, calcite and magnesian calcite (Mg-calcite). Aragonite and calcite are critical drivers for the production and solubility of shells and skeletons in many marine species, including oysters. Decreased saturation of these minerals has substantial implications for the calcification processes (Gattuso & Hansson 2011; Orr *et al.* 2005).

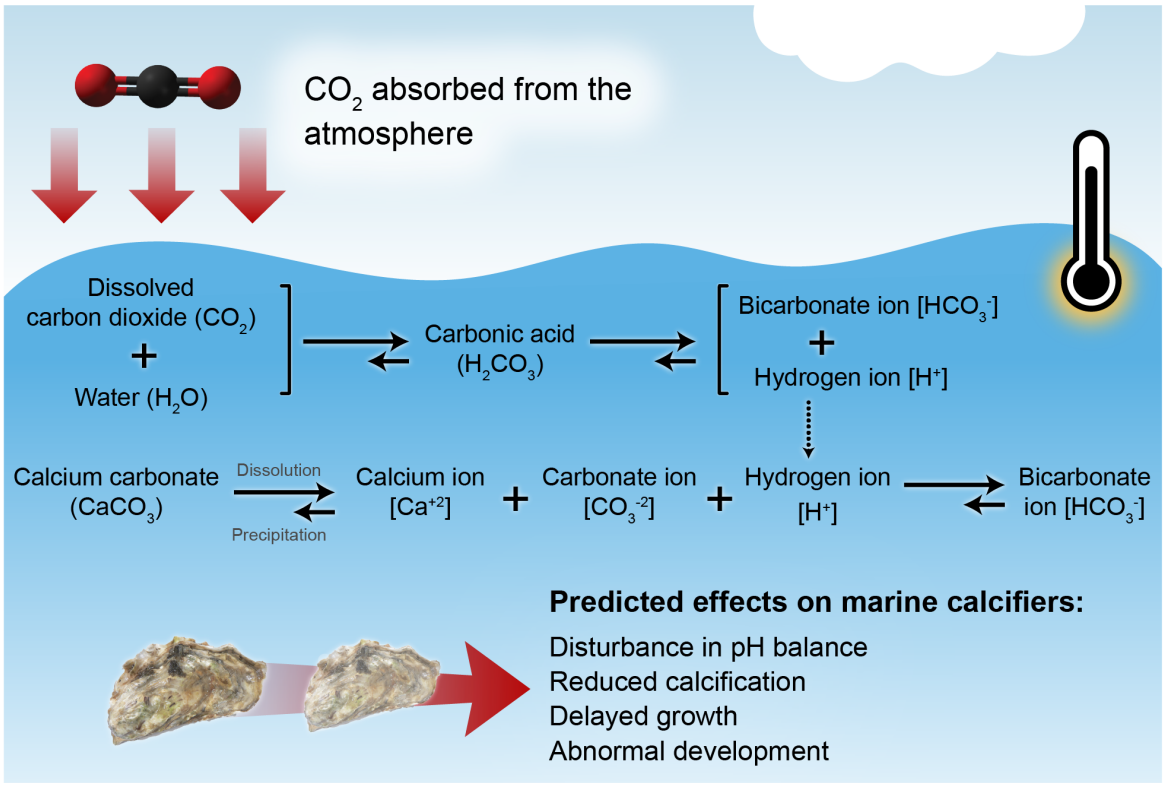

Figure 1.1. Simplified schematic of the major effects of increasing atmospheric CO_2 concentrations on seawater chemistry and physics and on marine calcifiers.

Although there are uncertainties about the magnitude of the environmental changes that will occur over this century, the available evidence clearly shows that the current rate of anthropogenic carbon perturbation exceeds that of preceding natural geological variability (Friedrich *et al.* 2012; Luthi *et al.* 2008). Many studies have shown that marine organisms will be at high risk even under low-emission scenarios for climate change (Gattuso *et al.* 2015; Pörtner *et al.* 2014). The most stringent emission-mitigation scenario proposed by the Intergovernmental Panel on Climate Change (IPCC) (Representative Concentration Pathway 2.6; RCP2.6) predicts an increase in ocean surface temperature of 1.2 °C and a decrease in ocean pH in 0.14 units by the year 2100 (IPCC 2013). However, the current high-carbon-emission business-as-usual scenario (RCP8.5) anticipates that ocean surface temperature will increase by 3.2 °C by the end of the century, while pH will decrease by 0.4 units (Fig. 1.2) (IPCC 2013).

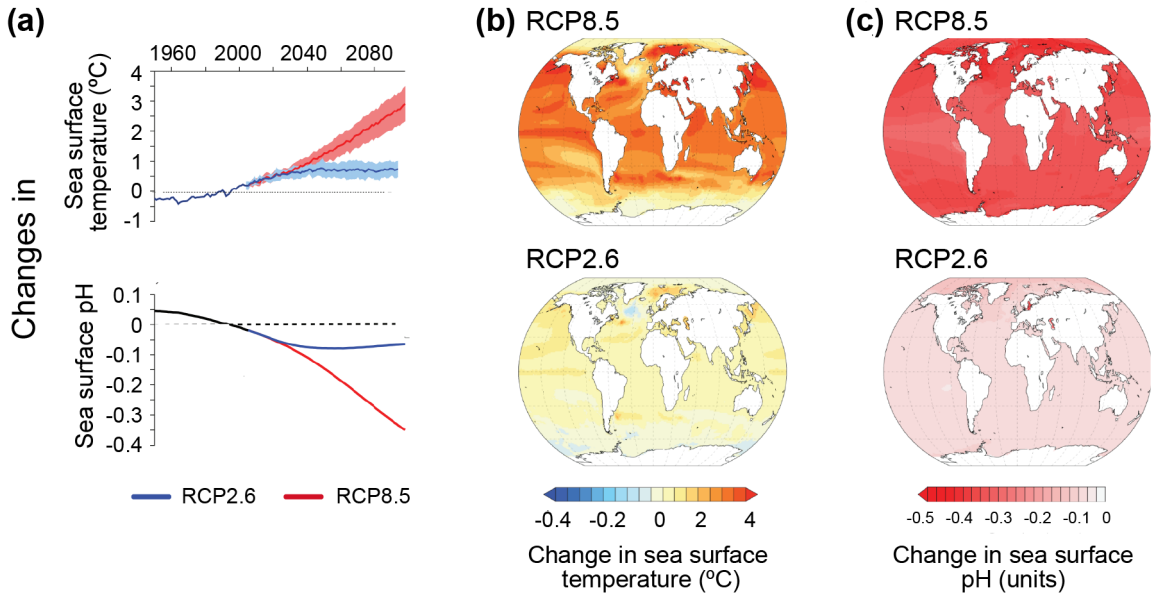

Figure 1.2. Changes in global sea surface temperature and pH over the industrial period and the current century. Projected temperature and pH are based on the current high-carbon-emission business-as-usual scenario (Representative Concentration Pathway 8.5; RCP8.5) and the stringent emission-mitigation scenario (RCP2.6) proposed by the Intergovernmental Panel on Climate Change (IPCC) (IPCC 2013). **(a)** Global mean changes in sea surface temperature and pH (total pH scale). Maps show the projected changes in sea surface temperature **(b)** and pH **(c)** by the end of the 21st century according to each scenario. Adapted from Gattuso *et al.* (2015).

1.2 EFFECTS OF CLIMATE-CHANGE STRESSORS ON CALCIFYING MARINE ORGANISMS

Temperature and carbonate chemistry (including pCO_2, pH and calcium carbonate saturation) are considered the most important climate change variables affecting the distribution, physiological performance, morphology and behaviour of marine organisms, especially calcifying species (Brierley & Kingsford 2009; Pörtner 2010; Pörtner 2008; Pörtner & Knust 2007). Temperature is a fundamental factor in regulating biological processes. It plays a central role in energy metabolism by governing enzymatic activities, molecular diffusion and transmembrane transport (Hochachka & Somero 2002). Even slight increases in temperature have been shown to produce substantial impacts on the performance of different marine species (Deutsch *et al.* 2015; Kroeker *et al.* 2013). For instance, an increase of only 0.5 °C (relative to the maximum temperature currently experienced in nature) makes the marine crab *Petrolisthes cinctipes* reach its upper critical temperature, beyond which its cardiac function collapses (Stillman 2002). At its extremes, increases in temperature beyond thermal tolerance limits cause a rapid deterioration of cellular processes and fitness of marine organisms. Temperature extremes have been implicated in mass mortality events, increased incidences of infectious disease, physiological limitation of oxygen supply (hypoxia), coral bleaching, invasion by exotic species, and changes in food chain dynamics (Burge *et al.* 2014; Edwards & Richardson 2004; Hoegh-Guldberg *et al.* 2007; O'Connor *et al.* 2009; Pörtner 2010; Pörtner & Knust 2007; Stachowicz *et al.* 2002).

OA is also a major threat to marine organisms in the face of climate change. Elevated concentrations of CO_2 (also known as hypercapnia) are particularly damaging to calcifying marine invertebrates due to the decreased availability of carbonate ions required for calcification and skeletogenesis (Fabry 2008; Gazeau *et al.* 2013; Orr *et al.* 2005). The deposition of $CaCO_3$ in a shell is a complex, biologically-controlled process. Shell growth relies

on an animal's ability to precipitate $CaCO_3$ from the seawater (carbonate minerals aragonite and calcite) onto an internal organic matrix, which acts as a basal structure for the rising shell (Marin *et al.* 2008). Even slight changes in seawater pH alter the calcification process making the shell smaller and weaker. Reduction of seawater pH by only 0.09 unit (relative to control) substantially decreased net calcification and caused net dissolution of the shells in the marine gastropods *Urosalpinx cinerea* (whelk) and *Littorina littorea* (periwinkle) (Ries *et al.* 2009). Similarly, in eastern oysters (*Crassostrea virginica*), small decreases in seawater pH (-0.15 pH unit relative to control) reduced the growth and thickness of their shells (Miller *et al.* 2009). Altered shell strength and architecture may lead marine calcifiers to become progressively vulnerable to predation and susceptible to environmental stress throughout this century (Gazeau *et al.* 2013; Hofmann *et al.* 2010; Kroeker *et al.* 2014). For instance, Sydney rock oysters (*Saccostrea glomerata*) from acidified environments (pH below 7.6 due to acid sulphate soil runoff) exhibit weaker shells and are consumed by the predatory snail *Morula marginalba* at a faster rate than oysters from control estuaries (Amaral *et al.* 2012). Such impacts may ultimately affect the survival and sustainability of entire populations.

Calcifying organisms are keystone species in many marine environments. They are responsible for the formation of complex habitat structures (*e.g.* mussel and oyster beds, and coral reefs) and water purification (due to their filter-feeding behaviour). Some calcifiers also serve as a major food source for other organisms in their ecosystem (Gutiérrez *et al.* 2003). Many calcifying species (*e.g.* mussels, edible and pearl oysters) also have a substantial economic value for fishing communities and aquaculture industries worldwide (Gazeau *et al.* 2013).

Oysters are particularly relevant to the broader environmental impacts of climate change because they are the predominant calcifiers in many coastal marine ecosystems (Gutiérrez *et al.* 2003). As well as other bivalves, oysters are highly dependent on seawater conditions for survival and growth due to their sessile and filter feeding lifestyle (Gosling 2003). Therefore,

any persistent perturbation in seawater quality is likely to have substantial effects on wild oyster populations (Gazeau *et al.* 2013; Przeslawski *et al.* 2008). Oysters also represent a valuable economic resource worldwide in addition to their ecological significance. Global aquaculture production of oysters is currently worth US$ 4 billion per year, with 4.9 million tonnes of oysters harvested worldwide in 2012 (FAO 2014). In Australia, the edible oyster industry is the oldest and most productive aquaculture activity (in value) in the state of New South Wales (NSW), where an average of 4.5 million dozen oysters are produced each year (Trenaman *et al.* 2015). Therefore, shifts in seawater physicochemical parameters have the potential to substantially impact the ecosystem goods and services, as well as the economic productivity provided by oysters (Doubleday *et al.* 2013; Gattuso *et al.* 2015).

In addition to its impacts on calcification, increased dissolved CO_2 is already known to have pervasive effects on other energy-demanding processes in marine calcifiers. Exposure to CO_2-driven OA disrupts a variety of physiological and cellular processes across a range of marine species and life-history stages. OA impacts fertilization, larval development and growth (Havenhand *et al.* 2008; Kurihara 2008), as well as different aspects of juvenile and adult performance (Gazeau *et al.* 2013; Harvey *et al.* 2013; Kroeker *et al.* 2013). Many marine species have complex life histories and each life stage often responds differently to shifting environmental conditions. In the context of OA, early life-history stages (embryos, larvae and juveniles) seem to be particularly sensitive to reducing seawater pH, generally experiencing abnormal development and skeletal dissolution (Byrne 2011; Parker *et al.* 2013; Ross *et al.* 2011). Compromised performance among a specific life-history stage could determine the sensitivity of the species as a whole, producing negative consequences for adult populations and marine communities (Byrne 2011; Kroeker *et al.* 2013).

OA is known to affect the acid–base balance and energy metabolism of numerous calcifying organisms, including oysters (Fabry 2008; Pörtner 2008; Pörtner *et al.* 2004). Elevated concentrations of CO_2 can disrupt internal pH balance, causing acidosis. Unless pH

disturbance is mitigated, it can lead to metabolic shifts and cause cellular dysfunction (Pörtner 2008). Acid-base balance can be preserved or restored though passive or active pH buffering. Passive buffering occurs through the binding of excess respiratory protons (originating from CO_2 hydration) on partially protonated regions of proteins from internal fluids. In contrast, active pH buffering is mediated by energetically costly ion exchange mechanisms and proton pumps (Pörtner *et al.* 2004).

Molluscs with limited mobility or sessile life styles and low metabolic rates generally show less efficient ion-regulatory and non-bicarbonate buffering mechanisms. As a result, their capacity to compensate for changes in acid–base balance derived from OA is limited (Melzner *et al.* 2009). Extracellular and intracellular acid–base variables are key mediators of energy metabolism. Therefore, uncompensated extracellular pH is often linked to substantial changes in the metabolic rate of molluscs (Pörtner 2008). For instance, exposure of the mussel *Mytilus galloprovincialis* to elevated CO_2 caused a permanent decrease in hemolymph pH, reducing their metabolic rate and leading to slower shell growth (Michaelidis *et al.* 2005).

Disturbances in acid-base balance and energy metabolism resulting from OA can further lead to downstream impacts on the development, survival and immune responses of marine calcifiers (Fabry 2008; Gazeau *et al.* 2013; Kurihara 2008; Miller *et al.* 2009; Parker *et al.* 2013). Oysters (*C. virginica*) exhibited higher mortality rates and lower soft tissue growth following exposure to CO_2 stress (Dickinson *et al.* 2012). Elevated CO_2 has also been shown to alter hemocyte parameters in the clam *Chamela gallina* and the mussel *M. galloprovincialis*, including total hemocyte count and phagocytic activity (Matozzo *et al.* 2012). In addition, OA caused an increase in apoptosis (programmed cell death) and reactive oxygen species (ROS) production in hemocytes of *Crassostrea gigas* (Wang *et al.* 2016), and affected the adhesive capacity and lysozyme activity of hemocytes in the clam *Mercenaria mercenaria* and the oyster *C. virginica* (Ivanina *et al.* 2014).

The impacts of environmental stressors associated with climate change have also been explored at the macromolecular level. A number of studies have described the effects of rising seawater temperature and decreasing pH on subcellular processes involving protein and transcriptional regulation. Increasing temperature has been shown to cause substantial changes in the molecular processes of marine organisms (Tomanek 2014). Heat shock proteins (Hsp) are one of the protein families most affected by thermal stress in different species of calcifying marine organisms. In the Pacific oysters (*C. gigas*), exposure to temperature extremes (15 °C to 19 °C increases relative to current ambient conditions) induced significant increases (up to 2,000-fold) in Hsp expression (Farcy *et al.* 2009; Zhang *et al.* 2012). Acute heat stress (12 °C increase) has also been shown to induce changes in the transcription of numerous other genes involved in cellular homeostasis, cell proliferation and differentiation, as well as protein synthesis (Meistertzheim *et al.* 2007). These included genes encoding cystatin B, cathepsin L and ribosomal proteins (*e.g.* 60S ribosomal protein L10/QM). At the protein level, exposure of oysters (*C. gigas*) to temperature extremes (20 °C increase) enhanced the abundance of Hsp, and altered the concentrations of proteins involved in energy metabolism, calcium binding and immune responses (Zhang *et al.* 2015). Although informative, these studies have focused on exposure to acute heat stress (12 °C to 20 °C increases relative to ambient conditions). Such temperature extremes do not reflect near-future climate change scenarios, where projected seawater temperature increases range between 1.2 °C (RCP2.6) and 3.2 °C (RCP8.5) by the end of this century (Fig. 1.2) (IPCC 2013).

Proteomic and transcriptional approaches have also revealed the response of oysters to decreasing seawater pH. CO_2-driven OA (pH 7.3 to 7.9) has been shown to alter the intracellular concentrations of proteins involved in antioxidant defence, metabolism and the cytoskeleton in the oysters *C. gigas*, *C. virginica*, *Crassostrea hongkongensis* and *S. glomerata* (Dineshram *et al.* 2015; Dineshram *et al.* 2013; Dineshram *et al.* 2012; Thompson *et al.* 2015; Timmins-

Schiffman *et al.* 2014; Tomanek *et al.* 2011; Wei *et al.* 2015a). At the transcriptional level, *C. virginica* adults exhibited changes in the expression of genes associated with biomineralisation, stress and immune responses under elevated CO_2 conditions (pH 7.5 to 7.6) (Beniash *et al.* 2010; Ivanina *et al.* 2014).

Some studies have also evaluated the response of oysters to the combination of heat stress and CO_2-driven OA. Increasing temperature and OA are unlikely to operate independently as climate change progresses (Fabry 2008). Therefore, studies that examine the combination of thermal and CO_2 stresses are an important adjunct to the analysis of individual stressors. In this context, comprehensive transcriptomic and proteomic analyses were used to assess the response of the oysters *C. virginica* and *C. gigas* to a combination of elevated temperature and low pH (Chapman *et al.* 2011; Clark *et al.* 2013; Harney *et al.* 2016). These studies showed that OA and the combination of both OA and increased temperature have distinct effects on the intracellular systems of these oyster species. In *C. virginica*, OA enhanced the expression of genes involved in antioxidant and metabolic processes, whereas combined OA and increasing temperature primarily affected the expression of genes related to protein synthesis, cell growth and metabolic processes (Chapman *et al.* 2011). In *C. gigas*, low pH also increased the expression of antioxidant genes, as well as of genes involved in immune responses, while the combination of both pH and thermal stresses induced the expression of genes encoding protease inhibitors and cytoskeletal proteins (Clark *et al.* 2013). At the protein level, Harney *et al.* (2016) recently described the effects of OA and increasing temperature on *C. gigas* larvae. They found that OA affected the expression of proteins mainly involved in metabolism and development. The combination of both stressors also reduced the abundance of proteins associated with metabolism and increased the concentrations of those involved in oxidative stress.

Overall, findings from previous studies indicate that OA, increasing temperature and the combination of both stressors alter the status of critical subcellular functions in oysters.

Genes and their encoded proteins involved in oxidative stress and antioxidant defence, energy metabolism and the cytoskeleton are often affected by disturbances in seawater temperature and/or pH. These responses are consistent with recent integrative studies that analysed the effects of a range of environmental stressors (such as temperature extremes, hypoxia, salinity and infectious diseases) on the molecular profiles of marine invertebrates, including oysters (Anderson *et al.* 2015; Tomanek 2014; Zhang *et al.* 2012). These studies identified key similarities in the subcellular functions affected by a broad range of different environmental factors. Both Anderson *et al.* (2015) and Tomanek (2014) concluded that a common induced response may be elicited by multiple environmental perturbations. In this consensus model of induced stress responses, changes in environmental factors beyond tolerance limits affect the mitochondrial electron transport chain, increasing energy production to provide ATP for adaptive reactions to stress. While potentially beneficial, increased mitochondrial energy production necessarily generates reactive oxygen species (ROS). Excessive ROS can disrupt the cytoskeleton and mitochondrial membranes resulting in cell death through apoptosis unless the effects of ROS are neutralised by the antioxidant system and molecular chaperones (Fig. 1.3). In agreement with this model, sequencing of the Pacific oyster (*C. gigas*) genome revealed an expanded set of genes involved in stress responses, particularly those encoding heat shock proteins (Hsps), superoxide dismutases (SODs) and inhibitors of apoptosis (IAPs) (Fig. 1.3) (Zhang *et al.* 2012). Such enlarged repertoires seem to be associated with the capacity of oysters to cope with fluctuating environmental conditions that are naturally found in their habitats. Hence, differential regulation of antioxidant enzymes, chaperones and cytoskeletal proteins represents a key feature of inducible intracellular stress responses.

The molecular responses of marine calcifiers to OA and ocean warming are not yet fully described. However, they may involve the same set of subcellular systems that are affected by other forms of stress. If this is the case, adaptation of these biological processes may form the

basis of transgenerational changes in the performance and overall fitness observed in many marine calcifying species responding to projected future ocean temperature and pH.

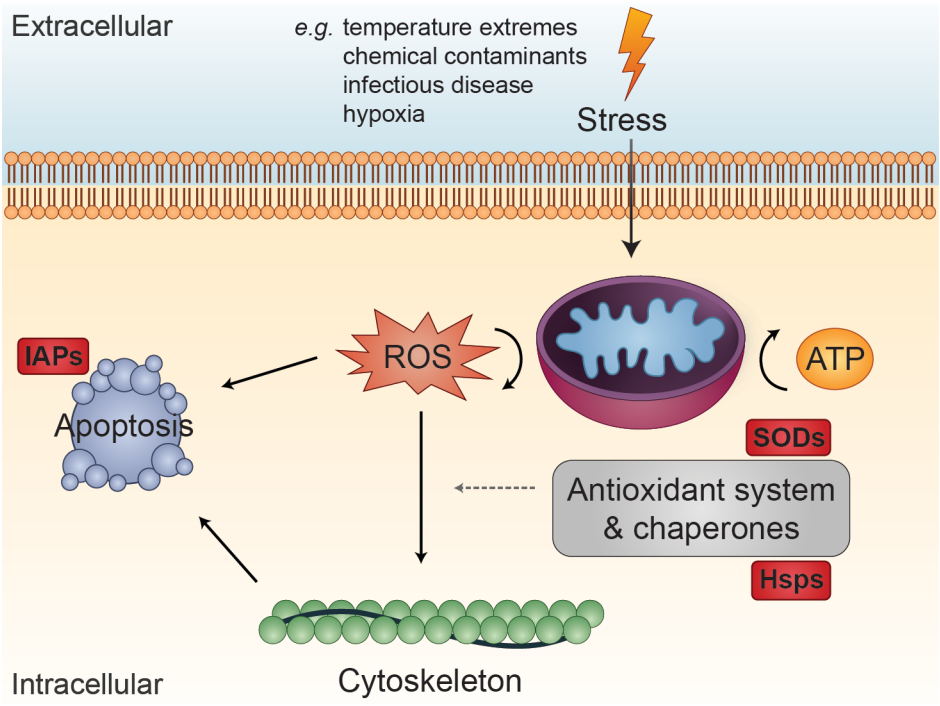

Figure 1.3. Schematic representation of the general stress response in oysters. In this model, environmental stress (biotic and abiotic factors) affects mitochondrial function, increasing energy production (ATP) and generating reactive oxygen species (ROS). Failure to control and neutralise excess ROS (via the antioxidant system or molecular chaperones) can lead to disruption of the cytoskeleton and mitochondrial membranes resulting in cell death through apoptosis. Red boxes associated with some biological functions indicate gene families that are expanded in the Pacific oyster (*Crassostrea gigas*) genome. IAPs: genes coding for inhibitor of apoptosis proteins; SODs: superoxide dismutases; Hsps: heat shock proteins. Adapted from Anderson *et al.* (2015) and Zhang *et al.* (2012).

1.3 ADAPTATION OF MARINE CALCIFIERS TO STRESSFUL ENVIRONMENTAL CONDITIONS

The ability of sessile marine organisms to grow and thrive in the near future will depend, at least in part, on their capacity to maintain and regulate their cellular and intracellular processes under modified environmental conditions (Evans & Hofmann 2012; Franks &

Hoffmann 2012). At the most fundamental level, these adaptive responses are likely to involve the differential regulation of specific molecular systems responsible for preserving cellular homeostasis, as well as preventing and/or repairing cellular and macromolecular damage (Evans & Hofmann 2012; Pörtner 2008; Somero 2010; Zhang *et al.* 2016).

The response of organisms to shifting environmental conditions can occur through both genetic (*i.e.* adaptation) and non-genetic (*i.e.* acclimation) processes. Genetic adaptation is defined in this thesis as a change in phenotype over time driven by natural (Darwinian) selection that involves heritable changes in allele frequencies. This is dependent on differential reproductive fitness within the parental generation. By contrast, acclimation (both natural acclimatisation and laboratory-induced acclimation) is defined here as a phenotypic response to variation in the environment that affects physiology, morphology and/or behaviour, but does not involve a classical Darwinian genetic change (Fig. 1.4a). Acclimation is mediated by the existing capacities of organisms to modify their phenotype to maintain fitness under new environmental conditions (*i.e.* phenotypic plasticity). Changes resulting from acclimation involve epigenetic processes and/or maternal provisioning (parental effects) and so can be transmitted between generations, although they are not permanently heritable (Danchin *et al.* 2011; Mirouze & Paszkowski 2011; Parker *et al.* 2015). Acclimation often represents a more rapid response to environmental disturbance compared with Darwinian genetic adaptation, and it may persist for several generations (Evans & Hofmann 2012; Sunday *et al.* 2014; van Oppen *et al.* 2015).

Acclimation can be further categorised into three groups: reversible, developmental and transgenerational (Fig. 1.4b). Reversible acclimation occurs over short periods of time (days to months) in response to daily and seasonal environmental fluctuations, often within a life stage, providing no trait evolution from one generation to the next. Developmental acclimation occurs when exposure of an early life-history stage to a stressful condition affects the physiological performance of juveniles and/or adults in the same environment. Such phenotypic carry-over

effects are also often limited to the life span of an organism. In contrast, transgenerational acclimation occurs across consecutive generations through non-genetic inheritance (parental effects or epigenetic processes), whereby the environment experienced by parents mediates the performance of offspring (and potentially following generations) in the same environment (Sunday *et al.* 2014).

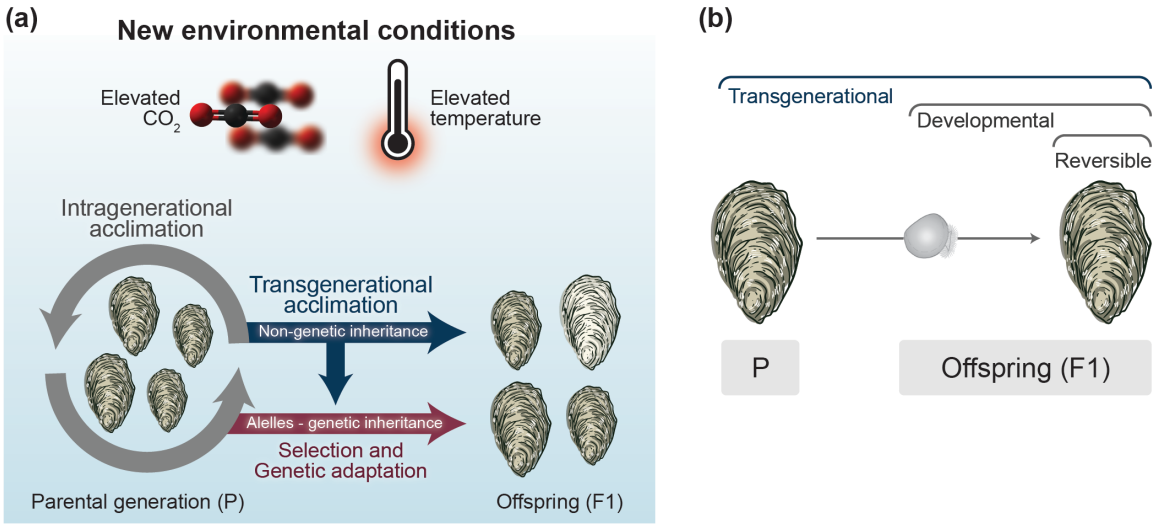

Figure 1.4. Mechanisms for adaptive changes in response to shifting environmental conditions. **(a)** Schematic diagram showing intragenerational acclimation through non-genetic processes, as well as transgenerational non-genetic (acclimation) and classical genetic (Darwinian; natural selection) inheritance. Adapted from van Oppen *et al.* (2015). **(b)** Categories of acclimation to environmental stress. Reversible and developmental acclimations correspond to subtypes of intragenerational acclimation. Adapted from Sunday *et al.* (2014).

Phenotypic plasticity in marine organisms is revealed by the broad variability of responses to OA and increasing temperature that have been observed between species and even between populations within a species. The latter suggests that inherent genetic variability and phenotypic plasticity within species may provide the requisite foundation for acclimation or adaptation to climate change variables (Evans & Hofmann 2012; Harley *et al.* 2006). Some marine species have a notable capacity for acclimation or adaptation to stressful environmental conditions, including those associated with climate change (Donelson *et al.* 2012; Dupont *et al.*

2013; Parker *et al.* 2015; Sanford & Kelly 2011; Schoville *et al.* 2012). Coastal organisms, including molluscs, have long evolutionary histories of exposure to rapid and substantial fluctuations in seawater variables. Hence, these organisms may have greater capacity for adaptation to stressful environmental conditions than those living in more stable oceanic environments. This capacity is supported by genomic analyses. For instance, the Pacific oyster (*C. gigas*) genome harbours an expanded repertoire of stress response genes, such as extensive sets of genes encoding heat shock proteins (Hsps), inhibitor of apoptosis (IAPs) and superoxide dismutases (SODs) (Fig. 1.3) (Zhang *et al.* 2012). By corollary, physiological tolerance to low pH and carbonate saturation have already been reported in some species. Molluscs originating from environments that are naturally enriched in CO_2 do not exhibit the negative physiological effects typically induced by OA when they are experimentally exposed to elevated CO_2 (Miller *et al.* 2009; Thomsen & Melzner 2010). Such compensatory or adaptive responses could have evolved gradually as a result of the continuous exposures of these organisms to disturbed conditions. Therefore, coastal regions represent potential hotspots for local adaptation to OA (Hofmann *et al.* 2010).

This agrees with recent studies investigating the effects of transgenerational exposure to OA or increased temperature in the laboratory. These experimental studies have also revealed the capacity of some marine organisms to acclimate or adapt across generations to the adverse effects of climate change. Assessing responses to OA and increased temperature across life-history transitions is extremely challenging, particularly for calcifying metazoans with long generation times (months to years) (*e.g.* molluscs and corals). Despite the difficulties, there are reports of altered larval or adult performance following transgenerational conditioning to OA in fish (Allan *et al.* 2014; Miller *et al.* 2012; Schade *et al.* 2014; Welch *et al.* 2014), corals (Putnam & Gates 2015), sea urchins (Dupont *et al.* 2013; Kelly *et al.* 2013; Suckling *et al.* 2015), mussels (Sunday *et al.* 2011) and oysters (Parker *et al.* 2015; Parker *et al.* 2012). In the context of ocean warming, transgenerational exposure to thermal stress has been performed in

fish (Donelson *et al.* 2012; Muñoz *et al.* 2015; Veilleux *et al.* 2015), corals (Putnam & Gates 2015) and sea urchins (Kelly *et al.* 2013; Suckling *et al.* 2015).

Most studies of transgenerational exposure have shown that the physiological impacts of OA and elevated temperature are ameliorated by conditioning parents to these stresses (Ross *et al.* 2016). For instance, larvae, juveniles and adults of Sydney rock oysters (*S. glomerata*) exposed to CO_2 stress over two consecutive generations are able to cope better with OA than non-exposed oysters or oysters exposed to elevated CO_2 over a single generation (Parker *et al.* 2015; Parker *et al.* 2012). CO_2-conditioned oysters exhibited faster growth, lower percentages of abnormality during development and a greater capacity to regulate their extracellular pH following exposure to elevated CO_2. These findings indicate that the environment experienced by parents mediates the response of offspring. As such, larvae and juveniles bred from CO_2- or temperature-conditioned parents are primed to exhibit improved performance when exposed to the same stressful environment (Sunday *et al.* 2014).

The majority of existing studies indicate that transgenerational conditioning to OA or increasing temperature is not linked to Darwinian natural selection (genetic adaptation) since the experimental conditions did not result in mortality (or overt changes in reproductive fitness) among parental groups. However, classical genetic adaptation cannot be entirely ruled out. The design of some studies included exposing both parents and their larvae to elevated CO_2 (*e.g.* Miller *et al.* 2012; Parker *et al.* 2015; Parker *et al.* 2012). In these studies, the differential reproductive success necessary for genetic adaptation could have gone undetected in early life-history stages. The exposure of gametes, eggs and larvae to elevated CO_2 may have actively selected those with beneficial genotypes, resulting in classical Darwinian adaptation.

Even though classical adaptation cannot be discounted, acclimation through non-genetic inheritance, involving nutritional, somatic, cytoplasmic or epigenetic transfers between generations, could explain the results of many transgenerational studies (Fig. 1.4) (Bonduriansky *et al.* 2012; Sunday *et al.* 2014). Epigenetics refers to inherited phenotypic

variation that is not due to changes in nucleotide sequence. Examples of epigenetic mechanisms include DNA methylation, post-translational modifications of histones, chromatin remodelling and biogenesis of small non-coding RNAs. These processes can activate, reduce or completely silence gene expression without altering the DNA sequence (Danchin *et al.* 2011). Such modifications can produce substantial effects in the performance and overall fitness, and may even contribute to rapid adaptive evolution (Danchin *et al.* 2011; Somero 2010).

Regardless of the mechanism by which transgenerational change occurs, such modifications of phenotype are likely to involve large-scale rearrangements in gene and protein networks (Franks & Hoffmann 2012; Somero 2010). Veilleux *et al.* (2015) recently described the molecular mechanisms associated with transgenerational conditioning of a reef fish (*Acanthochromis polyacanthus*) to ocean warming. Transgenerational exposure to thermal stress (1.5 °C and 3 °C increase relative to ambient temperature) enhanced the expression of genes involved in metabolism, and immune and stress responses, suggesting that elevated temperature alters constitutive energy production and allocation in this species. This evidence suggests that the potential for heritable responses to climate change across generations may lie in the capacity of marine organisms to modify transcriptional processes in the face of temperature and CO_2 increases (Somero 2010; Veilleux *et al.* 2015).

Although beneficial in the context of the selective stressor, transgenerational change may also lead to adaptive trade-offs with long-term consequences for population viability. The higher maintenance costs for marine organisms under conditions of acidifying oceans could result in energy budget constraints for other energy-demanding processes. Metabolic depression is a common consequence of uncompensated extracellular pH during hypercapnia for some marine organisms (Pörtner 2008). However, increases in metabolic activity in response to elevated CO_2 have been reported in different oyster species (Beniash *et al.* 2010; Lannig *et al.* 2010; Parker *et al.* 2012). Such increased metabolic rates may represent an adaptive strategy for tolerance to elevated CO_2. Melzner *et al.* (2009) showed that many

organisms with higher (specific) metabolic rates (*e.g.* fish, cephalopods, decapod crustaceans) appear to be less affected by CO_2 stress. This may be due to the highly efficient ion-regulatory machinery for CO_2 excretion and acid-base regulation that is associated with the high levels of mobility/activity in these organisms (Melzner *et al.* 2009). The increased metabolic activity observed in oysters in response to CO_2 stress (in spite of their sessile lifestyle) may contribute to pH compensation and to the different levels of tolerance found in these animals.

Some evidence also suggests energy allocation trade-offs between climate-change resilience and other physiological energy-demanding processes (Holcomb *et al.* 2010; Parker *et al.* 2015; Pedersen *et al.* 2014; Sunday *et al.* 2014; Thomsen *et al.* 2013). For example, maintenance of pH balance in CO_2-enriched environments can divert energy from other fundamental processes, such as reproduction and immune responses (Kelly & Hofmann 2013; Pörtner *et al.* 2004). When food is abundant, some species of molluscs seem to be able to acquire the additional energy necessary to regulate pH balance and calcification by increasing feeding rates, without compromising growth and reproduction (Melzner *et al.* 2011; Thomsen *et al.* 2013). However, under food shortage, energy limitation often results in either reduced calcification or the maintenance of calcification processes at the cost of growth, reproduction and/or other processes (Kroeker *et al.* 2014).

Hence, it is unclear whether resilience to a specific trait and the costs associated with it could compromise the capacity of the organism to cope with other forms of stress. Genetic correlations between traits and evolutionary trade-offs between environmental drivers can have major impacts on how evolution proceeds (Sunday *et al.* 2014). Despite this, it is widely recognised that both acclimation and adaptation can help organisms to persist as environmental changes accumulate and accelerate. Understanding the intracellular basis and interactions between these processes will be critical for predicting evolutionary responses to climate change.

1.4 SELECTIVE BREEDING AS A STRATEGY TO MITIGATE CLIMATE CHANGE IMPACTS

The evident capacity of some marine species for acclimation or adaptation to climate change variables suggests that selective breeding programs represent viable management strategies in some circumstances (Doubleday *et al.* 2013; van Oppen *et al.* 2015). For instance, they could be used to future proof aquaculture industries or introduce beneficial traits into wild populations, helping marine species to persist in a rapidly changing ocean. Traditionally, breeding programs for organisms such as oysters have selected parents with specific phenotypic traits (such as large body size or disease resistance) to found the next generation (Conner 2003). Some evidence suggests that this approach can be used to diminish sensitivity to OA and rising temperature. Genetically distinct populations of oysters produced through selective breeding differ in their responses to climate-change stressors compared to wild type (non-selected) populations. Among Pacific oysters (*C. gigas*), populations selectively bred for tolerance to acute heat stress (higher survival rates during experimentally-induced summer mortality events) show distinctive transcriptional profiles relative to populations sensitive to rising temperatures. The heat-tolerant oyster population had lower expression levels of Hsp27, collagen, peroxinectin and S-crystallin, and higher expression of cystatin B following exposure to thermal stress (Lang *et al.* 2009).

Similarly, resilience to elevated CO_2 has been observed among selectively bred Sydney rock oysters (*S. glomerata*). Sydney rock oysters are an Australian iconic species found in estuarine and coastal areas along Southeast Australia, where they form the basis of a long-established aquaculture industry (Fig. 1.5). This species has been subjected to mass selection for fast growth and resistance to the most significant oyster diseases in Australia (QX disease and winter mortality) over the past 25 years (Dove *et al.* 2013). Surprisingly, seven generations of artificial selection for these traits appear to have coincidentally resulted in resilience against

CO_2-driven OA. One of the selectively bred populations, the B2 breeding line, stands out for their unique capacity to better cope with the effects of CO_2 stress (Parker *et al.* 2012; Parker *et al.* 2011). Oysters from this population are far less affected by CO_2 stress than wild type (non-selected) oysters at the physiological level (Parker *et al.* 2012; Parker *et al.* 2011). Larvae from B2 line oysters grow faster (2.6-fold) and have higher survival rates (1.7-fold) than wild type larvae following exposure to elevated CO_2 (pH 7.84 to 7.9) (Fig. 1.6a) (Parker *et al.* 2012; Parker *et al.* 2011). In addition, B2 adults have higher standard metabolic rates (SMR) than wild type oysters under ambient conditions, and this difference is further amplified by CO_2 exposure (pH 7.9) (Fig. 1.6b) (Parker *et al.* 2012).

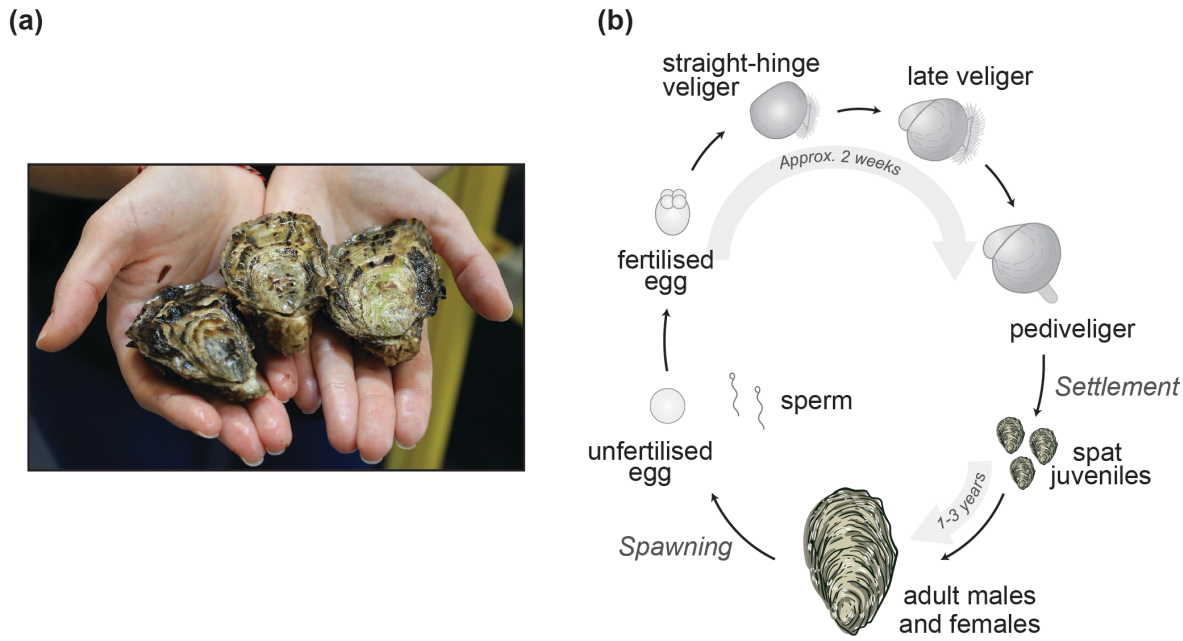

Figure 1.5. Life cycle of Sydney rock oysters. **(a)** Examples of adult Sydney rock oysters (*Saccostrea glomerata*). Photo credit: Gabriel Fonseca. **(b)** Simplified life cycle of Sydney rock oysters. Diagram adapted from Karen R. Swanson/COSEE SE/NSF.

Differential responses to OA between the B2 and non-selected populations of Sydney rock oysters are also evident at the protein level. Thompson *et al.* (2015) recently showed that the majority of differentially regulated proteins are down-regulated in B2 oysters but up-regulated in wild type oysters after exposure to elevated CO_2 (pH 7.84). Proteins involved in

the mitochondrial electron transport chain and oxidative stress were among the most affected by CO_2 stress. Such findings suggest that the distinctive performance of B2 oysters might derive from their capacity to regulate metabolic activities and control oxidative stress in face of pathogens or elevated CO_2, probably through differential regulation of molecules involved in these processes. This fits with recent models (see Section 1.2) which predict that acclimation or adaptation to one form of environmental stress (disease in the context of the B2 line) may confer resilience against a range of other stressors (Anderson *et al.* 2015).

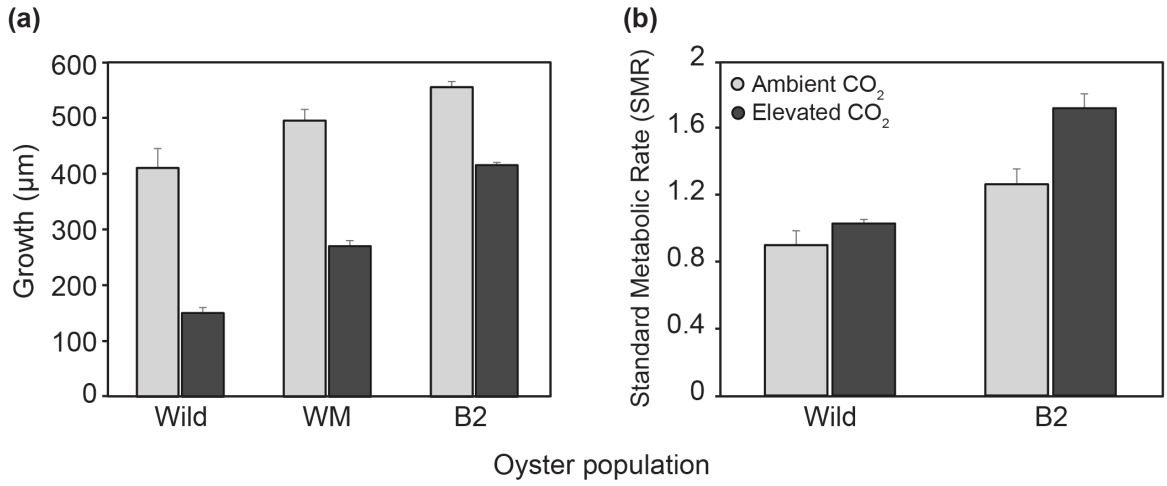

Figure 1.6. Differential performance of Sydney rock oyster breeding lines to elevated CO_2. **(a) S**hell growth (Mean ± SEM) of spat after 4 days at ambient (pH 8.2) and elevated CO_2 (pH 7.84). Adapted from Parker *et al.* (2011). **(b)** Standard metabolic rate (mg O_2 g^{-1} dry tissue mass h^{-1}) (Mean ± SEM) of adults following 5-week exposure to ambient (pH 8.2) and elevated CO_2 (pH 7.9). Adapted from Parker *et al.* (2012). Wild: wild type, non-selected oysters; WM: fast growing, Winter Mortality resistant oysters; B2: fast growing, QX and Winter Mortality resistant oysters.

The breeding program for Sydney rock oysters (*S. glomerata*) was developed by the New South Wales (NSW) Department of Primary Industries (DPI) across different sites in the Georges River (NSW, Australia). It was established in 1990 in an effort to overcome a gradual decline in production (40% decline since 1970) (Nell *et al.* 2000). Oysters were initially selected based on their ability to grow faster than wild caught oysters and their resistance to

winter mortality syndrome (causative agent to be confirmed, currently under investigation). Substantial losses (up to 97% mortality) of selected line oysters resulting from the occurrence of QX disease outbreaks (causative agent: *Marteilia sydneyi*) were observed in the Georges River between 1994 and 1996 (Nell *et al.* 2000). The program was then modified to include breeding for resistance to both infectious diseases, winter mortality and QX disease (Fig. 1.7). The base population for the mass selected breeding lines comprised 25 wild type oysters from each of the four major Sydney rock oyster growing estuaries in NSW: Wallis Lake, Port Stephens, Hawkesbury River and Georges River (100 oysters total). Upon reaching spat stage (mean shell height = 12 mm) (Fig. 1.5b), oysters were divided into four breeding lines that were reared in different sites in the Georges River. Breeding was based on mass selection, such that for each successive generation, the largest survivors were selected for breeding (Fig. 1.7) (Nell *et al.* 2000).

Of the four populations selected for fast growth and disease resistance in the Georges River, two also showed substantial levels of tolerance to elevated CO_2 (Figs. 1.6 and 1.7) (Parker *et al.* 2011). These CO_2-resilient oysters comprised a winter mortality-resistant line held at Quibray Bay (downstream of the Georges River) and a winter mortality- and QX-resistant line (the B2 line) held at Lime Kiln Bar (upstream of the Georges River). Both of these lines exhibited less pronounced changes in shell growth under OA when compared to wild type (non-selected, control) oysters. Oysters from the winter mortality-resistant and B2 lines showed reductions of 45% and 25% in shell growth when exposed to elevated CO_2, compared to a 64% reduction in wild type oysters (Fig. 1.6a) (Parker *et al.* 2011). Additional analyses of larval development and metabolic rate (SMR) in adults confirmed the distinctive resilience of B2 oysters, in particular, to OA (Fig. 1.6b) (Parker *et al.* 2012).

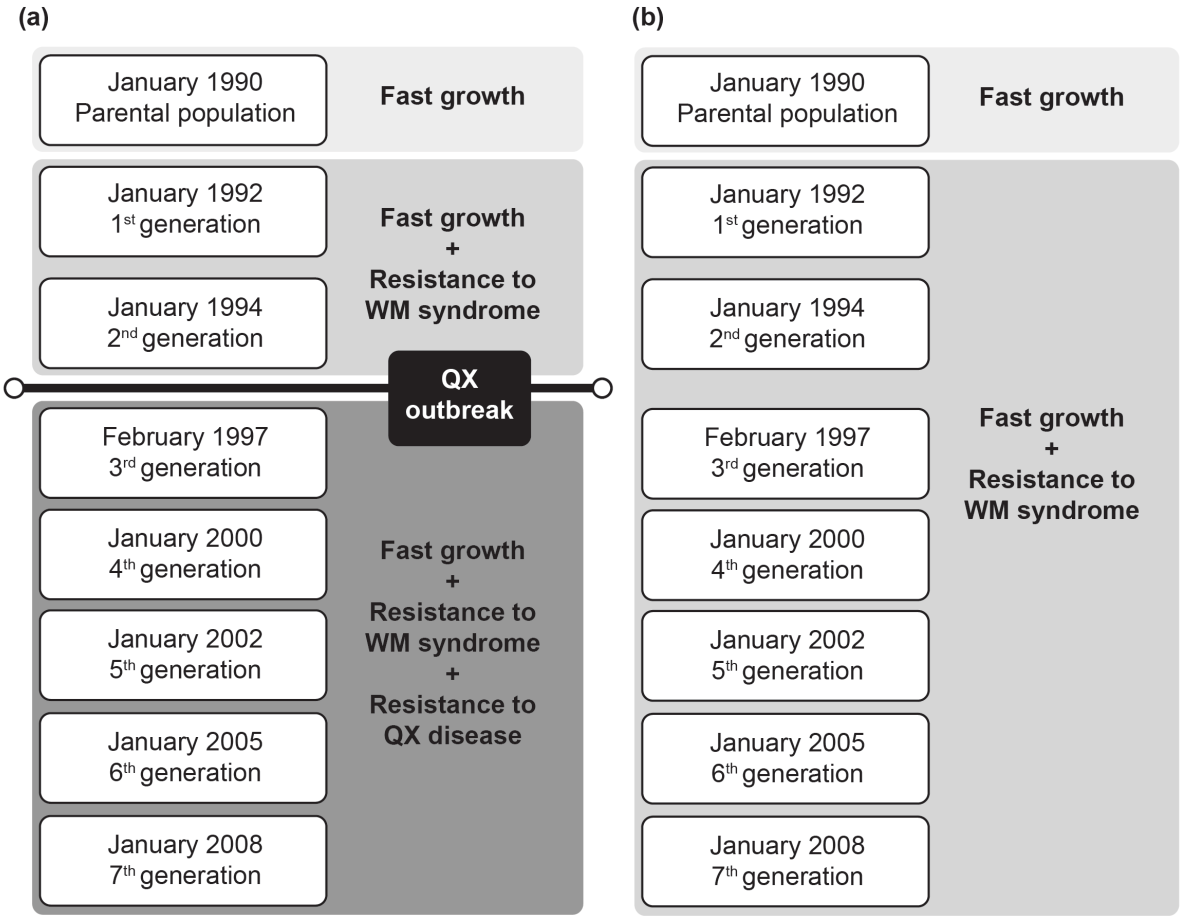

Figure 1.7. Selective breeding program for fast growth and disease resistance of Sydney rock oysters. Diagram summarises the breeding period, generation and selected traits for the B2 **(a)** and Winter Mortality **(b)** breeding lines. Selective bred oysters show faster growth and resistance to Winter Mortality (WM) syndrome and/or QX disease (compared to wild type, non-selected oysters). This program has been developed by the NSW Department of Primary Industries across different sites in the Georges River (NSW, Australia). Adapted from Nell (2006).

1.5 TOOLS FOR THE DISCOVERY OF STRESS RESPONSES

This thesis exploits the unique genetic resource provided by the opportunistic resilience of selectively bred Sydney rock oysters to OA. It aims to identify the molecular processes underlying their distinctive performance to CO_2 stress by investigating their intracellular responses at the transcriptional, proteomic and cytological levels. The rationale for studying selectively bred oysters at the molecular level is that changes in physiological parameters and performance in response to climate change (*e.g.* reduced calcification, delayed growth,

mortality) are a reflection of large-scale, multifactorial rearrangements at the intracellular level (Evans & Hofmann 2012; Somero 2010). As such, marine organisms respond to environmental stress through differential regulation of genes and proteins, which ultimately result in changes in cellular functions, physiological parameters and overall performance. Hence, cellular and molecular analyses need to be performed in order to understand the fundamental basis of climate change resilience, and to elucidate interactions between organisms and their environment. This understanding will be crucial to ensuring the ecological and economic health of rapidly changing oceans.

Rapid advances in technology have expanded the repertoire of experimental approaches available to investigate subcellular responses to environmental stress in marine organisms. These quantitative and qualitative tools can be applied at the level of individual genes and proteins, or at a global scale ('omics). The relevant technologies include analysis of DNA, RNA, proteins and metabolites. Many different techniques for assessing these molecules and compounds have been developed (Table 1.1). The most recent of these include global protein and transcription analyses, through shotgun proteomics and next-generation sequencing (NGS), respectively. Analyses of gene expression and protein abundance represent powerful tools for high-throughput phenotyping of marine organisms responding to modified environmental conditions, providing an essential link between phenotype and genotype. This link between desirable phenotypic characteristics and the genome enables the application of transcriptomic and proteomics approaches in the identification of key targets for marker-assisted selective breeding programs and for the development of conservation strategies (Evans & Hofmann 2012; Franks & Hoffmann 2012; Kelly & Hofmann 2013).

Transcriptional analyses represent an extremely useful approach to examine the regulation of gene expression in response to specific environmental stressors (Kelly & Hofmann 2013). Changes in the transcriptome can be evaluated using expressed sequence tag (EST) libraries, suppression subtractive hybridization (SSH), real-time quantitative PCR

(qPCR), microarray profiling and, more recently, next-generation RNA sequencing (RNA-seq). For instance, transcriptomic profiling using microarrays of native and invasive mussels (*Mytilus trossulus* and *M. galloprovincialis*, respectively) revealed genes that were differentially expressed in response to thermal stress and potentially responsible for the success of the invasive species in a warming ocean (Lockwood *et al.* 2010). The advent of high-throughput next-generation sequencing (NGS) technologies has allowed deeper genome-wide surveys of the transcriptome. This methodology identifies the complete set of transcripts affected in organisms exposed to different environmental conditions. As such, NGS techniques have been essential in understanding the large-scale molecular rearrangements driven by exposure to stress (Evans & Hofmann 2012; Li *et al.* 2013; Suárez-Ulloa *et al.* 2013; Zhang *et al.* 2012). One early example of the utility of NGS technology was an extensive characterisation of the molecular responses of Pacific oysters (*C. gigas*) to a variety of environmental stressors (including temperature, salinity, air exposure and heavy-metal stress) (Table 1.1) (Zhang *et al.* 2012). That study identified an expanded set of genes that were highly responsive to those stressors, including some that reflected the genetic adaptation of oysters to highly fluctuating environments.

Table 1.1. Summary of high-throughput studies analysing the impacts of ocean acidification (CO_2) and warming (heat) on edible oysters. This list includes studies that have analysed gene expression or proteomic profiling at broad (> 50 genes or proteins) or global scales ('omics). Changes in CO_2 concentration and temperature are relative to control, ambient conditions.

Species	Stress	Approach	Reference
Crassostrea gigas	CO_2 (-0.5 pH units)	Proteomics (2D electrophoresis)	Dineshram *et al.* (2012)
Crassostrea gigas	CO_2 (up to -0.7 pH units)	Proteomics (shotgun)	Timmins-Schiffman *et al.* (2014)
Crassostrea gigas	CO_2 (-0.5 pH units)	Proteomics (shotgun)	Dineshram *et al.* (2015)
Crassostrea gigas	CO_2 (up to -0.6 pH units)	Proteomics (2D electrophoresis)	Wei *et al.* (2015a) and Wei *et al.* (2015b)

Table 1.1 continued

Species	Stress	Approach	Reference
Crassostrea gigas	CO_2 (-0.4 pH units) Heat (+5 °C)	Transcriptomics (qPCR and NGS)	Clark *et al.* (2013)
Crassostrea gigas	CO_2 (-0.2 pH units) Heat (+2 °C)	Proteomics (2D electrophoresis)	Harney *et al.* (2016)
Crassostrea gigas	Heat (+12 °C)[a]	Transcriptomics (SSH and qPCR)	Meistertzheim *et al.* (2007)
Crassostrea gigas	Heat (+29 °C) [a]	Transcriptomics (microarray)	Lang *et al.* (2009)
Crassostrea gigas	Heat (+15 °C) [a]	Transcriptomics (NGS)	Zhang *et al.* (2012) and Zhu *et al.* (2016)
Crassostrea gigas	Heat (+20 °C) [a]	Proteomics (shotgun)	Zhang *et al.* (2015)
Crassostrea hongkongensis	CO_2 (up to -0.6 pH units)	Proteomics (2D electrophoresis)	Dineshram *et al.* (2013)
Crassostrea hongkongensis	CO_2 (-0.5 pH units)	Proteomics (shotgun)	Dineshram *et al.* (2015)
Crassostrea virginica	CO_2 (-0.8 pH units)	Proteomics (2D electrophoresis)	Tomanek *et al.* (2011)
Crassostrea virginica	CO_2 (up to -0.8 pH units) Heat (up to +3 °C)	Transcriptomics (microarray)	Chapman *et al.* (2011)
Saccostrea glomerata	CO_2 (-0.4 pH units)	Proteomics (2D electrophoresis)	Thompson *et al.* (2015)
Saccostrea glomerata	CO_2 (-0.4 pH units)	Proteomics (2D electrophoresis)	Thompson *et al.* (2016)

[a] Exposures to acute heat stress (+12 to 29 °C relative to control conditions). Such extreme conditions do not represent realistic near-future scenarios for ocean warming.

Proteomics provides a broad picture of how proteins respond to stressful environmental conditions (Tomanek 2014; Vogel & Marcotte 2012). Global protein analysis can be performed using shotgun (mass spectrometry-based) or two-dimensional (2D) electrophoresis. It is a useful complement to transcriptomic approaches because it relates more directly to intracellular

function (Altelaar *et al.* 2013). Changes in gene expression may not always result in changes in protein expression due to post-transcriptional modifications, translational regulation and protein degradation (Hegde *et al.* 2003). Likewise, altered intracellular function can arise from complex protein-protein interactions that are not necessarily related to transcription. Thus, measurements of gene expression alone do not always reflect physiological outcomes, while proteins promote functional cellular changes and so can more directly reveal impacts at the phenotypic level (Vogel & Marcotte 2012).

Both high-throughput transcriptomics and proteomics rely heavily on genome sequencing to provide valuable information on target molecules, as well as insights into adaptation and evolution. The recent sequencing of the Pacific oyster (*C. gigas*) genome revealed that this species is notable for its proliferation of genes involved in stress responses (Fig. 1.3) (Zhang *et al.* 2012). Such an expanded repertoire of stress-related genes could provide the basis for rapid evolutionary changes in response to environmental disturbance in oysters.

Even though transcriptomics and proteomics provide a detailed picture of the dynamics of response within a cell, cytology is also required to confirm the cellular alterations that can be predicted from those molecular approaches. The assessment of cell structure in response to stress can reveal the functional outcomes derived from complex arrays of altered genes and proteins (Weis *et al.* 2008). By exploring and integrating innovative approaches (transcriptomics, proteomics and cytology), it is possible to gain an expanded, more comprehensive view of the changes in subcellular networks that occur when organisms experience environmental disturbances. Collectively, analyses of these different levels of biological organisation accurately reflect an organism's adaptive response to the environment.

1.6 RESEARCH AIMS

Given the rapid rate of environmental change, understanding the phenotypic plasticity of contemporary populations and their potential to acclimate or adapt to projected near-future conditions is crucial to minimise the effects of OA and ocean warming. This thesis explores the effects of climate change on populations of Sydney rock oysters (*S. glomerata*) that show different levels of tolerance to CO_2 stress. The availability of these populations is a unique resource because they appear to be pre-adapted for climate change resilience through selective breeding for other characteristics (fast growth and disease resistance). Comparative analyses using these unique populations may allow the identification of molecular processes involved in acclimation or adaptation to climate change. Even though numerous studies have assessed the effects of climate change variables on other oyster species (Table 1.1), only one has compared resilient and wild type populations within the same species at the intracellular level. This one exception is a study by Thompson *et al.* (2015), who described differences in protein abundance between two populations of *S. glomerata* responding to elevated CO_2.

In my thesis, the intracellular processes driving the potential resilience of selectively bred oysters are investigated through a combination of cellular and molecular techniques, including confocal microscopy, transcriptomics (qPCR and RNA sequencing) and proteomics (2D electrophoresis). This project was conducted sequentially so each chapter corresponds to an independent experimental exposure. This resulted in different samples and slightly different experimental conditions, particularly for the elevated CO_2 treatments (625-933 μatm pCO_2, pH 7.70-7.87). Oysters were assessed in response to a single exposure to elevated CO_2 (Chapter 2) or following transgenerational conditioning to this stress (Chapters 3 and 4). CO_2-resilient oysters were also exposed to a combination of elevated CO_2 and elevated temperature in order to analyse the concurrent effects of OA and ocean warming, and to evaluate the potential

implications of CO_2 resilience for responses to another stressor (Chapter 5) (Fig. 1.8). The objectives of these studies are to:

- Determine the differential effects of elevated CO_2 on the intracellular functions of selectively bred and wild type oysters (Chapter 2).
- Evaluate whether selectively bred and wild type oysters have the capacity for transgenerational conditioning to elevated CO_2, and whether this adaptive potential differs between populations (Chapter 3).
- Identify the molecular basis of the distinctive performance of selectively bred oysters to elevated CO_2 (Chapter 4).
- Characterise the transcriptional and proteomic responses of selectively bred oysters exposed to a combination of elevated CO_2 and elevated temperature (Chapter 5).

By meeting these objectives, the thesis provides novel insights into the intracellular processes underlying the response and tolerance of marine organisms to OA and ocean warming. It describes the integrative subcellular profiles of oysters responding to these environmental stressors and reveals the biological functions that may enable marine calcifiers to cope with such conditions in the face of climate change.

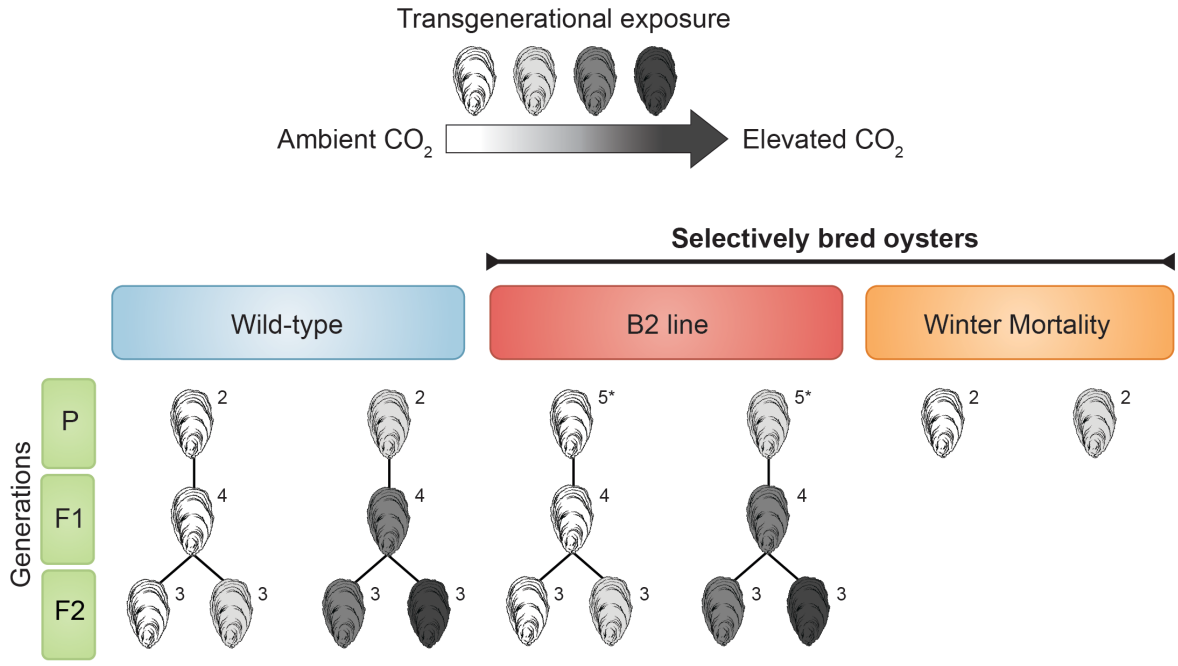

Figure 1.8. Summary of the experimental approach of this thesis. Different populations of Sydney rock oysters were exposed to elevated CO_2 over a single (parental; P) or multiple consecutive generations (parental to F1 and/or F1 to F2). Numbers associated with each oyster condition indicate the Chapter in this thesis where that analysis is reported. Chapter 5 (*) includes a single experimental exposure of B2 oysters to both elevated CO_2 and elevated temperature.

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CHAPTER 2

THE CAPACITY OF OYSTERS TO REGULATE INTRACELLULAR OXIDATIVE STRESS MAY BE KEY TO THEIR RESILIENCE AGAINST OCEAN ACIDIFICATION

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ABSTRACT

Marine calcifiers, such as oysters, are threatened by shifts in seawater chemistry resulting from climate change. However, the magnitude of impacts induced by climate change-associated stressors can vary substantially between species, and even between populations within a species. Understanding the intracellular basis of differential responses in genetically distinct populations can contribute to the development of strategies to mitigate the impacts of changing oceans. In the current study, we explored intracellular responses to ocean acidification in selectively bred and wild type populations of Sydney rock oysters (*Saccostrea glomerata*). We found that the two populations had markedly different subcellular responses to elevated CO_2 in terms of oxidative stress, as reflected by changes in mitochondrial integrity (mitochondrial membrane potential) and in the production of reactive oxygen species (ROS). Analysis of these intracellular parameters after 4 and 15 days of exposure to elevated CO_2 indicated that the onset of oxidative stress occurred earlier in the selectively bred oysters when compared to the wild type population. This may be due to an inherent capacity for increased intracellular energy production or adaptive energy reallocation in the selectively bred population. The differences observed in the oxidative stress responses between genetically distinct oyster populations may reveal candidate biological processes that underlie resilience or susceptibility to ocean acidification.

2.1 INTRODUCTION

The acidity and temperature of world's oceans are increasing as a result of climate change (IPCC 2013). Much of the anthropogenic carbon dioxide (CO_2) released into the atmosphere is absorbed by oceans, altering the fundamental chemistry of seawater. Elevated CO_2 concentrations are predicted to reduce the pH of the oceans from 8.1 to 7.8 and substantially alter the availability of carbonate ions by the year 2100 (IPCC 2013; Orr *et al.* 2005). Such near-future conditions can be particularly damaging to oysters because the production of their calcium carbonate shells is hampered by changes in ocean chemistry (Fabry 2008; Gazeau *et al.* 2007; Miller *et al.* 2009). Ocean acidification (OA) also has impacts (generally deleterious) on the metabolism, immune responses, reproduction, development and growth of oysters (*e.g.* Gazeau *et al.* 2013; Gazeau *et al.* 2007; Kroeker *et al.* 2013; Kurihara 2008; Kurihara *et al.* 2007; Miller *et al.* 2009; Parker *et al.* 2013; Parker *et al.* 2009; Parker *et al.* 2012; Parker *et al.* 2010). The susceptibility of oysters to OA has important commercial ramifications as well. Global aquaculture production of oysters, which is currently worth US$ 4 billion per year (FAO 2014), is predicted to decline rapidly due to climate change (Doubleday *et al.* 2013).

The potential impacts of OA on oysters may be mitigated by their generalised capacity to respond to a range of environmental stressors. The genome of the Pacific oyster (*Crassostrea gigas*) is notable for its proliferation of genes involved in stress responses (Zhang *et al.* 2012). Disturbances in seawater temperature, pH, salinity and chemical contaminants have all been shown to alter the energy metabolism, oxidative stress reactions, cytoskeletal activity and immune functions of oysters (Dineshram *et al.* 2013; Dineshram *et al.* 2012; Holmstrup *et al.* 2010; Matoo *et al.* 2013; Thompson *et al.* 2012a; Thompson *et al.* 2012b; Tomanek 2014; Tomanek *et al.* 2011; Zhang *et al.* 2012). Transcriptome analyses have revealed that many genes involved in those biological functions are differentially regulated by multiple

environmental stressors, including temperature extremes, low salinity, heavy metals and low pH (Anderson *et al.* 2015; Chapman *et al.* 2011; Clark *et al.* 2013; Zhang *et al.* 2012). In the context of OA, Wang *et al.* (2016) recently identified increases in the number of apoptotic cells and in the production of reactive oxygen species (ROS) in Pacific oysters (*C. gigas*) in response to elevated CO_2 (2,062 ppm CO_2, pH 7.55). At the transcriptional level, *C. virginica* showed changes in the relative expression of genes involved in biomineralisation, stress and immune functions under CO_2 stress (3,523 µatm pCO_2, pH 7.5 and 2,000 µatm pCO_2, pH 7.6) (Beniash *et al.* 2010; Ivanina *et al.* 2014). Tomanek *et al.* (2011) have also shown that elevated CO_2 induces a strong oxidative stress response in the eastern oyster, *Crassostrea virginica*. After two weeks of exposure to hypercapnic conditions (357 Pa pCO_2, pH 7.5), the majority of differentially regulated proteins in *C. virginica* were associated with the cytoskeleton and antioxidant defences (Tomanek *et al.* 2011). Differential expression of cytoskeletal and metabolic proteins has also been observed in *Crassostrea hongkongensis* larvae and in *C. gigas* larvae and juveniles exposed to elevated CO_2 (1,697 µatm pCO_2, pH 7.87; 2,275 µatm pCO_2, pH 7.5; and 2,013 ppm CO_2, pH 7.57, respectively) (Dineshram *et al.* 2013; Dineshram *et al.* 2012; Wei *et al.* 2015b). Similarly, exposure to extremely high CO_2 concentrations (2,800 µatm pCO_2, pH 7.3) induced changes in the relative abundance of proteins associated with metabolism, oxidative stress and nucleotide regulation in *C. gigas* adults (Timmins-Schiffman *et al.* 2014).

The responsive capacity of oysters to climate change stressors can vary between species, and even between populations within a species (Fabry 2008). The deleterious effects of elevated CO_2 are far less severe in larvae of Sydney rock oysters (*Saccostrea glomerata*) produced through selective breeding for fast growth and disease resistance when compared to wild type (non-selected) oysters (Parker *et al.* 2012; Parker *et al.* 2011). Parker *et al.* (2011) found that larvae from selectively bred oysters had significantly higher survival rates and grow twice as fast as wild type larvae when exposed to elevated CO_2. Adult oysters from the same selective

breeding line also have higher standard metabolic rates (SMR) than wild oysters under ambient conditions, and their SMR is further increased upon exposure to elevated concentrations of CO_2 (Parker *et al.* 2012). These findings suggest that selective breeding has led to enhanced metabolic activity, and that this adaptation may have a protective effect on larvae in terms of OA.

The current study further explores the capacity of selectively bred oysters to withstand OA by investigating the cytological effects of elevated CO_2. Based on previous studies that identified differences between the SMR of selectively bred and wild type populations (Parker *et al.* 2012), we predict that differential effects of elevated CO_2 will be evident in the mitochondria, and associated systems that result in oxidative stress and changes in the cytoskeleton. Hence, our study analyses the effects of elevated CO_2 on mitochondrial integrity, the actin cytoskeleton, and the production of reactive oxygen species (ROS).

2.2 Materials and Methods

2.2.1 Animals and experimental CO_2 exposure

Selectively bred (S) and wild type (W) Sydney rock oysters (*Saccostrea glomerata*) (7.54 ± 1.00 cm shell length) were provided by the Port Stephens Fisheries Institute (PSFI, Taylors Beach, NSW, Australia) of the New South Wales Department of Primary Industries (NSW DPI). The selectively bred oysters used in this study were mass selected for fast growth and resistance to winter mortality (causative agent currently under review) over seven generations (Dove *et al.* 2013). A recent study analysing ten microsatellite loci in oysters from these two populations found significant reductions in sequence diversity in the selected line (In *et al.* 2016). Disease-associated mortalities in the selectively bred line are also significantly lower than those of wild type oysters (23% versus 52%) (Dove *et al.* 2013). Therefore, this selectively bred line is considered a genetically distinct subset of the wild population.

Oysters were exposed to elevated CO_2 at the Sydney Institute of Marine Science (Chowder Bay, NSW) in a flow-through seawater system (0.5 L per minute, seawater filtered at 20 µm). The nutritional supply from the flow-through seawater was supplemented every three days with a concentrated blend of microalgae (Shellfish Diet® 1800, Reed Mariculture Inc., 4.3×10^8 algal cells per oyster), containing *Isochrysis*, *Pavlova*, *Tetraselmis*, *Chaetocerous calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. Temperature, salinity and carbonate chemistry (pH and total alkalinity) were monitored (Table 2.1) and no mortality was observed throughout the experiment.

Table 2.1. Seawater chemistry parameters during the fifteen-day experimental exposure. Salinity, temperature and pH were measured once a day using a YSI 63 probe, total alkalinity was determined with an automatic titrator (Metrohm 888 Titrando) and CO_2 was calculated using co2sys software (Lewis & Wallace 1998). Data are presented as means ± SEM (n = 9 days).

Parameter	**Control (C)**	**Elevated CO_2 (E)**
pH	8.09 ± 0.01	7.78 ± 0.04
Temperature (°C)	18.39 ± 0.16	19.26 ± 0.17
Salinity (ppt)	33.79 ± 0.47	33.70 ± 0.55
Total alkalinity (mmol kg^{-1} SW)	2.20 ± 0.03	2.14 ± 0.01
pCO_2 (µatm)	324.02 ± 5.64	760. 36 ± 68.37

After 10 days of acclimation to aquarium conditions, oysters from both populations were exposed to ambient (324 µatm pCO_2, pH 8.09) (control; C) and near-future elevated (760 µatm pCO_2, pH 7.78) (E) pCO_2 concentrations. Elevated seawater pCO_2 concentrations were controlled by a custom-made CO_2 system that included pneumatic components from Parker Hannifin (Castle Hill, NSW), CO_2 sensors from Vaisala (Hawthorn, VIC) and a control system from Greenstar Building Automation & Citywide Electrical Services (Marrickville, NSW). CO_2-enriched air was bubbled into four header tanks, each of which supplied a single replicate tank. CO_2-enrichement and the water flow were regulated to maintain the target seawater pH

during the experiment. Four replicate tanks (each containing a mix of 3 wild type and 3 selectively bred oysters) were used for either control (ambient CO_2) or elevated CO_2 conditions. These exposures produced four treatments: wild type oysters under control conditions (**WC**); wild type oysters exposed to elevated CO_2 (**WE**); selectively bred oysters under control conditions (**SC**); and selectively bred oysters exposed to elevated CO_2 (**SE**). Five oysters per treatment were randomly selected and sampled after 4 and 15 days of CO_2 exposure. A different set of oysters was sampled in each time point.

2.2.2 Hemocyte collection

To assess cellular stress induced by OA, we analysed the response of oyster hemocytes to elevated CO_2 concentrations. Hemocytes are tractable and abundant cells, and optimised protocols specifically for oyster hemocytes have been developed for different cytological stains (Aladaileh *et al.* 2007; Aladaileh *et al.* 2008a). On days 4 and 15 of CO_2 exposure, oysters were shucked and hemolymph was withdrawn from the pericardial cavity of each oyster into ice-cold marine anticoagulant (MAC; 100 mM glucose, 15 mM trisodium citrate, 13 mM citric acid, 20 mM EDTA, 450 mM NaCl, pH 7.0). Hemocyte concentrations were adjusted to 1×10^6 cells ml^{-1} and cells were kept on ice. Hemocytes were centrifuged (500 × g, 4 min at 4 °C) and resuspended in ice-cold modified Hank's balanced salt solution (mHBSS; 450 mM NaCl, 0.6 mM $MgSO_4\cdot7H_2O$, 0.5 mM $MgCl_2\cdot6H_2O$, 1.3 mM $CaCl_2$, 5.4 mM KCl, 5.6 mM D-glucose, 0.3 mM $Na_2HPO_4\cdot7H_2O$, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, pH 7.4). These cell suspensions from each oyster were dotted as 100 µl droplets onto round coverslips (22 mm; previously cleaned with acid-alcohol solution; 1% HCl in 70% ethanol) held in the wells of 6-well cell culture plates. Hemocytes were allowed to adhere to coverslips for 30 min in a moist chamber. Assays were performed in duplicate at room temperature. Differential hemocyte counts to identify different sub-populations of hemocytes were also undertaken on the adhered, fixed cells (Supplementary Figure 2.1).

2.2.3 Mitochondrial and actin staining

Hemocytes were simultaneously stained with the mitochondrion-selective probe MitoTracker® Orange CMTMRos (Molecular Probes®) and the F-actin stain Alexa Fluor® 488 Phalloidin (Molecular Probes®). Adhered hemocytes on coverslips were incubated with 100 nM of MitoTracker® Orange CMTMRos (100 µl in mHBSS) for 15 min in the dark. After incubation, cells were washed (3 × 5 min) by adding 2 ml of mHBSS into the wells of the 6-well plates holding coverslips. The hemocytes were then fixed for 10 min with 2% paraformaldehyde in mHBSS (100 µl), and further permeabilised with 0.1% Triton X-100 in phosphate buffered saline (PBS, Amresco) for 2 min (100 µl). Nonspecific binding was blocked by incubating cells with 1% BSA in PBS (100 µl) for 5 min before staining for F-actin. The fixed hemocytes were incubated with 250 nM Alexa Fluor® 488 Phalloidin (100 µl in PBS) for 15 min. After washing (3 × 5 min, mHBSS), nuclei were counter-stained with 20 µg ml^{-1} Hoechst 33342 for 10 min (Molecular Probes®, 100 µl in PBS). Coverslips were then washed as previously described and mounted using 12 µl of Fluoro-Gel (ProSciTech) onto acid-alcohol washed slides.

2.2.4 Reactive oxygen species (ROS)

After adhesion, hemocytes were stained with 5 µM CellROX® Green Reagent (Molecular Probes®, 100 µl in mHBSS) for 30 min in the dark. This cell-permeant fluorogenic probe is a reliable method for measuring oxidative stress in cells (Kaczmarzyk *et al.* 2014). Upon oxidation by reactive oxygen species (ROS), the reagent binds to DNA. Thus, its signal is localised primarily in the nucleus and mitochondria. Following staining, cells were washed (3 × 5 min) with mHBSS (2 ml) and fixed with 2% paraformaldehyde (100 µl in mHBSS) for 10 min. Nuclear counter-staining and mounting onto slides were performed as described in section 2.2.3.

2.2.5 Confocal data acquisition and image analyses

Hemocytes stained for mitochondria, F-actin or ROS were visualised using an Olympus Fluoview Fv1000 Ix81 inverted confocal microscope in the Macquarie University Faculty of Science Microscopy Unit. Captured micrographs were analysed by measuring the fluorescence intensity (arbitrary units) for each probe using ImageJ software (Schneider *et al.* 2012). For each treatment, the fluorescence intensities of at least 100 individual hemocytes (20 cells from each of the 5 oysters per treatment) were analysed by measuring the integrated density of the sum of confocal slices per image (10 slices of 1 μm each for mitochondria and F-actin, and 20 slices of 0.5 μm each for ROS). Fluorescence intensity measurements were corrected for background fluorescence by dividing the measured fluorescence intensities by the average intensity of five cell-free regions in each image. The frequency of F-actin foci (aggregates of actin filaments, see Results for description) was assessed by counting the number of foci per cell and measuring the area and fluorescence intensity of each focus using Adobe Photoshop CC software.

2.2.6 Statistical analyses

Statistical analyses for fluorescence intensity data were performed using a linear mixed-effects model, considering treatment as a fixed effect (four groups) and individual oysters (5 oysters per treatment) as a random factor. Due to the high inter-individual variability found among oysters within treatments, we have included individual oysters as a random effect in the model. Post-hoc analysis was performed using Tukey's test. Filamentous actin distribution (number of F-actin foci per cell) was analysed by one-way ANOVA, followed by Tukey's test. Fluorescence intensity and F-actin distribution data were transformed to natural logarithm values (ln) prior to analysis. Differences were considered statistically significant if $p < 0.05$. Complete statistical tables can be found in Supplementary Table 2.1.

2.3 RESULTS

2.3.1 Characteristics of mitochondrial and actin staining in hemocytes

Oyster hemocytes were stained for intact mitochondrial membranes (in red, MitoTracker® Orange CMTMRos), actin (in green, Alexa Fluor® 488 Phalloidin) and nuclear DNA (in blue, Hoechst 33342) (Fig. 2.1). Cells ranged from 6.5 to 15 µm in diameter, and varied in shape from discoidal to articulated and filopodial. MitoTracker® Orange CMTMRos staining resolved individual mitochondria (40 to 120 mitochondria per cell) in oyster hemocytes (Fig. 2.1a). F-actin was found either as linear filaments, structural networks or as condensed punctate foci mainly located near the cell periphery (Fig. 2.1b). In the majority of cells, filamentous actin also defined filopodia.

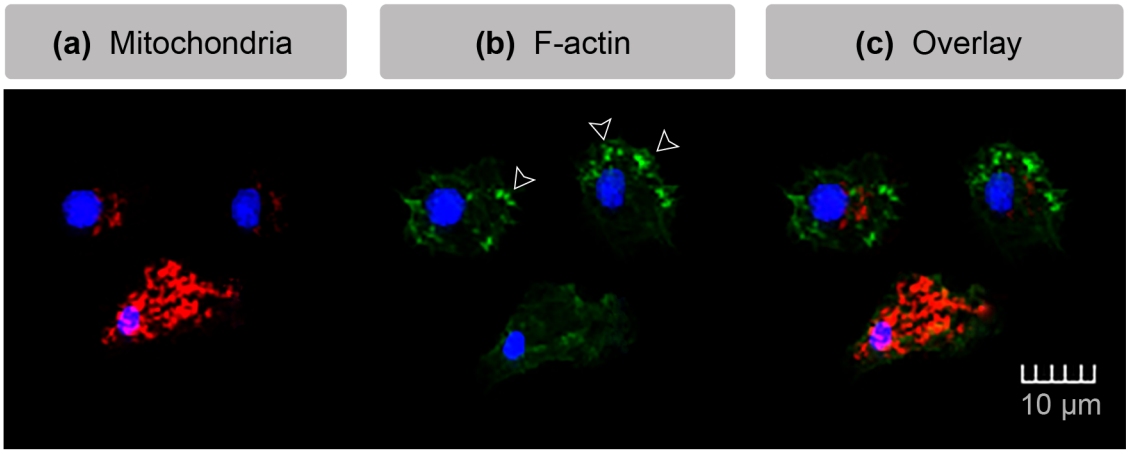


Figure 2.1. Confocal fluorescence micrographs of oyster hemocytes illustrating mitochondria and actin cytoskeleton. Hemocytes mitochondrial membranes in red (MitoTracker® Orange CMTMRos) **(a)**, filamentous actin (F-actin) in green (Alexa Fluor® 488 Phalloidin) **(b)**, and nuclei in blue (Hoechst 33342). Overlay of fluorescent stains is shown in **(c)**. Arrowheads indicate actin foci.

2.3.2 Changes in mitochondrial staining resulting from elevated CO_2 differ between oyster populations

Elevated CO_2 affected mitochondrial viability in hemocytes from the two Sydney rock oyster populations. Mitochondrial fluorescence intensities did not differ significantly between selectively bred (SC) and wild type (WC) oysters under ambient conditions on either day of analysis (4 or 15 days of CO_2 exposure) (Fig. 2.2). However, significant differences were evident between the two populations when oysters were exposed to elevated CO_2. Selectively bred oysters (SE) had significantly lower (4.6-fold) mitochondrial fluorescence intensities relative to wild type oysters (WE) on day 4 of elevated CO_2 exposure (Fig. 2.2a,b). A contrasting response was found on day 15 of elevated CO_2 exposure, where the selectively bred oysters (SE) had higher (3-fold) mitochondrial fluorescence intensities compared to wild type oysters (WE). However, that difference between populations on day 15 was not statistically significant ($p > 0.05$).

Differences in mitochondrial staining between populations were also revealed by comparing CO_2-exposed oysters from a particular population and the corresponding ambient controls from the same population. After 4 days of CO_2 exposure, hemocytes from wild type oysters (WE) had higher (2.9-fold) mitochondrial fluorescence intensities when compared to wild type oysters under ambient conditions (WC) (Fig. 2.2a,b). Conversely, the hemocytes of selectively bred oysters (SE) had significantly lower (2.8-fold) mitochondrial fluorescence intensities after exposure to elevated CO_2 than oysters from the same population that were held under ambient condition for 4 days (SC) (Fig. 2.2a,b). After 15 days of exposure, mitochondrial fluorescence intensities were significantly lower (5.4-fold) among CO_2-exposed wild type oysters (WE) compared to ambient controls (WC) (Fig. 2.2c,d). In contrast, mitochondrial fluorescence intensities did not differ significantly between selectively bred oysters exposed to elevated (SE) and ambient (SC) CO_2 conditions for 15 days (Fig. 2.2c,d).

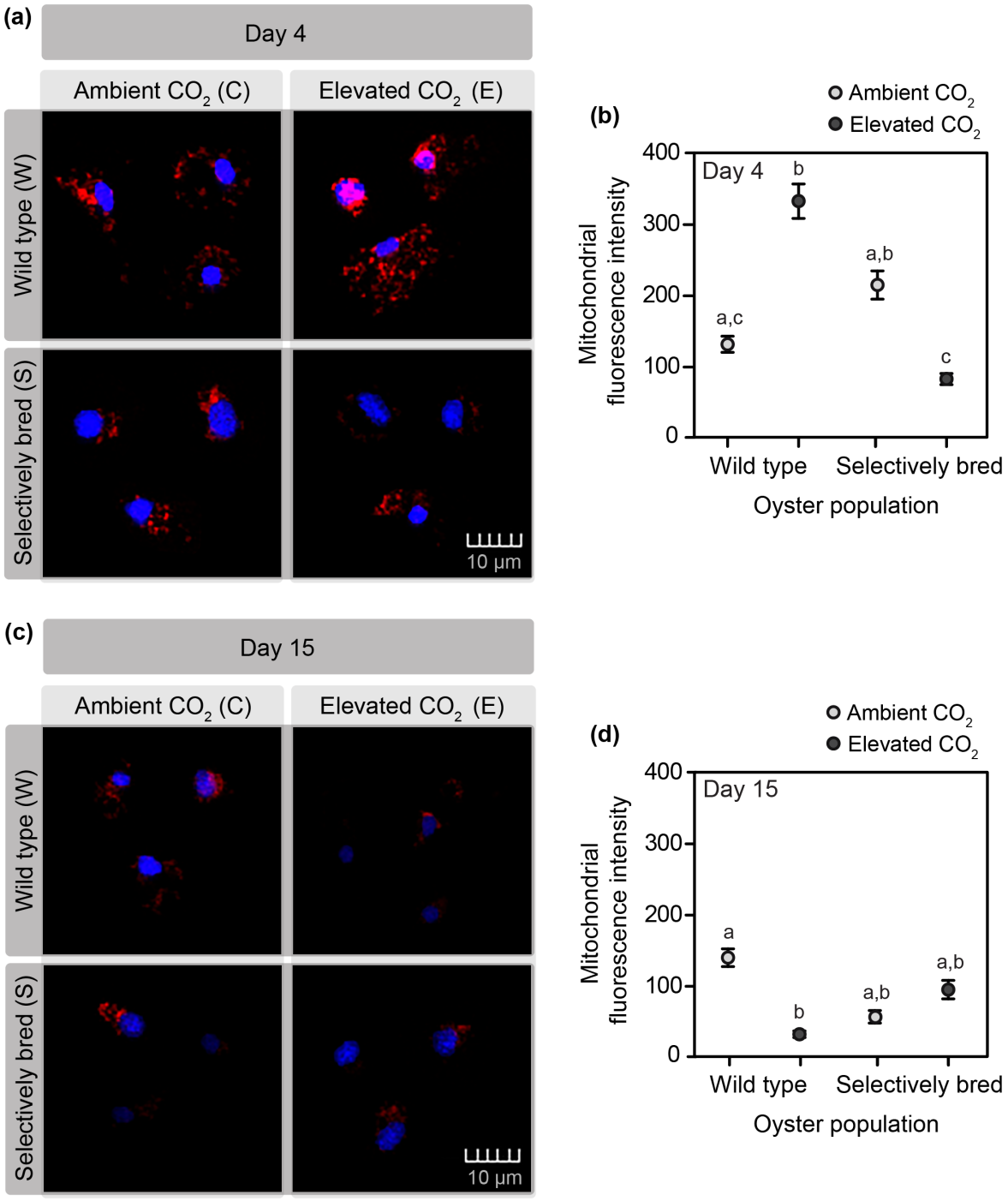


Figure 2.2. Effects of elevated CO_2 on mitochondrial fluorescence intensity of *Saccostrea glomerata* hemocytes. Confocal fluorescence micrographs showing mitochondrial membranes (in red, MitoTracker® Orange CMTMRos) and nuclei (in blue, Hoechst 33342) in hemocytes from the four different treatments on day 4 **(a)** and 15 **(c)** of CO_2 exposure. Mitochondrial fluorescence intensities (arbitrary units) after 4 **(b)** and 15 **(d)** days of exposure to elevated CO_2 ($n \geq 100$ cells per treatment; bars = SEM). Different letters indicate statistically significant differences between treatments ($p < 0.05$; linear mixed-effects model/Tukey's test). **WC**: wild type oysters under ambient conditions (324 µatm pCO_2, pH 8.09); **WE**: wild type oysters exposed to elevated CO_2 (760 µatm pCO_2, pH 7.78); **SC**: selectively bred oysters under ambient conditions; **SE**: selectively bred oysters exposed to elevated CO_2.

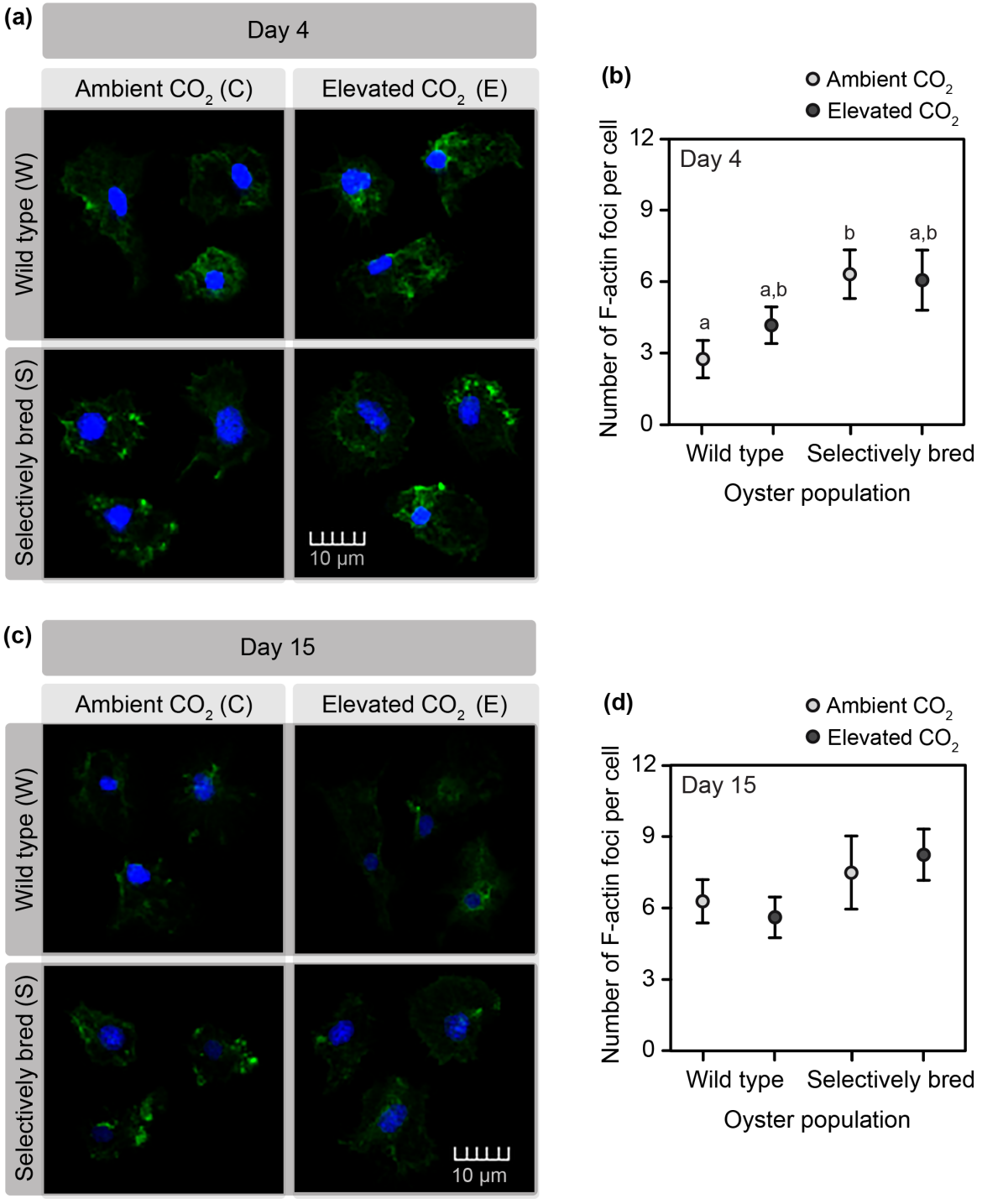
Mitochondrial fluorescence intensities were found to be lower in both CO_2-exposed wild type oysters (WE) and selectively bred oysters held under ambient conditions (SC) at day 15 when compared to day 4. However, the mitochondrial fluorescence intensities of CO_2-exposed selectively bred oysters (SE) and control wild type oysters (WC) did not change (stayed low) between days 4 and 15 (Fig. 2.2).

The differences evident in fluorescence intensities between different treatments and populations were not related to variation in the types of hemocytes present in hemolymph. Elevated CO_2 exposure did not alter the frequency of granulocytes and hyalinocytes in either oyster population at either time point (Supplementary Figure 2.1). A higher proportion of hemoblasts was found in selectively bred oysters (SC) compared to wild type oysters (WC) under ambient CO_2 conditions at day 15 of analysis. However, this difference did not affect our data for mitochondrial fluorescence intensity, because fluorescence signals were not measured in hemoblasts. Moreover, tests of the phagocytic activity of oyster hemocytes did not show clear differences between populations in response to CO_2 exposures (Supplementary Figure 2.2).

2.3.3 Filamentous actin morphology differs between oyster populations but is not affected by elevated CO_2

The morphology of filamentous actin differed between the two oyster populations at the 4 day time point. F-actin networks in selectively bred oysters appeared to be disordered, accumulating into aggregates (foci) close to the cell surface (Fig. 2.3a). Under ambient conditions, selectively bred oysters (SC) had 2.3 times more of these actin foci than wild type oysters (WC) (Fig. 2.3b). The number of actin foci also appeared to be higher (1.5-fold) in selectively bred oyster hemocytes (SC) at day 15 of CO_2 exposure. However, inter-individual

variability was higher at this time point, so no statistically significant difference was observed between selectively bred (SC) and wild type (WC) treatments (Fig. 2.3c,d; $p > 0.05$).



Despite the difference between populations under ambient conditions on day 4 of analysis, no statistically significant differences in the number of foci per cell were evident after either 4 or 15 days of exposure to elevated CO_2 relative to ambient conditions (Fig. 2.3). Similarly, the mean fluorescence intensities of total F-actin staining per cell (foci + filaments) and the size F-actin foci did not differ between populations or CO_2 treatments (data not shown).

2.3.4 Production of ROS is affected by exposure to elevated CO_2 in selectively bred oysters

The production of ROS was significantly affected by elevated CO_2 in selectively bred but not in wild type oysters on both days of analysis (Fig. 2.4). ROS production was significantly greater (3-fold) in selectively bred oysters (SE) after exposure to elevated CO_2 for 4 days, compared to ambient conditions (SC) (Fig. 2.4a,b). Conversely, on day 15 of CO_2 exposure, ROS production in selectively bred oysters (SE) was significantly lower (4.5-fold) relative to the control group (SC) (Fig. 2.4c,d). No statistically significant differences in ROS production were found between CO_2-exposed selectively bred and wild type oysters (SE versus WE) or among wild type oysters (WE versus WC) after 4 and 15 days of exposure.

Figure 2.3. (opposite page) Effects of elevated CO_2 on actin distribution in *Saccostrea glomerata* hemocytes. Hemocytes stained for filamentous actin (F-actin) in green (Alexa Fluor® 488 Phalloidin) and nuclear DNA in blue (Hoechst 33342) on day 4 **(a)** and 15 **(c)** of CO_2 exposure. Mean numbers of F-actin foci per hemocyte after 4 **(b)** and 15 **(d)** days of exposure to elevated CO_2 ($n \geq 20$ cells per treatment; bars = SEM). Different letters indicate statistically significant differences between treatments ($p < 0.05$; one-way ANOVA/Tukey's test). **WC**: wild type oysters under ambient conditions (324 µatm pCO_2, pH 8.09); **WE**: wild type oysters exposed to elevated CO_2 (760 µatm pCO_2, pH 7.78); **SC**: selectively bred oysters under ambient conditions; **SE**: selectively bred oysters exposed to elevated CO_2.

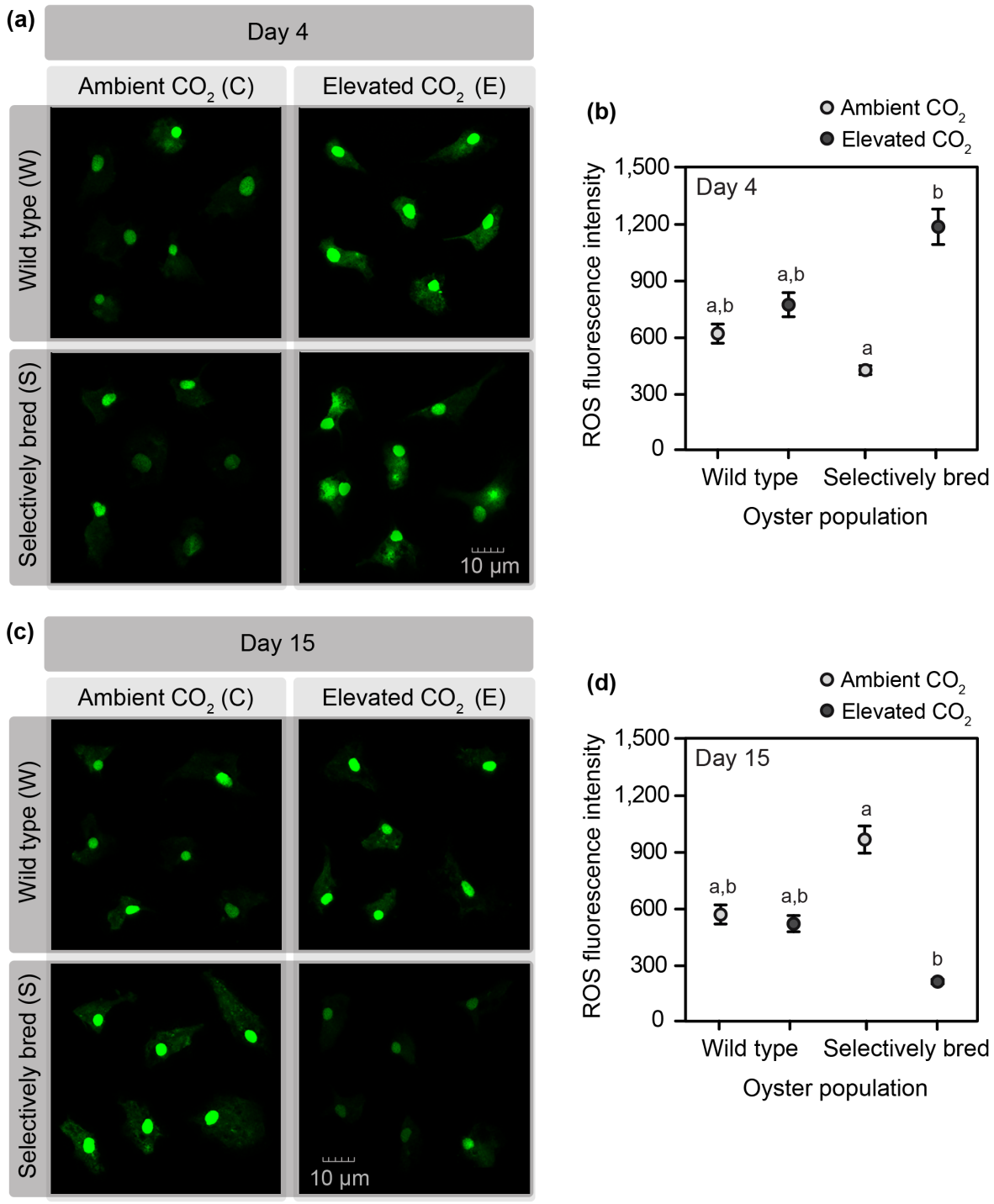


Figure 2.4. Effects of elevated CO_2 on the production of reactive oxygen species (ROS). Hemocytes from wild type and selectively bred oysters were stained with CellROX® Green Reagent on day 4 **(a)** and 15 **(c)** of CO_2 exposure. Mean ROS fluorescence intensities (arbitrary units) after 4 **(b)** and 15 **(d)** days of elevated CO_2 exposure ($n \geq 100$ per treatment; bars = SEM). Different lower case letters indicate statistically significant differences between treatments ($p < 0.05$; linear mixed-effects model/Tukey's test). **WC**: wild type oysters under ambient conditions (324 µatm pCO_2, pH 8.09); **WE**: wild type oysters exposed to elevated CO_2 (760 µatm pCO_2, pH 7.78); **SC**: selectively bred oysters under ambient conditions; **SE**: selectively bred oysters exposed to elevated CO_2.

2.4 DISCUSSION

This study has identified substantial differences in the intracellular responses of two genetically distinct Sydney rock oyster populations to elevated CO_2. CO_2-driven acidification induced changes in mitochondrial viability and the production of reactive oxygen species (ROS) in hemocytes from selectively bred and wild type (non-selected) oysters. Both the magnitude and direction of these changes varied depending on the population and the time of exposure.

A brief (4 day) exposure to elevated CO_2 substantially increased the production of ROS and decreased mitochondrial membrane potential in hemocytes from oysters that had been selectively bred for disease resistance and fast growth over seven generations. The increased production of ROS in hemocytes from selectively bred oysters after 4 days of CO_2 exposure is indicative of oxidative stress resulting from an enhanced demand for cellular energy. The mitochondrial electron transport chain is a major intracellular source of ROS production in oyster hemocytes (Donaghy *et al.* 2012). Unless neutralised by antioxidant mechanisms, ROS can damage essential cellular components, including mitochondria, DNA and the cytoskeleton (Bocchetti & Regoli 2006; Finkel & Holbrook 2000; Tedesco *et al.* 2010). In mammals, accumulation of ROS within mitochondria is followed by the opening of the mitochondrial permeability transition pore (MPTP) and the inner membrane anion channel (IMAC), decreasing mitochondrial membrane potential (Lemasters *et al.* 1998). These effects are reflected in the current study by increased CellROX® Green Reagent fluorescence intensities along with decreased MitoTracker® Orange CMTMRos fluorescence intensities in hemocytes from selectively bred oysters exposed to elevated CO_2. The high levels of ROS observed in selectively bred oysters under CO_2 stress may reflect an induced response by hemocytes to increase mitochondrial energy production within the first few hours following CO_2 exposure, with the subsequent generation of ROS and reduction of mitochondrial integrity.

In addition to impeding mitochondrial function, increased ROS production can also lead to structural changes in the actin cytoskeleton. Excess ROS may cause actin networks to disassemble and form aggregates that accumulate into punctate foci (Dalle-Donne *et al.* 2001; Farah *et al.* 2011; Franklin-Tong & Gourlay 2008; Green & Reed 1998). We found that selectively bred oysters have a higher number of such F-actin foci per hemocyte than wild type oysters. However, this difference was not exacerbated by exposure to elevated CO_2.

Similar oxidative stress responses involving ROS production and the loss of mitochondrial viability have been previously described in oysters following exposure to different environmental conditions, including OA. Aladaileh *et al.* (2008a; 2008b) have shown that a range of environmental stressors (extreme temperature, altered salinity and physical agitation) increased levels of the stress hormone noradrenaline in Sydney rock oyster hemolymph. This led to increased ROS production and the induction of apoptotic markers in hemocytes, including the loss of mitochondrial membrane potential and reorganisation of the actin cytoskeleton. CO_2-driven acidification also resulted in increased ROS production and in the number of apoptotic cells in the Pacific oyster, *C. gigas* (2,062 ppm CO_2, pH 7.55). Higher transcript levels of immune- and antioxidant-related genes were also observed following exposure to CO_2 stress (7 to 28 days) (Wang *et al.* 2016). In the eastern oyster, *C. virginica*, proteomic analyses identified a typical oxidative stress response involving the induction of antioxidant enzymes after oysters were exposed to elevated CO_2 (357 Pa pCO_2, pH 7.5) for two weeks (Tomanek *et al.* 2011). Similarly, CO_2-exposed (1,000 - 3,000 µatm pCO_2, pH 7.9 - 7.6) *C. gigas* (4 day exposure) and *C. hongkongensis* (30 day exposure) larvae exhibited altered concentrations of proteins associated with mitochondrial energy metabolism and stress responses relative to non-exposed controls (Dineshram *et al.* 2013; Dineshram *et al.* 2012). Elevated CO_2 also affected the proteomic profiles of juvenile and adult *C. gigas*. Proteins involved in carbohydrate metabolism and responses to ROS were found to be differentially abundant in adults (Timmins-Schiffman *et al.* 2014) and metabolism-related proteins were

down-regulated in juveniles (Wei *et al.* 2015a; Wei *et al.* 2015b) after one month of acute CO_2 stress (2,800 µatm pCO_2, pH 7.29, and 2,013 ppm CO_2, pH 7.57, respectively). Thompson *et al.* (2015) recently found that the majority of differentially regulated proteins, including those involved in mitochondrial electron transport chain and oxidative stress, were also down-regulated in selectively bred Sydney rock oysters (*S. glomerata*) exposed to elevated CO_2 (856 µatm pCO_2, pH 7.84). Such findings suggest that low pH changes energy metabolism and causes dysfunction in the same cellular systems that were analysed in the current study.

In contrast to the earlier (4 day) time point, 15 days of exposure to elevated CO_2 substantially reduced the production of ROS in hemocytes from selectively bred oysters, and their mitochondrial fluorescence intensities remained low in response to continuing CO_2 stress. These findings suggest that the mitochondrial damage resulting from excess ROS production in the early stages (4 day) of response to elevated CO_2 subsequently decreases ROS production by mitochondria in the later stages of the response as the number of viable mitochondria decreases. In addition, mitochondrial ROS production depends on the magnitude of the mitochondrial membrane potential (Abele *et al.* 2007; Keller *et al.* 2004). Hence, the drop in oxygen consumption resulting from the reduced number of viable mitochondria and/or the reduced mitochondrial membrane potential prevent the formation of ROS, which decreased to levels lower than baseline (selectively bred oysters under ambient conditions) on day 15 of CO_2 exposure.

Our data also suggest that wild type Sydney rock oysters have a delayed and/or less pronounced stress response to CO_2 stress relative to selectively bred oysters. Elevated CO_2 increased mitochondrial membrane potential in wild type oyster hemocytes on day 4, but reduced this cellular parameter on day 15 of exposure. Moreover, ROS production in their hemocytes was not affected by elevated CO_2 at either time point. Even though wild type oysters triggered an immediate response to CO_2 stress reflected by the elevated mitochondrial

fluorescence intensities in their hemocytes, this response was transient, such that mitochondrial fluorescence intensities fell below baseline levels after 15 days of CO_2 exposure.

These differences in response between oyster populations are in line with previous studies of CO_2 stress in Sydney rock oysters. Differential responses within this species have been reported previously by Thompson *et al.* (2015), who analysed different populations of adult Sydney rock oysters at the protein level. The majority of the differentially regulated proteins were found at lower concentrations in selectively bred oysters under CO_2 stress relative to ambient conditions, indicating cellular dysfunction. In contrast, wild type oysters exhibited an up-regulation of their differential proteins in response to elevated CO_2. A number of studies have found that elevated CO_2 decreases the SMR of marine invertebrates (Fernández-Reiriz *et al.* 2011; Liu & He 2012; Michaelidis *et al.* 2005; Navarro *et al.* 2013; Wang *et al.* 2015). However, Parker *et al.* (2012) have shown that the SMR of selectively bred Sydney rock oysters increases following exposure to elevated CO_2 (856 µatm pCO_2, pH 7.9), while no major increase is observed in wild type oysters. The higher and more responsive SMR of selectively bred Sydney rock oysters might explain the apparent resilience of their larvae to elevated CO_2. The ability of these oysters to regulate their metabolic activity may allow their early developmental stages to survive the CO_2 stress without affecting their survival, developmental and growth rates. However, our data suggest that the enhanced SMR of selectively bred oysters might also lead to production of sufficient ROS to initiate deleterious oxidative stress. Therefore, while selectively bred larvae experience some physiological benefits from enhanced SMR, adults may not be able to escape its potential side effects, including excess ROS production and resulting mitochondrial damage.

Although our findings clearly show that elevated CO_2 has different subcellular effects in selectively bred and wild type oysters, it is impossible to predict the long-term physiological or ecological consequences of these impacts based on these data alone. Some evidence suggests that the impacts of elevated CO_2 on oxidative stress and metabolism may be transient in

molluscs (Clark *et al.* 2013; Dickinson *et al.* 2012; Ivanina *et al.* 2013; Lannig *et al.* 2010; Matoo *et al.* 2013; Thomsen & Melzner 2010). Hence, long-term studies linking subcellular processes to population level outcomes are now required, particularly those investigating whether diverting energy allocation into stress responses compromises reproduction or any other physiological mechanisms that affect population fitness. It is also important to analyse the transgenerational effects of elevated CO_2 on subcellular processes, as the population level differences identified in the current study suggest that there is genetic diversity in subcellular responses that might provide the basis for heritable adaptation to elevated CO_2. In this context, Parker *et al.* (2015) recently reported the persistence of positive carryover effects (*e.g.* faster growth and development rate) following transgenerational exposure of wild type Sydney rock oysters to elevated CO_2 (856 µatm pCO_2, pH 7.9). This may shed light on the capacity of oysters for heritable changes and allow us to predict impacts of OA across generations.

In summary, we have demonstrated that genetically distinct populations within the same species of oyster have different acute intracellular responses to OA. The onset of oxidative stress induced by OA occurred earlier and was more pronounced in selectively bred oysters when compared to the wild type population, as reflected by changes in mitochondrial integrity (mitochondrial membrane potential) and in the production of reactive oxygen species (ROS). This may be due to an inherent capacity for enhanced intracellular energy production or adaptive energy reallocation in the selectively bred population which could confer resilience to elevated CO_2 conditions. This study is the first step toward identifying the subcellular basis of differences in performance between breeding lines. This understandingcould provide a functional framework to underpin breeding programs designed to minimise the impacts of climate change on marine organisms.

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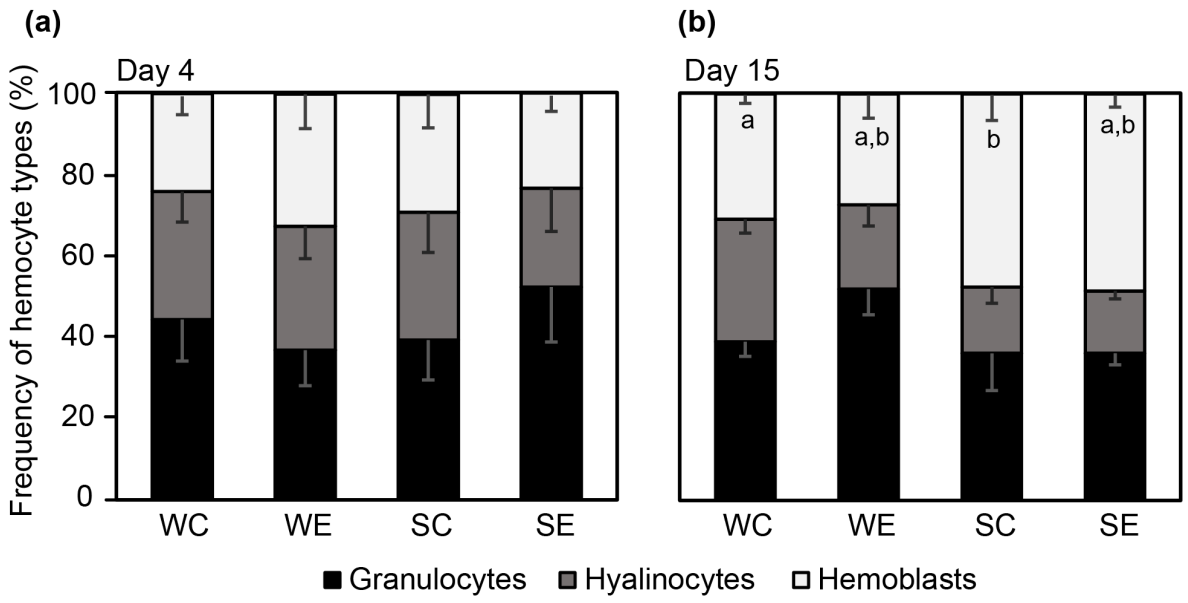
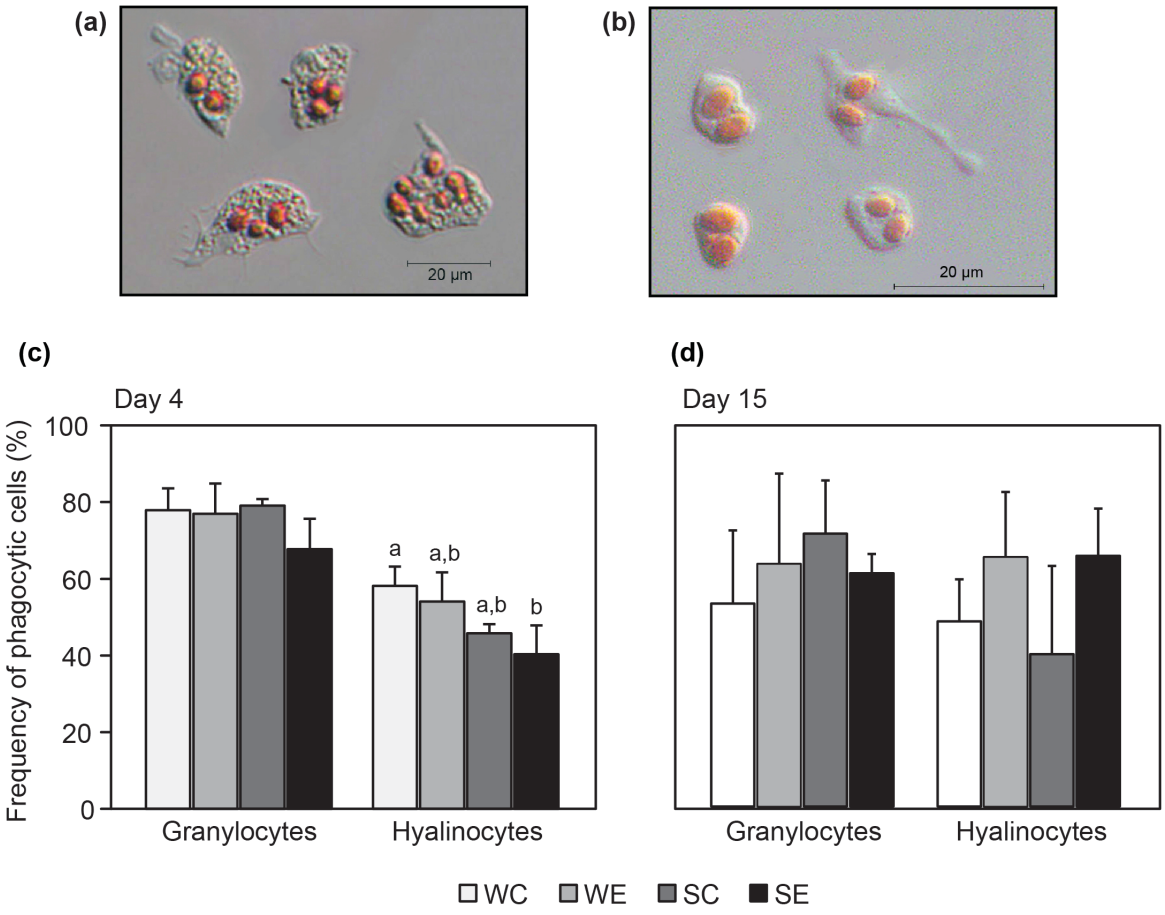
SUPPLEMENTARY INFORMATION



Supplementary Figure 2.1. Differential hemocyte frequencies of oyster hemocytes exposed to elevated CO_2. Differential hemocyte counts of two populations of Sydney rock oysters after 4 **(a)** and 15 **(b)** days of CO_2 exposure. The frequencies of three different hemocyte types (granulocytes, hyalinocytes and hemoblasts) were determined on the adhered, fixed hemocytes. Cells were examined under differential interference contrast (DIC) on a Leica DM5500 B microscope, and differential cell counts were based on the descriptions of Aladaileh *et al.* (2007). Bars represent the mean frequencies of each cell type (n ≥ 350 per treatment; bars = SEM). Different letters indicate statistically significant differences between treatments within each hemocyte population ($p < 0.05$; linear mixed-effects model/Tukey's test). **WC**: wild type oysters under ambient conditions (324 µatm pCO_2, pH 8.09); **WE**: wild type oysters exposed to elevated CO_2 (760 µatm pCO_2, pH 7.78); **SC**: selectively bred oysters under ambient conditions; **SE**: selectively bred oysters exposed to elevated CO_2.



Supplementary Figure 2.2. Phagocytic activity of oyster hemocytes following exposure to elevated CO_2. Differential interference contrast (DIC) micrographs showing granulocytes **(a)** and hyalinocytes **(b)** with engulfed yeast cells (red). Proportion of phagocytic hemocytes in two populations of Sydney rock oysters after 4 **(c)** and 15 **(d)** days of CO_2 exposure. Phagocytic activity was evaluated by incubating adhered hemocytes with Congo red-stained *Saccharomyces cerevisiae* (5×10^4 cells in filtered seawater, for 30 min). Bars represent the mean frequencies of phagocytic granulocytes or hyalinocytes (n = 16 to 26 per cell type per treatment; bars = SEM). Different letters indicate statistically significant differences between treatments within a hemocyte population ($p < 0.05$; linear mixed-effects model/Tukey's test). **WC**: wild type oysters under ambient conditions (324 µatm pCO_2, pH 8.09); **WE**: wild type oysters exposed to elevated CO_2 (760 µatm pCO_2, pH 7.78); **SC**: selectively bred oysters under ambient conditions; **SE**: selectively bred oysters exposed to elevated CO_2.

Supplementary Table 2.1. Statistical results from the analyses performed in this study. Summary of the statistical results from the linear mixed-effects model (lme), One-Way ANOVA and Tukey's post-hoc tests.

Linear mixed-effects model (lme)

Mitochondrial fluorescence intensity – day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	4.531936	0.236281	19.1803	0
WC – WE	0.966869	0.335176	2.884659	0.012
WC – SC	0.600486	0.354701	1.692936	0.1126
WC – SE	-0.68816	0.355075	-1.93807	0.073
Random effects (oyster)				
Std. deviation	0.4935273			
Mitochondrial fluorescence intensity – day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	4.438008	0.483059	9.187293	0
WC – WE	-2.06068	0.682503	-3.0193	0.0081
WC – SC	-1.43478	0.682502	-2.10223	0.0517
WC – SE	-0.89884	0.682537	-1.31691	0.2064
Random effects (oyster)				
Std. deviation	1.040968			
ROS fluorescence intensity – day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	6.055974	0.256422	23.61721	0
WC – WE	0.242546	0.361812	0.670364	0.5122
WC – SC	-0.19543	0.361593	-0.54046	0.5963
WC – SE	0.772069	0.362129	2.132032	0.0489
Random effects (oyster)				
Std. deviation	0.5537782			

ROS fluorescence intensity – day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	6.066819	0.282343	21.48739	0
WC – WE	-0.07515	0.399495	-0.18811	0.8532
WC – SC	0.41948	0.399414	1.050238	0.3092
WC – SE	-0.88061	0.399216	-2.20585	0.0424
Random effects (oyster)				
Std. deviation	0.6214985			

Differential cell count – Granulocytes at day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	3.701423	0.1619059	22.861573	0.0000
WC – WE	-0.351896	0.2377957	-1.479826	0.1627
WC – SC	-0.143071	0.2989736	-0.478542	0.6402
WC – SE	0.023892	0.2352669	0.101551	0.9207
Random effects (oyster)				
Std. deviation	0.316197			

Differential cell count – Granulocytes at day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	3.470831	0.198098	17.5208	0
WC – WE	0.474505	0.305797	1.551703	0.143
WC – SC	-0.04282	0.301243	-0.14213	0.889
WC – SE	-0.02741	0.286595	-0.09563	0.9252
Random effects (oyster)				
Std. deviation	0.3726133			

Differential cell count – Hyalinocytes at day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	3.398822	0.1280826	26.536167	0.0000
WC – WE	-0.003902	0.1874606	-0.020816	0.9837
WC – SC	0.191238	0.2352603	0.812877	0.4309
WC – SE	-0.162893	0.1859071	-0.876206	0.3968
Random effects (oyster)				
Std. deviation	0.211644			

Differential cell count – Hyalinocytes at day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	3.323161	0.262323	12.66819	0
WC – WE	-0.40815	0.487334	-0.83752	0.4174
WC – SC	-0.49475	0.389696	-1.26959	0.2265
WC – SE	-0.49528	0.380649	-1.30115	0.2158
Random effects (oyster)				
Std. deviation	0.5286198			

Differential cell count – Hemoblasts at day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	3.281814	0.112121	29.27034	0
WC – WE	0.274253	0.169892	1.614277	0.1305
WC – SC	0.15251	0.20296	0.751427	0.4658
WC – SE	0.077053	0.165093	0.466725	0.6484
Random effects (oyster)				
Std. deviation	0.1124215			

Differential cell count – Hemoblasts at day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**t-value**	**P-value**
Fixed effects (treatment)				
WC	3.392932	0.129154	26.27053	0
WC – WE	0.359396	0.216059	1.663412	0.1184
WC – SC	0.546648	0.190353	2.871754	0.0123
WC – SE	0.444199	0.187298	2.371616	0.0326
Random effects (oyster)				
Std. deviation	0.2513157			

Tukey's post-hoc test

Mitochondrial fluorescence intensity – day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	0.9669	0.3352	2.885	0.01999
SC – WC	0.6005	0.3547	1.693	0.32694
SE – WC	-0.6882	0.3551	-1.938	0.21185
SC – WE	-0.3664	0.3557	-1.03	0.73146
SE – WE	-1.655	0.356	-4.648	< 0.001
SE – SC	-1.2886	0.3745	-3.441	0.00315

Mitochondrial fluorescence intensity – day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	-2.0607	0.6825	-3.019	0.0133
SC – WC	-1.4348	0.6825	-2.102	0.1525
SE – WC	-0.8988	0.6825	-1.317	0.5521
SC – WE	0.6259	0.6819	0.918	0.7953
SE – WE	1.1618	0.6819	1.704	0.3216
SE – SC	0.5359	0.6819	0.786	0.8609

ROS fluorescence intensity – day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	0.2425	0.3618	0.67	0.908
SC – WC	-0.1954	0.3616	-0.54	0.949
SE – WC	0.7721	0.3621	2.132	0.143
SC – WE	-0.438	0.3608	-1.214	0.618
SE – WE	0.5295	0.3613	1.466	0.458
SE – SC	0.9675	0.3611	2.679	0.037
ROS fluorescence intensity – day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	-0.07515	0.39949	-0.188	0.99764
SC – WC	0.41948	0.39941	1.05	0.71978
SE – WC	-0.88061	0.39922	-2.206	0.12135
SC – WE	0.49463	0.39962	1.238	0.60275
SE – WE	-0.80546	0.39942	-2.017	0.1818
SE – SC	-1.30009	0.39934	-3.256	0.00592
Differential cell count – Granulocytes at day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	-0.35190	0.23780	-1.480	0.446
SC – WC	-0.14307	0.29897	-0.479	0.963
SE – WC	0.02389	0.23527	0.102	1.000
SC – WE	0.20882	0.30579	0.683	0.902
SE – WE	0.37579	0.24387	1.541	0.409
SE – SC	0.16696	0.30382	0.550	0.946

Differential cell count – Granulocytes at day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	0.47451	0.3058	1.552	0.406
SC – WC	-0.04282	0.30124	-0.142	0.999
SE – WC	-0.02741	0.2866	-0.096	1
SC – WE	-0.51732	0.32523	-1.591	0.383
SE – WE	-0.50191	0.31171	-1.61	0.372
SE – SC	0.01541	0.30724	0.05	1
Differential cell count – Hyalinocytes at day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	-0.0039	0.187461	-0.021	1
SC – WC	0.191238	0.23526	0.813	0.847
SE – WC	-0.16289	0.185907	-0.876	0.815
SC – WE	0.19514	0.240164	0.813	0.847
SE – WE	-0.15899	0.192075	-0.828	0.84
SE – SC	-0.35413	0.238953	-1.482	0.445
Differential cell count – Hyalinocytes at day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	-0.40815	0.487334	-0.838	0.835
SC – WC	-0.49475	0.389696	-1.27	0.579
SE – WC	-0.49528	0.380649	-1.301	0.559
SC – WE	-0.0866	0.501727	-0.173	0.998
SE – WE	-0.08713	0.494733	-0.176	0.998
SE – SC	-0.00053	0.39891	-0.001	1

Differential cell count – Hemoblasts at day four of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	0.27425	0.16989	1.614	0.368
SC – WC	0.15251	0.20296	0.751	0.875
SE – WC	0.07705	0.16509	0.467	0.966
SC – WE	-0.12174	0.21193	-0.574	0.939
SE – WE	-0.1972	0.176	-1.12	0.674
SE – SC	-0.07546	0.2081	-0.363	0.984
Differential cell count – Hemoblasts at day 15 of CO_2 exposure				
Parameter	**Estimate**	**Std. Error**	**z-value**	**Adjusted p-value (>|z|)**
WE – WC	0.3594	0.2161	1.663	0.3417
SC – WC	0.5466	0.1903	2.872	0.0208
SE – WC	0.4442	0.1873	2.372	0.0821
SC – WE	0.1872	0.2226	0.841	0.834
SE – WE	0.0848	0.22	0.385	0.9804
SE – SC	-0.1024	0.1948	-0.526	0.9526

One-way ANOVA, followed by Tukey's test

Number of F-actin foci – day four of CO_2 exposure					
Parameter	**Degrees of freedom**	**Sum Sq[a]**	**Mean Sq[b]**	**F-value**	**Adjusted P-value (>F)**
Group	3	146	48.68	3.346	0.0248
Residual	61	887.4	14.55		

[a] Sum Sq = sum of square values; [b] Mean Sq = mean of square values

Parameter	**Difference**	**Lower**	**Upper**	**Tukey adj. p-value**
SE – SC	-0.5	-4.06178	3.061781	0.982436
WC – SC	-3.8125	-7.37428	-0.25072	0.031376
WE – SC	-2.26838	-5.77739	1.240629	0.328738
WC – SE	-3.3125	-6.87428	0.249281	0.07731
WE – SE	-1.76838	-5.27739	1.740629	0.547016
WE – WC	1.544118	-1.96489	5.053129	0.652673

Number of F-actin foci – day 15 of CO_2 exposure					
Parameter	**Degrees of freedom**	**Sum Sq[a]**	**Mean Sq[b]**	**F-value**	**Adjusted P-value (>F)**
Group	3	67.1	22.36	1.095	0.358
Residual	61	1245.4	20.42		

[a] Sum Sq = sum of square values; [b] Mean Sq = mean of square values

Parameter	**Difference**	**Lower**	**Upper**	**Tukey adj. p-value**
SE – SC	0.75	-3.46945	4.969451	0.96547
WC – SC	-1.20221	-5.35914	2.954731	0.87034
WE – SC	-1.875	-6.09445	2.344451	0.645582
WC – SE	-1.95221	-6.10914	2.204731	0.603891
WE – SE	-2.625	-6.84445	1.594451	0.362564
WE – WC	-0.67279	-4.82973	3.484143	0.97355

CHAPTER 3

POPULATION-SPECIFIC MOLECULAR RESPONSES FOLLOWING TRANSGENERATIONAL CONDITIONING OF OYSTERS TO OCEAN ACIDIFICATION

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ABSTRACT

Marine organisms need to acclimate or adapt in order to cope with the adverse effects of climate change. Transgenerational exposure to CO_2 stress has been shown to enhance resilience to ocean acidification in offspring from a number of species. However, the molecular basis underlying such responses is largely unknown. Here, we compared the transcriptional profiles of two genetically distinct oyster breeding lines following transgenerational exposure to elevated CO_2. This is the first study to explore the molecular basis of transgenerational acclimation or adaptation to ocean acidification in these organisms. Transgenerational exposure to elevated CO_2 resulted in changes to both basal and inducible expression of key target genes associated with antioxidant defence, metabolism and the cytoskeleton, particularly in oysters derived from the disease-resistant, fast-growing B2 line. Transgenerational CO_2 exposure produced opposite and less evident effects on transcription in a second population that was derived from wild type (non-selected) oysters. Our findings suggest that the acute responses of oysters to CO_2 stress are affected by population-specific genetic and/or phenotypic traits, and by the CO_2 conditions to which their parents had been exposed. This supports the contention that the capacity for heritable change in response to ocean acidification varies between oyster breeding lines and is mediated by parental conditioning.

3.1 INTRODUCTION

Rising emissions of anthropogenic CO_2 are altering the fundamental chemistry of the oceans, substantially reducing their pH and the availability of carbonate ions (IPCC 2013). Marine calcifiers, including oysters, are particularly threatened by this changing ocean. Increases in the partial pressure of CO_2 (pCO_2) in seawater have been shown to induce changes in the acid–base balance, biomineralisation and energy metabolism of oysters (Fabry 2008; Pörtner *et al.* 2004). Such impacts also have downstream effects on immune responses, reproduction and development (Fabry 2008; Gazeau *et al.* 2013; Kurihara 2008; Miller *et al.* 2009; Parker *et al.* 2013). The majority of these effects are more pronounced in early life-history stages (larvae and juveniles), resulting in reduced fertilisation success, abnormal development and decreased growth (reviewed in Parker *et al.* 2013; Ross *et al.* 2011).

Despite these detrimental effects, recent studies have found that molluscs may be able to acclimate or adapt to near-future scenarios for ocean acidification (OA) (Miller *et al.* 2009; Parker *et al.* 2012; Thomsen & Melzner 2010). Oysters and mussels from naturally CO_2-enriched waters have not shown the adverse effects typically induced by experimental exposures to elevated CO_2 (Miller *et al.* 2009; Thomsen & Melzner 2010). *Crassostrea virginica* and *Crassostrea ariakensis* larvae that were conditioned under fluctuating pH environments and then exposed to elevated CO_2 exhibited normal development, growth and calcification rates (Miller *et al.* 2009). Similar responses were observed for the mussel *Mytilus edulis* conditioned at elevated CO_2 for two months. Even though the calcification process was reduced after exposure to elevated CO_2, somatic growth in conditioned *M. edulis* was not affected by this stressor. In addition, CO_2-conditioning of *M. edulis* elicited an increase in metabolic rates during moderate hypercapnia (Michaelidis *et al.* 2005; Thomsen & Melzner 2010). Based on such results, Pörtner *et al.* (2004) and Melzner *et al.* (2009) have proposed that the level of resilience in marine organisms to OA is often associated with increased baseline

metabolic rates and energy demand. While this may be the case, little is known about how acclimation and adaptation to OA are mediated at a transcriptional level in molluscs and how these energy-demanding physiological processes may change depending on genetic differences between species and between populations within species.

Numerous studies have described the response of different oyster species to OA at the protein level. Elevated CO_2 triggered a strong oxidative stress response in the eastern oyster, *C. virginica*. After two weeks of exposure to CO_2 stress (357 Pa pCO_2, pH 7.5), proteins involved in antioxidant defences and the cytoskeleton were found to be differentially regulated (Tomanek *et al.* 2011). Differential regulation of cytoskeletal and metabolic proteins has also been observed in *Crassostrea hongkongensis* larvae and in *Crassostrea gigas* larvae and juveniles exposed to elevated CO_2 (1,697 µatm pCO_2, pH 7.87; 2,275 µatm pCO_2, pH 7.5; and 2,013 ppm pCO_2, pH 7.57, respectively) (Dineshram *et al.* 2013; Dineshram *et al.* 2012; Wei *et al.* 2015). Another extensive proteomic study revealed differential abundance of proteins associated with metabolism, oxidative stress and nucleotide regulation in the gills of *C. gigas* exposed to extremely high CO_2 concentrations (2,800 µatm pCO_2, pH 7.3) (Timmins-Schiffman *et al.* 2014).

Few studies have used transcriptional approaches to understand the response of oysters to OA. *C. virginica* adults exhibited changes in the expression of genes involved in biomineralisation (carbonic anhydrase; up-regulation in mantle, down-regulation in gills), stress (down-regulation of Hsp70) and immune (down-regulation of integrin) responses under CO_2 stress (3,523 µatm pCO_2, pH 7.5 and 2,000 µatm pCO_2, pH 7.6, respectively) (Beniash *et al.* 2010; Ivanina *et al.* 2014). Chapman *et al.* (2011) and Clark *et al.* (2013) have also performed comprehensive transcriptomic analyses assessing the response of *C. virginica* and *C. gigas*, respectively, to a combination of OA and ocean warming. Decreasing pH was shown to increase the immune functions and production of antioxidants in *C. gigas*, while the combination of both pH and thermal stress induced the expression of protease inhibitors and

cytoskeleton-related genes (Clark *et al.* 2013). The combination of decreased pH and increased temperature also enhanced the expression of genes involved in antioxidant and metabolic processes in *C. virginica* (Chapman *et al.* 2011). Overall, both proteomic and transcriptional responses are extremely complex particularly when considered in terms of up- or down-regulation of specific genes or proteins. This hinders the interpretation of such results with regard to their physiological consequences. For instance, down-regulation of some proteins may result in an overall increase in the activity of the molecular pathway to which they contribute.

Despite the complex and dynamic regulation of molecules under OA, the consensus of these studies suggests that genes and proteins involved in oxidative stress, energy metabolism and the cytoskeleton are often affected by OA. This is consistent with models of adaptive stress responses proposed by Anderson *et al.* (2015) and Tomanek (2014). In these models, a range of environmental stressors (such as temperature extremes, hypoxia, salinity and infectious disease) have common cellular targets and elicit similar intracellular responses in marine invertebrates, including oysters. Disturbances in environmental conditions affect the mitochondrial electron transport chain, increasing energy production and generating reactive oxygen species (ROS). Unless neutralised by the antioxidant system or molecular chaperones, excessive ROS can disrupt the cytoskeleton and mitochondrial membranes resulting in cell death through apoptosis (Anderson *et al.* 2015). In support of this model, sequencing of the Pacific oyster (*C. gigas*) genome revealed an expanded set of genes involved in stress responses, particularly heat shock proteins (Hsps), superoxide dismutases (SODs) and inhibitors of apoptosis (IAPs) (Zhang *et al.* 2012).

Although extremely informative, previous studies assessing the molecular response of molluscs to OA do not provide insights into their adaptive potential against climate change-associated stressors. In addition to variation in sensitivity to OA observed among species, the potential of molluscs to acclimate or adapt to disturbances in seawater pH is supported by

differential responses observed in genetically distinct populations within species. Larvae from the B2 breeding line of Sydney rock oysters (*Saccostrea glomerata*) produced through selective breeding for fast growth and disease resistance are less affected by elevated CO_2 than wild type (W, non-selected) oysters (Parker *et al.* 2012; Parker *et al.* 2011). B2 larvae have significantly higher survival rates and grow twice as fast as wild type larvae when exposed to elevated CO_2 (Parker *et al.* 2011). Moreover, adult oysters from the B2 line have higher standard metabolic rates (SMR) than wild type oysters under ambient conditions, and this difference is exacerbated by exposure to elevated CO_2 (Parker *et al.* 2012). The intracellular stress responses of B2 and wild type Sydney rock oyster populations have also been shown to differ under CO_2 stress. Thompson *et al.* (2015) analysed the proteomes of adult B2 oysters and found that the majority of differentially regulated proteins, including those involved in the mitochondrial electron transport chain and oxidative stress, were down-regulated in selectively bred oysters exposed to elevated CO_2 (856 µatm pCO_2, pH 7.84). In contrast, proteins in wild type oysters were largely up-regulated in response to CO_2 stress. Based on their results, OA appears to induce a universal intracellular stress response in wild type oysters, but not in selectively bred oysters. B2 oysters showed signs of cellular dysfunction following exposure to elevated CO_2, potentially resulting from their capacity for rapid induction of metabolic activity. Elevated CO_2 (760 µatm pCO_2, pH 7.78) also induced substantial changes in mitochondrial integrity and ROS production in another population of potentially CO_2-resilient oysters, but not in wild type oysters (Chapter 2). These findings suggest that low pH alters metabolism and resulting oxidative stress in selectively bred oysters, and that these responses might reflect increased intracellular energy production or adaptive energy reallocation. However, the genomic and transcriptional bases of differential physiological and intracellular responses to OA in these oyster populations remains unclear.

Another major limitation to our understanding of the potential for molluscs to acclimate or adapt to acidifying oceans is the lack of long-term, transgenerational studies. The long

generation time of oysters (several months to years) has hindered the development of multigenerational experiments. One exception is the work of Parker *et al.* (2012), who found that the continuous exposure of adults of *S. glomerata* and their offspring to elevated CO_2 resulted in larvae with faster development and growth rates when compared to parents conditioned at ambient CO_2. More recently, Parker *et al.* (2015) showed such positive carryover effects of parental conditioning over two generations (F1 and F2). Adults with histories of transgenerational exposure to CO_2 stress had improved capacities to regulate extracellular pH and their offspring developed and grew faster, with lower levels of abnormality, compared to controls reared under ambient conditions. Such responses could be due to either non-Mendelian acclimation (epigenetic change or maternal provisioning) or classical Darwinian/Mendelian selection. The current study takes these observations further by analysing the transcriptional responses of two Sydney rock oyster populations (B2 and wild type) that were reared under ambient or elevated CO_2 conditions over two consecutive generations and then exposed to CO_2 stress immediately prior to gene expression profiling. The work focuses on a set of genes that contribute to the universal intracellular stress response pathway described by Anderson *et al.* (2015) to test the hypothesis that this pathway is also involved in responses to OA. By comparing the transcriptional responses of different breeding lines to CO_2 stress, we were able to investigate whether the potential for adaptive changes vary between populations within species. As such, the current study is one of the first to explore the heritable potential of oysters for acclimation or adaptation to OA. This understanding of the genetic systems responsible for heritable changes in oysters will be crucial for the development of marker-assisted breeding programs to future proof oyster aquaculture industries worldwide. It may also be important for predicting the future trajectories of wild oyster populations as climate change progresses.

3.2 MATERIALS AND METHODS

3.2.1 Animals and transgenerational CO_2 exposures

Selectively bred (B2) and wild type (W) Sydney rock oysters, *Saccostrea glomerata* (Gould 1850), (2.65 ± 0.41 cm shell length, 1.64 ± 0.65 g total weight) were provided by the Port Stephens Fisheries Institute (PSFI, Taylors Beach, NSW, Australia) of the New South Wales Department of Primary Industries (NSW DPI). The B2 breeding line has been mass selected for fast growth and resistance to QX disease (causative agent: *Marteilia sydneyi*) and winter mortality (causative agent currently under review) over seven generations. B2 and W oysters were reared at ambient (385 µatm pCO_2, pH 8.20) or elevated (856 µatm pCO_2, pH 7.90) CO_2 over two consecutive generations (Fig. 3.1) (Parker *et al.* 2015; Parker *et al.* 2012). Founding parents (300 per population) were collected from the field at the beginning of their reproductive season and then held in aquaria for five weeks at ambient or elevated CO_2 until they reached gravid stage. This protocol was likely to encompass a substantial proportion of the gametogenic process in the exposed adults. Gametes from a minimum of 10 females and 10 males from each founding population (ambient or elevated CO_2) were collected by strip spawning and fertilised. The resulting F1 larvae were grown under the same conditions (ambient or elevated CO_2) as their parents. Spat (approx. 5 mm retained) were then transferred into the field (Port Stephens, NSW, Australia) at ambient CO_2 conditions (385 µatm pCO_2, pH 8.20) where they remained for 14 months until they reached reproductive maturity. Oysters were transferred into the field for maturation to ensure sufficient food supply during their growth-out phase. F1 adults (n = 300 per population and CO_2 treatment) were returned to the laboratory, held under ambient or elevated CO_2 and an F2 generation produced as describe above. F2 larvae were again reared under ambient or elevated CO_2 and the spat transferred into the field. Upon reaching juvenile size (2 to 3 cm shell length), F2 oysters were returned to the

laboratory and held in elevated or ambient CO_2 for one week before their tissues (whole body) were sampled for transcriptomic analysis (Fig. 3.1).

3.2.2 CO_2 exposure prior to transcriptional analysis

Juvenile F2 oysters (males and females) from the transgenerational CO_2 (and ambient) exposures (as described in section 3.2.1) were transferred to the Sydney Institute of Marine Science (Chowder Bay, NSW). They were acclimated to aquarium conditions for 10 days in a flow-through seawater system (0.5 L per minute, seawater filtered at 20 µm). Every three days, the nutritional supply from the flow-through seawater was supplemented with a concentrated blend of microalgae (Shellfish Diet® 1800, Reed Mariculture Inc., 4.3×10^8 algal cells per oyster).

Following acclimation, the F2 juveniles were exposed to ambient (387 µatm pCO_2, pH 8.03) and near-future elevated (625 µatm pCO_2, pH 7.87) CO_2 concentrations for one week prior to transcriptional analysis. This exposure period was selected to assess acute responses to elevated CO_2. Elevated seawater CO_2 concentrations were controlled by a custom-made CO_2 system that included pneumatic components from Parker Hannifin (Castle Hill, NSW), CO_2 sensors from Vaisala (Hawthorn, VIC) and a control system from Greenstar Building Automation & Citywide Electrical Services (Marrickville, NSW). CO_2-enriched air was bubbled into three header tanks, each of which supplied a single elevated CO_2 replicate tank (independent water supply for each replicate tank). Each replicate tank (three for each CO_2 condition; ambient and elevated) contained 6 oysters from each of the different founding parental populations and CO_2 treatments (n = 18 oysters per treatment). CO_2-enrichement and water flow were regulated to maintain the target seawater pH during the experiment. Temperature, salinity and carbonate chemistry (pH and total alkalinity) were monitored (Table 3.1). No mortality was observed throughout the experiment.

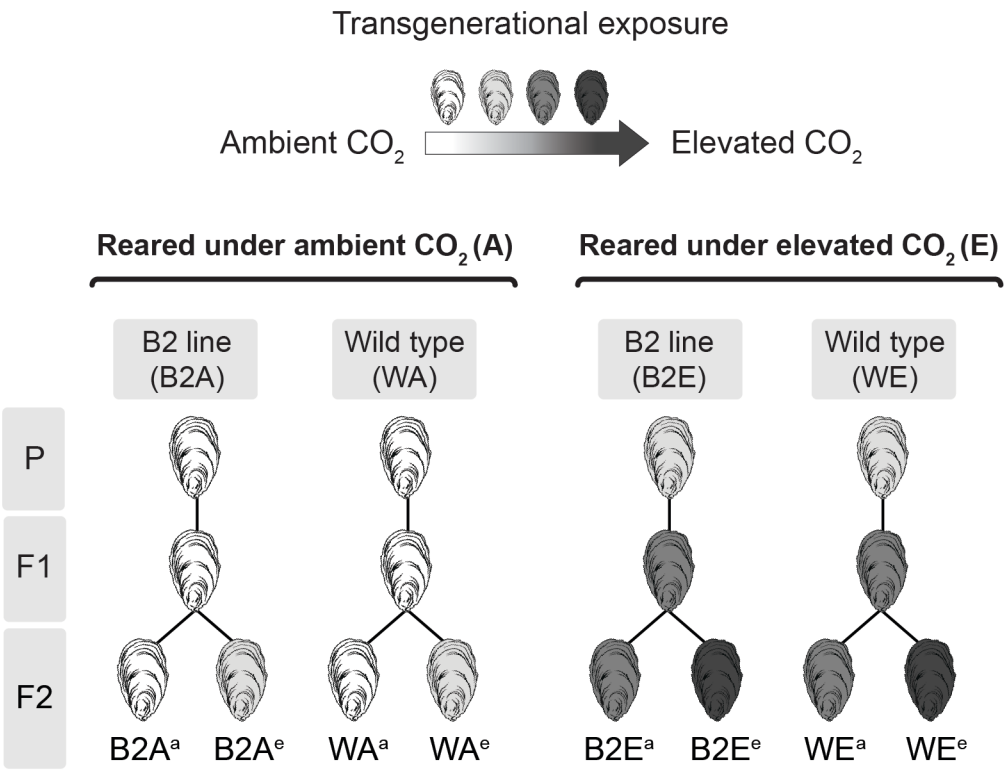

Figure 3.1. Transgenerational experimental design. Selectively bred (B2) and wild type (W) oysters were reared under either ambient or elevated CO_2 conditions over two consecutive generations (parental to F1 and F1 to F2). Exposures to elevated CO_2 conditions were performed during reproductive stage of adults, fertilisation and larval development (adult field grow-out at ambient conditions). Juveniles of the F2 generation were then exposed to ambient or elevated CO_2 immediately prior to transcriptional analysis. The designations used throughout this study for the different treatments are shown at the bottom of the diagram. In these designations, B2 and W represents the two founding parental populations, upper case A or E designates whether that breeding line had been reared under ambient (A) or elevated (E) CO_2 conditions over the two generations, and superscript lower case letters reflects whether F2 juveniles had been exposed to ambient (^{a}) or elevated (^{e}) CO_2 conditions immediately prior to gene expression analysis.

These exposures produced a total of eight treatments (Fig. 3.1). The treatments comprised the two founding parental populations (B2 or W) that had been reared over two consecutive generations under either ambient or elevated CO_2. For instance, B2A represents B2 oysters reared under ambient (A) conditions for two generations and B2E represents B2 oysters reared under elevated (E) CO_2 conditions for two generations. Each of the breeding lines were then exposed to either ambient or elevated CO_2 immediately prior to gene expression analysis,

as designated by the lower case letters [a] for ambient conditions or [e] for elevated CO_2 conditions. Hence, the eight treatments subjected to gene expression analysis were: $B2A^a$, $B2A^e$, $B2E^a$ $B2E^e$, WA^a, WA^e, WE^a and WE^e (Fig. 3.1).

Table 3.1. Seawater chemistry in tanks during ocean acidification trial. Salinity, temperature and pH (NBS scale) were determined once a day using a YSI 63 probe, total alkalinity was measured in an automatic titrator (Metrohm 888 Titrando) and pCO_2 was calculated using co2sys software (Lewis & Wallace 1998). Data are presented as mean ± SD (n = 7 days).

Parameter	Ambient CO_2 (A)	Elevated CO_2 (E)
pH	8.03 ± 0.01	7.87 ± 0.07
Temperature (°C)	22.70 ± 0.30	22.83 ± 0.08
Salinity (ppt)	36.61 ± 1.37	36.15 ± 0.55
Total alkalinity (mmol kg^{-1} SW)	2.20 ± 0.03	2.14 ± 0.02
pCO_2 (µatm)	387.41 ± 9.90	625.21 ± 114.37

3.2.3 RNA extraction and cDNA synthesis

After one week of exposure to elevated or ambient CO_2, oysters were shucked (n = 18 per treatment) and their whole bodies were individually stored in RNA later (Ambion) at -20 °C. The entire body rather than specific tissues was sampled and used for RNA extraction due to the small size of the oysters (juveniles). In addition, oysters were assessed individually (*i.e.* no pooling of tissue from different oysters was performed). Total RNA was extracted using a combination of TRI reagent (Sigma) and illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare Lifesciences). Approximately 100 mg of tissue (whole body) was homogenised in TRI reagent and the upper clear phase was transferred to a spin column after phase separation. RNA was then purified following the illustra RNAspin protocol. Total RNA concentration and purity were checked with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000). Reverse transcription was performed using SuperScript® III First-Strand Synthesis System

(Invitrogen) in a 20 µl reaction volume containing 1 µg of purified total RNA and 50 µM oligo$(dT)_{12–18}$, according to the manufacturer's instructions.

3.2.4 Gene selection and primer design

To assess the transcriptional responses of oysters from the different treatments to CO_2-driven OA, we evaluated the relative expression profiles of 16 genes associated with oxidative stress, mitochondrial energy production and the cytoskeleton. Genes were selected based on models of adaptive responses to environmental stress (Anderson *et al.* 2015; Tomanek 2014; Zhang *et al.* 2012) and on the biological functions shown to be affected by elevated CO_2 (Thompson *et al.* 2015 and Chapter 2 of this thesis). Primers for ecSOD and peroxiredoxin 6 were sourced from Green *et al.* (2009). All other qPCR primers were designed using Primer3 software (Untergasser *et al.* 2012) and were based on contigs from *S. glomerata* 454 sequencing (Hook *et al.* 2014) (Table 3.2).

Table 3.2. Primer sequences used in this study for qPCR.

Gene	Sequence (Fw/Rv: 5'→ 3')	Amplicon (bp)	E[1] (%)	Functional annotation[2]
Catalase	CGCTGACGTGGAGCAGATTG GGCGATGGGTGTCCGAATAA	113	102.1	AD
Glutathione peroxidase	TGGTGGCCGAACTGGTTACA TCAGTACCACCAACTGAATGCA	148	96.5	AD
Glutathione S-transferase omega	CGCTGGAGAAGGACGGAAAG TCCCGAGCTTGTTGGTATGG	115	95.5	AD
Superoxide dismutase (ecSOD)	AACTCTACCACGGCGAGCAT CCACGGTCGTCATCATGAAG	76	98.4	AD
Peroxiredoxin 6	GAAGGATGGAAGGACGGTGAT CACCTGTGGAAACACCTTCTC	82	104.6	AD
Hsp70	TGAATGGACACTCCTGGTTGG TGGGCATTGAAACTGCTGGA	121	99.5	CS
Hsp90	CCCAGAGGATGAGGAGGAGA CAATACAGCAGGGCGATGTC	146	97.1	CS
NADH dehydrogenase	TCCTCCGGTACCCCAGTCAG TGCATCAAGGGGCTATTCCA	150	98.5	EM
Cytochrome c oxidase I (COX1)	TTTCCTACCACGGGATGTG TGAGCTAATACCAGCCAAGTGA	65	104.2	EM
ATP synthase alpha-subunit	CCTCCACTCTCGTCTGTTGG GAGATGACGTTGGTTGGGATG	132	97.5	EM
β-tubulin	GCCATGACGAGGATCACAGG TGTCCCAGAACTCACACAGCAG	70	99.1	C
Myosin	TGGTGGTTGCGACGAGTGTT GGAGGAAGCAGAGGCTGCAC	141	99.6	C
TATA-binding protein (TBP)	GGACTTTGGCTCCTGTAAGCAC AGAATGGTGAAGCCTCGTATTG	73	97.4	PR/TF
Peptidylprolyl isomerase A (PPIA)	CGGAGAAGACCACTTGGCTAGA ATCCATGCCCTCGACGACT	65	104.0	PR/PF
Ribosomal protein 40S SA	GTCTGATGTGGTGGCTGTTGG CAAGGATGGTCACGGCTAATG	70	103.7	PR/RP
Ribosomal protein RL27	GAACCACGATGATGGCACAC TGGGTAACGGTCGATTCCTG	70	104.2	PR/RP
EF1α	CCATAGCGGCATCTCCACTC CCTTGATTGCCACACTGCTC	125	96.9	R/TF
β-actin	GCACCTGAATCGCTCGTTG CAGCAGCATCGTCATCATCC	86	95.3	R/C
GAPDH	ACCGCGCCAGTCTTTGTTG GGCATTGTTGAGGGTCTGATG	90	93.9	R/EM

[1]E = amplification efficiency

[2]AD = Antioxidant defence; CS = Cellular stress; EM = energy metabolism; C = Cytoskeleton; TF = Transcription factor; PF = Protein folding; RP = ribosomal protein; PR= Potential reference gene; R = Reference gene.

3.2.5 qPCR cycling conditions

qPCR assays were conducted on Bio-Rad CFX Real-time system (Bio-Rad). The 10 µl reaction mixtures contained 5 µl KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 300 nM each primer, 3.4 µl PCR grade water and 1 µl cDNA template (diluted 1:2). The cycling program used consisted of 3 min at 95 °C followed by 40 cycles of 95 °C for 10 s, 30 s at 58 or 60 °C and 30 s at 72 °C. Melting curve analysis was performed at the end of the qPCR cycles to confirm primer specificity by collecting fluorescence data between 65 - 95 °C at 0.5 °C increments. Standard curves were generated in triplicate for each primer pair using two-fold serially diluted cDNA as template, and reaction efficiencies (E) were subsequently calculated (Table 3.2). Amplification data were analysed using the Bio-Rad CFX Real-time system software to obtain Cq values.

Reference gene stability was assessed using the web-based RefFinder platform, which integrates results from different available algorithms (Xie *et al.* 2012). We tested the expression levels of seven potential reference genes on oysters from both populations exposed to either ambient or elevated CO_2: TATA-binding protein (TBP), peptidylprolyl isomerase A (PPIA), ribosomal protein 40S SA, ribosomal protein RL27, elongation factor 1 alpha (EF1α), β-actin and GAPDH. After this evaluation, elongation factor 1 alpha (EF1α), β-actin and GAPDH were selected as reference genes (geNorm stability values = 0.908, 0.908 and 0.988; NormFinder stability values = 1.304, 0.789 and 0.872; Stability values by ΔCt method / Average SD = 1.88, 1.70 and 1.77, respectively).

Data are presented as changes in relative expression normalized with the geometric mean of the Cq values of the three reference genes (Livak & Schmittgen 2001). An inter-run calibrator was analysed in every plate to account for technical variation. Statistical significance of differences between treatments was assessed by Student's t test and one-way ANOVA followed by Tukey's test, at $p < 0.05$. To visualise overall differences between populations and CO_2 exposure histories, we performed a non-metric multi-dimensional scaling (NMDS)

analysis including the expression levels of differentially expressed genes (absolute fold-change ≥ 1.4). NMDS was based on Bray-Curtis similarity coefficients calculated from gene expression levels of oysters from the same exposure tank (3 tanks with 6 oysters each per condition).

3.3 RESULTS

3.3.1 Effects of conditioning and CO_2 exposure on oyster transcriptional profiles

The transcriptional responses of B2 and wild type oysters reared under ambient or elevated CO_2 were analysed to study the molecular basis of transgenerational responses to OA. Exposure to elevated CO_2 immediately prior to transcriptional analysis induced different responses in oysters that had been reared under elevated CO_2 relative to those with no history of conditioning to CO_2 stress. Such differences in gene expression profiles were evident in both breeding lines (Fig. 3.2). Following exposure to CO_2 stress, B2 oysters reared under elevated CO_2 ($B2E^e$; transgenerational conditioning) exhibited higher expression of all differentially regulated genes compared to non-conditioned B2 oysters ($B2A^e$). The differentially expressed genes included cytochrome c oxidase I (COX1; 5.3-fold), peroxiredoxin 6 (2.8-fold), extracellular superoxide dismutase (ecSOD; 2.4-fold), NADH dehydrogenase (2.3-fold) and glutathione peroxidase (1.9-fold) (Fig. 3.2b). These differences resulted in a clear spatial separation of the transcriptional profiles of $B2E^e$ and $B2A^e$ oysters in NMDS plots (Fig. 3.2a).

Transgenerational conditioning to CO_2 stress also affected the transcriptional responses of wild type oysters. However, contrary to what was observed in B2 oysters, exposure to elevated CO_2 immediately prior to qPCR analysis (^{e}) resulted in lower expression of all differentially regulated genes in CO_2-conditioned wild type oysters (WE^e) when compared to non-conditioned oysters (WA^e). Five out of the 10 genes (50%) found in higher concentrations in $B2E^e$ (relative to $B2A^e$) were found in lower concentrations in WE^e (relative to WA^e). The genes with a contrasting response between populations included COX1 (5.3-fold in $B2E^e$ and

0.6-fold in WE^e), NADH dehydrogenase (2.3-fold in $B2E^e$ and 0.62-fold in WE^e) and glutathione peroxidase (1.9-fold in $B2E^e$ and 0.5-fold in WE^e) (Fig. 3.2b). In addition to those genes, transcription levels for Hsp90 (0.6-fold), Hsp70 (0.6-fold) and glutathione transferase omega (0.5-fold) were also lower in WE^e compared to WA^e (Supplementary Figure 3.1). As a result of these contrasting responses between breeding lines, NMDS indicated that the transcriptional profile of B2 oysters that had been reared under ambient conditions and exposed to elevated CO_2 immediately before gene expression analysis ($B2A^e$) was more closely related to that of wild type oysters that had been conditioned and exposed to elevated CO_2 prior to qPCR (WE^e). Similarly, the cumulative expression profile for the WA^e treatment resembled that of $B2E^e$ oysters (Fig. 3.2a).

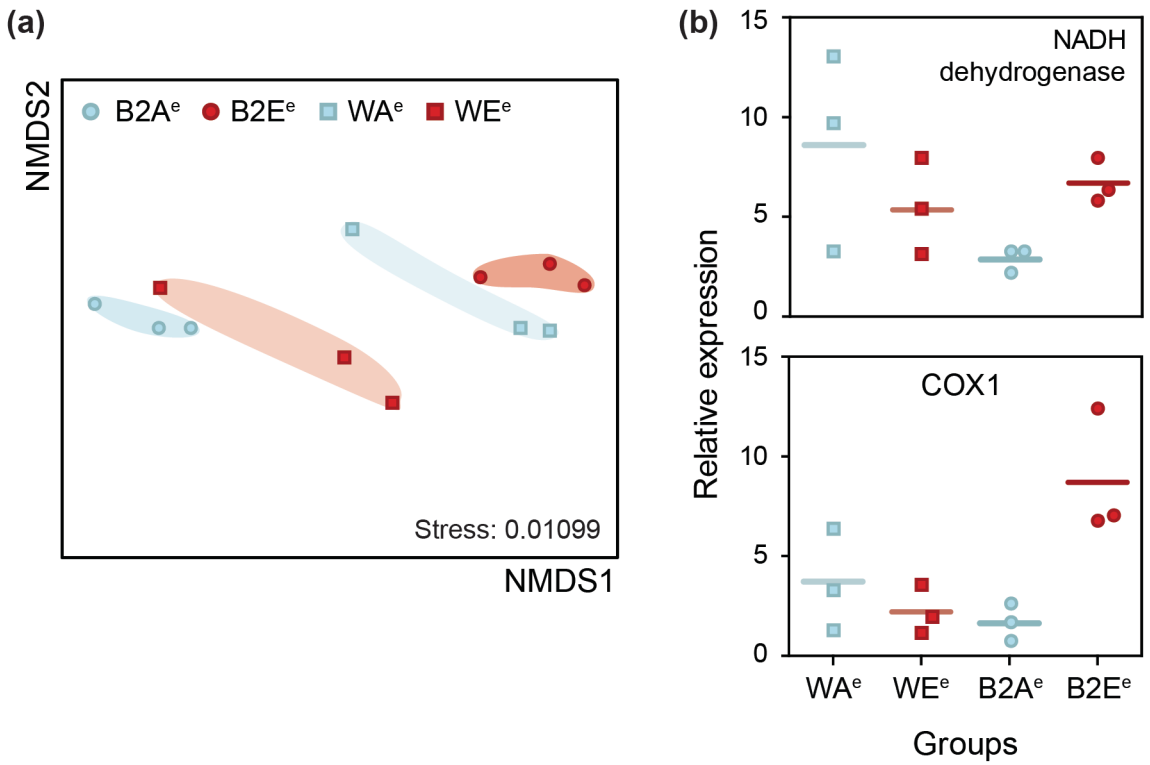

Figure 3.2. Effects of conditioning and CO_2 exposure on the transcriptional profiles of oysters derived from the B2 and wild type (W) lines. **(a)** Non-metric multidimensional scaling plot (NMDS) summarises the cumulative expression profiles of differentially regulated genes among B2 and W breeding lines that had been reared under ambient (B2A and WA) or elevated CO_2 (B2E and WE) and then exposed to elevated CO_2 immediately before analysis by qPCR. Each point represents the mean expression levels in oysters from the same exposure tank (3 tanks with 6 oysters each per treatment). **(b)** Relative expression levels for two arbitrarily selected genes (bar = mean).

3.3.2 Differential effects of transgenerational CO_2 conditioning between breeding lines

B2 and wild type oysters exhibited different gene expression profiles independent of their histories of CO_2 exposure (Fig. 3.3a). Differences in gene expression profiles between populations were evident even under full control conditions, when F2 juveniles and their preceding generations had never been exposed to elevated CO_2 (Fig. 3.3a,b). In this comparison, the expression of TATA-binding protein (TBP) (2.2-fold), ribosomal protein RL27 (1.5-fold), glutathione transferase omega (1.4-fold), COX1 (1.4-fold) and NADH dehydrogenase (1.4-fold) was higher in the B2 line ($B2A^a$) compared to wild type oysters (WA^a; Fig. 3.3a,b). Conversely, transcript levels of ecSOD (0.6-fold), peroxiredoxin 6 (0.7-fold) and Hsp90 (0.7-fold) were lower in $B2A^a$ oysters relative to WA^a oysters. As a result of these differences, NMDS plots showed spatial separation of the gene expression profiles for the $B2A^a$ and WA^a treatments (Fig. 3.3a).

Such differences in transcriptional responses were even more evident in breeding lines reared under elevated CO_2 and exposed again to this stress immediately prior to qPCR. The transgenerational conditioning of B2 oysters to elevated CO_2 ($B2E^e$) resulted in higher expression levels for all differentially expressed genes (fold-change $\geq$ 1.4) relative to the corresponding wild type treatment (WE^e). This higher transcript abundance was observed for genes from all three functional categories; antioxidant defence (ecSOD, 2.3-fold; catalase, 1.8-fold; peroxiredoxin 6, 1.6-fold; and glutathione peroxidase, 1.5-fold), energy metabolism (COX1, 4-fold) and the cytoskeleton (tubulin, 1.5-fold) (Fig. 3.3c,d). Interestingly, two genes that were initially tested as potential reference genes also had higher expression levels in $B2E^e$ oysters. These were a ribosomal protein (RL27; 4.2-fold) and a molecule involved in protein folding, peptidylprolyl isomerase A (PPIA; 3.2-fold). The differences between breeding lines resulted in a clear spatial delineation between $B2E^e$ and WE^e oysters in NMDS plots (Fig. 3.3a).

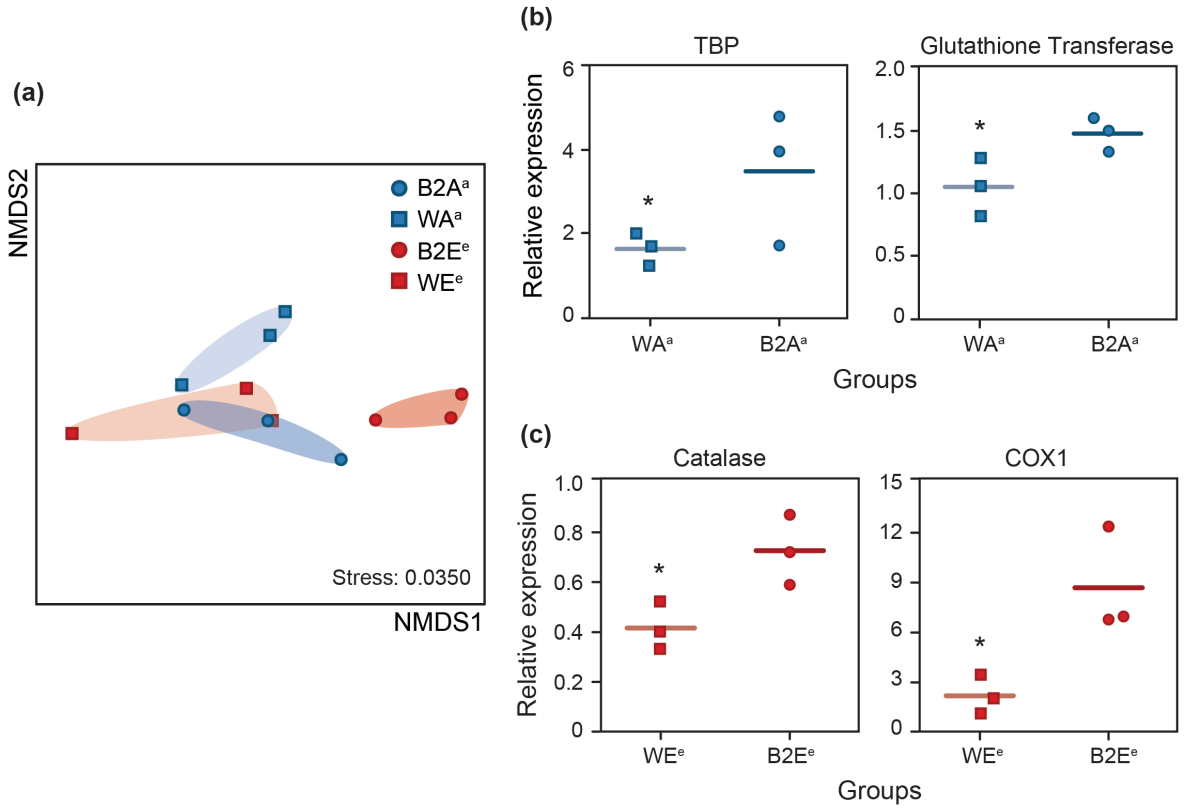

Figure 3.3. Differential gene expression profiles of B2 and wild type (W) oyster breeding lines following transgenerational conditioning to elevated CO_2. Data for differentially expressed genes is summarised in a non-metric multidimensional scaling (NMDS) plot **(a)**. Examples of differential expression for specific genes are shown for oysters reared under ambient conditions ($B2A^a$ and WA^a) **(b)** and oysters reared under elevated CO_2 conditions ($B2E^e$ and WE^e) **(c)**. Results are presented in univariate scatterplots as mean relative expression levels (bars) within each condition and each point represents the relative mean expression levels of oysters from the same exposure tank (3 tanks with 6 oysters each per condition). Asterisks indicate significant differences (Student's t-test, $p < 0.05$).

3.3.3 Transgenerational effects of elevated CO_2 on B2 oysters

Comparisons of gene expression between groups of oysters originating from the B2 parental line are shown in Figure 3.4. Substantial differences in gene expression were observed between B2 oysters that had never been exposed to elevated CO_2 ($B2A^a$) and those in which F2 juveniles and their preceding generations had been exposed to elevated CO_2 ($B2E^e$) (Fig. 3.4a). In this comparison, the expression of most differentially expressed genes was higher in $B2E^e$ oysters than in the $B2A^a$ treatment. The differential genes in this comparison included COX1

(3.9-fold), RL27 (2.8-fold), catalase (2-fold), PPIA (2-fold), ecSOD (1.9-fold) and peroxiredoxin 6 (1.4-fold) (Fig. 3.4b). Only two genes exhibited higher expression levels in the $B2A^a$ treatment when compared to $B2E^e$ oysters (glutathione S-transferase omega, 1.5-fold; and TBP, 1.7-fold) (Supplementary Figure 3.1). These differences resulted in a clear spatial separation between $B2A^a$ and $B2E^e$ oysters in NMDS plots (Fig. 3.4a).

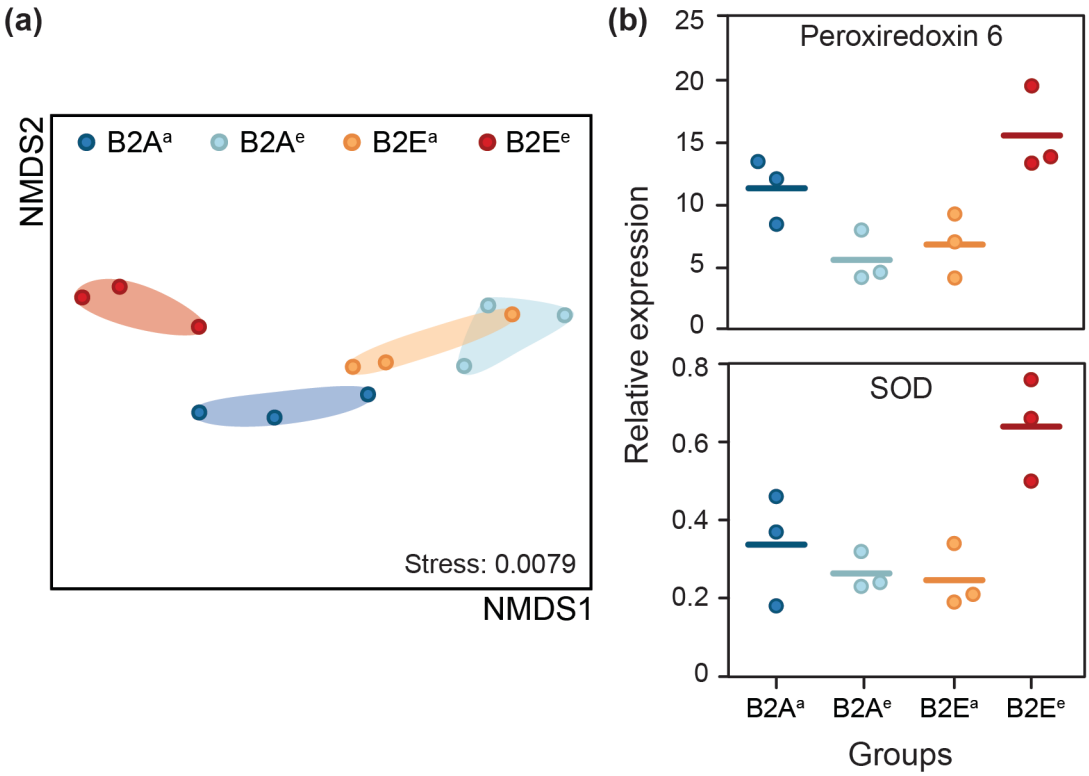

Figure 3.4. Effects of elevated CO_2 conditioning and exposure on gene expression in B2 oysters. **(a)** Non-metric multidimensional scaling plot (NMDS) showing the cumulative expression profiles of differentially regulated genes among B2 breeding lines that had been reared under elevated CO_2 and then exposed to either ambient ($B2E^a$) or elevated CO_2 ($B2E^e$) immediately before analysis by qPCR. Also shown are data for B2 oysters reared under ambient conditions and then exposed to either ambient ($B2A^a$) or elevated CO_2 ($B2A^e$) immediately before gene expression analysis. Each point represents the mean expression levels in oysters from the same exposure tank (3 tanks with 6 oysters each per condition). **(b)** Relative expression levels for two arbitrarily selected genes (bar = mean).

B2 oysters reared for two generations under ambient conditions and then exposed to elevated CO_2 immediately prior to gene expression analysis ($B2A^e$) had very similar expression profiles to B2 oysters reared under elevated CO_2 but exposed to ambient conditions prior to qPCR ($B2E^a$). This is reflected by the substantial overlap between these two treatments in NMDS plots (Fig. 3.4a). Eleven out of the 16 genes tested (69%) did not differ significantly in expression between $B2E^a$ and $B2A^e$ oysters, including peroxiredoxin 6, ecSOD, catalase and COX1 (Fig. 3.4b). As a result, the cumulative expression profiles for the $B2E^a$ and $B2A^e$ treatments revealed in NMDS plots were distinct from those for $B2A^a$ and $B2E^e$ oysters. $B2E^e$ oysters were the most spatially separated from the $B2E^a$/$B2A^e$ cluster in NMDS plots (Fig. 3.4a).

3.3.4 Transgenerational effects of elevated CO_2 on wild type oysters

Wild type breeding lines reared under elevated or ambient CO_2 over two generations exhibited more inter-individual variability to CO_2 exposure than B2 oysters. This is reflected by the lower number of genes that exhibited significant differences in expression and the substantial overlap between the treatments in NMDS plots (Fig. 3.5a).

Overlapping NMDS ordinates were evident between all of the wild type treatments. NMDS plots also revealed that gene expression was highly variable in this breeding line, especially in the WE^a treatment (Fig. 3.5a). Wild type oysters that had never been exposed to elevated CO_2 (WA^a) were most distinct from the other treatments in NMDS plots. This distinction was a reflection of differential expression of individual genes. For instance, the expression of ecSOD (2-fold) and peroxiredoxin 6 (1.8-fold) were higher in WA^a compared to the WE^e treatment. In addition, glutathione peroxidase (0.6-fold) and NADH dehydrogenase (0.6-fold) were found in lower levels in WA^a relative to the WA^e and WE^a treatments (Fig. 3.5b) (Supplementary Figure 3.1).

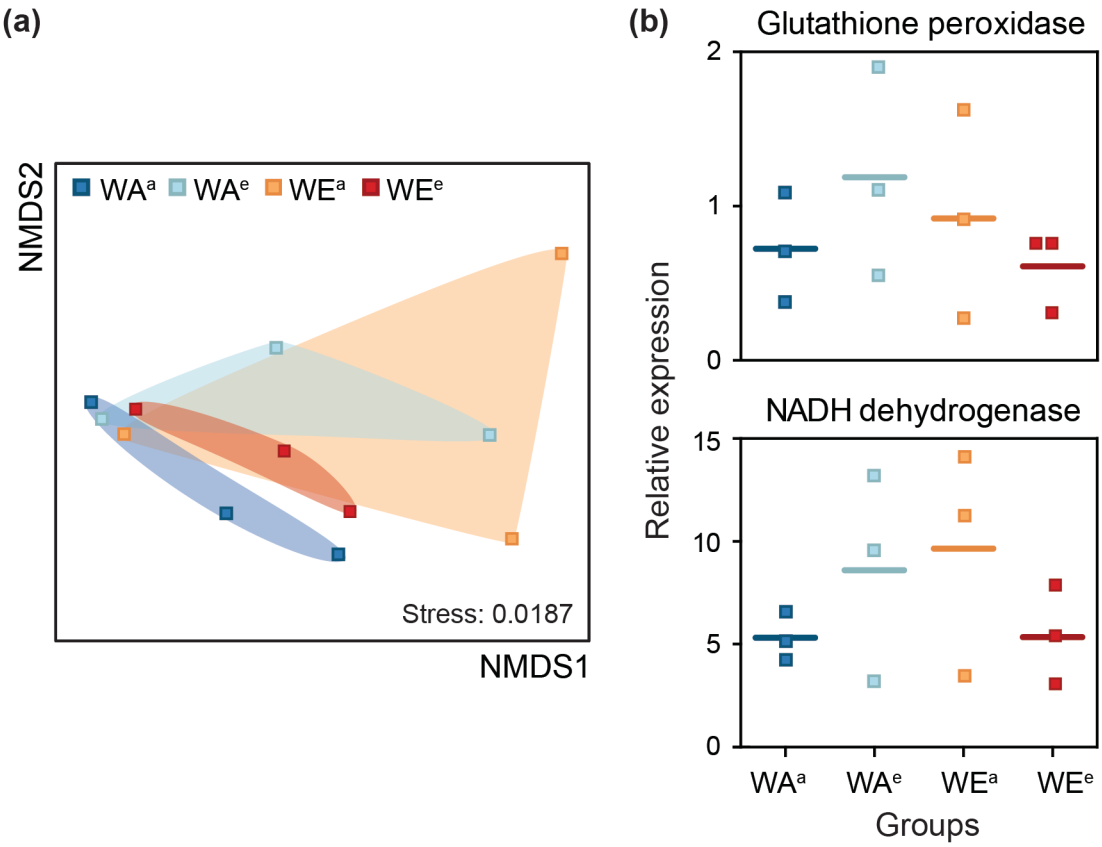

Figure 3.5. Effects of elevated CO_2 conditioning and exposure on gene expression in wild type (W) oysters. **(a)** Non-metric multidimensional scaling plot (NMDS) showing the cumulative expression profiles of differentially regulated genes among W breeding lines that had been reared under elevated CO_2 and then exposed to either ambient (WE^a) or elevated CO_2 (WE^e) immediately prior to qPCR analysis. Data for W oysters reared under ambient conditions and then exposed to either ambient (WA^a) or elevated CO_2 (WA^e) immediately before gene expression analysis are also shown. Each point represents the mean gene expression levels of oysters from the same exposure tank (3 tanks with 6 oysters each per condition). **(b)** Relative expression levels for two arbitrarily selected genes (bar = mean).

3.4 Discussion

Our study begins to explore the potential of oysters for acclimation or adaptation to OA, by describing the transgenerational effects of elevated CO_2 on their transcriptional responses. Previous work has shown that more than twenty years (seven generations) of artificial selection in Sydney rock oysters for fast growth and disease resistance has coincidentally resulted in resilience against CO_2 stress (Parker *et al.* 2012; Parker *et al.* 2011). The emergence of CO_2

resilience in oysters bred for other phenotypic traits suggests that responses to elevated CO_2 may be mediated by the same intracellular system that is responsible for responses to a range of environmental stressors (Anderson *et al.* 2015; Tomanek 2014; Zhang *et al.* 2012). If that is the case, CO_2 resilience, disease resistance and fast growth would all rely on the differential regulation of a common set of genes involved in energy production, antioxidant defence and the cytoskeleton.

In the current study, we tested this concept by performing transgenerational exposures of the same selectively bred (B2 line) and wild type (non-selected) oysters to elevated CO_2 in order to assess their phenotypic plasticity and adaptive capacity in response to OA. We evaluated the transcriptional profiles of these oysters using a panel of genes involved in the universal stress response, testing the hypothesis that this subcellular pathway contributes to transgenerational CO_2 acclimation or adaptation, as it does for other forms of environmental stress. By analysing the two genetically distinct populations using this targeted set of genes, we found that transgenerational exposure to CO_2 stress induces rapid and substantial changes in gene expression. In addition, these changes were shown to vary between breeding lines and to be mediated by parental conditioning.

Transgenerational exposure to elevated CO_2 resulted in divergent expression profiles of key target genes in B2 line and wild type oysters. Exposing transgenerationally-conditioned B2 oysters to CO_2 stress led to higher levels of expression among genes involved in multiple stress-related functions compared to non-conditioned B2 oysters. This up-regulation of key target genes in B2 oysters may be an indicative of increased metabolic activity and concurrent oxidative cellular stress responses induced by exposure to elevated CO_2 over three consecutive generations. In contrast, transgenerationally-conditioned wild type oysters exhibited lower expression of numerous stress-related genes following exposure to CO_2 relative to non-conditioned wild type oysters. These findings indicate that the acute impacts (within the F2 generation) of elevated CO_2 on oyster transcriptional responses is affected by transgenerational

(parental) conditioning, though in opposite directions between populations. While CO_2-conditioned B2 oysters show up-regulation of a variety of genes, CO_2-conditioned wild types exhibit widespread down-regulation in gene expression under CO_2 stress.

This contrasting response between populations is consistent with our recent work, which has revealed that CO_2 stress has distinct subcellular effects in wild type and selectively bred oysters. Exposure to elevated CO_2 (760 µatm pCO_2, pH 7.78; single exposure) induced changes in the mitochondria and systems associated with oxidative stress in selectively bred oysters, but not in wild type oysters (Chapter 2). In addition, Thompson *et al.* (2015) recently showed that the majority of proteins differentially regulated by CO_2 stress (856 µatm pCO_2, pH 7.84; single exposure) were found at lower concentrations in B2 oysters, but at higher concentrations in wild types, relative to their respective controls (ambient CO_2). The differentially regulated proteins included a number involved in mitochondrial electron transport chain, oxidative stress and protein synthesis. The current study complements those findings by revealing the differential expression of genes involved in intracellular energy production and antioxidant defence. The data indicate that low seawater pH affects biological functions in oysters at the cellular, protein and transcriptional levels in different magnitudes and directions. They also suggest that the differences identified in the gene expression profiles of B2 and wild type oysters may be due to an inherent capacity for increased intracellular energy production or adaptive energy reallocation in the B2 population, which could explain their distinctive performance to OA previously reported by Parker *et al.* (2011).

Differences in gene expression were also evident among B2 oysters that had differing histories of exposure to elevated CO_2. The expression of ecSOD, catalase and peroxiredoxin 6 were significantly higher in B2 oysters that had been exposed to elevated CO_2 over three generations (parental, F1 and F2) compared to oysters from the same breeding line that had no history of exposure to elevated CO_2. The extent of this transgenerational change was such that the gene expression profiles of oysters reared under elevated CO_2 but exposed to ambient

conditions immediately prior to molecular analysis were equivalent to those of oysters reared under ambient conditions and then exposed to elevated CO_2. These findings indicate that transgenerational exposure to CO_2 stress alters the transcriptional profile of B2 oysters, shifting both the basal and inducible expression of important genes. More importantly, our data suggest that the acute effects of elevated CO_2 (within the F2 generation) were determined by the CO_2 conditions that the parents experienced. This phenomenon is reflected in the similar gene regulation patterns found in oysters that were held under ambient conditions immediately prior to qPCR after their parents had been conditioned to CO_2 stress, and those exposed to elevated CO_2 immediately before qPCR after their parents had been conditioned under ambient CO_2 conditions. Such comparisons make it clear that the exposure histories of their parents to CO_2 stress mediated the molecular response of the F2 juveniles.

Amelioration of OA impacts by parental conditioning has been previously reported in fish (Allan *et al.* 2014; Donelson *et al.* 2012; Miller *et al.* 2012; Schade *et al.* 2014) (but not by (Welch *et al.* 2014)), and in oysters (Parker *et al.* 2015; Parker *et al.* 2012). However, these previous studies have analysed such effects at the physiological level only, particularly investigating how the parental environment alters the survival, growth and development of their offspring. Our study is the first to explore the biological mechanisms underlying transgenerational response to OA. In a similar context, Veilleux *et al.* (2015) recently described the molecular processes involved in transgenerational conditioning of a reef fish to thermal stress using transcriptome sequencing. Metabolism-, immune- and stress-related genes were found to be up-regulated transgenerationally, indicating shifts in energy production and allocation in response to elevated temperature. Our work focused on a targeted set of genes to test the hypothesis that a previously defined intracellular pathway was involved in CO_2 acclimation or adaptation. However, more comprehensive studies similar to that undertaken by Veilleux *et al.* (2015) using next generation transcriptomics should also be performed to

identify the broader molecular basis of transgenerational conditioning of marine organisms in the context of OA.

Wild type oysters responded differently to elevated CO_2 compared to the B2 line. While most genes were found at higher expression levels in CO_2-exposed B2 oysters relative to ambient CO_2, the transcript abundance of most differentially expressed genes in CO_2-exposed wild type oysters was lower than under ambient conditions. Wild type F2 juveniles whose preceding generations had been exposed to elevated CO_2 exhibited a down-regulation of genes encoding antioxidant proteins (ecSOD and peroxiredoxin 6) relative to wild types that had no history of exposure to elevated CO_2. In contrast, higher expression was observed for a number of genes in CO_2-conditioned oysters that were held under ambient conditions before qPCR analysis, as well as among non-conditioned oysters exposed to elevated CO_2, relative to wild types with a full or no history of exposure to CO_2 stress. These effects of transgenerational conditioning of wild type oysters to CO_2 stress were much less clear than those in B2 oysters, primarily due to inter-individual variability among wild type oysters. This suggests that the two parental populations have different potentials for heritable change. Their different trajectories in response to transgenerational exposure to elevated CO_2 could result from their distinct starting positions in terms of gene regulation. Substantial differences in gene expression were evident between wild type and B2 oysters even when they had no previous exposure to elevated CO_2. These differences in baseline gene expression no doubt reflect the long history of mass selection of B2 oysters for disease resistance and faster growth. Further studies assessing the expression of these genes in adults and in following generations should be performed in order to evaluate whether such transcriptional responses have a genetic basis and are heritable.

In addition to characterising transcriptional responses to elevated CO_2, we have identified eight putative stress-related genes that are differentially expressed between breeding lines in which F2 juveniles and their parental generations had never been exposed to elevated CO_2. The differential expression of these genes confirms our hypothesis that the intracellular

stress response to which they contribute plays a role in conditioning to OA. The baseline expression of cytochrome c oxidase I (COX1) and NADH dehydrogenase, genes involved in energy metabolism, was higher in the B2 line compared with wild type oysters. In contrast, genes associated with antioxidant stress, including extracellular superoxide dismutase (ecSOD) and a non-selenium glutathione peroxidase, peroxiredoxin 6, had lower basal expression levels in the B2 line than in wild type oysters. Differences in expression of antioxidant proteins between these two populations of Sydney rock oysters have been previously analysed using suppression subtractive hybridisation. Green *et al.* (2009) found a lower baseline expression for peroxiredoxin 6, but increased levels for ecSOD in QX disease resistant oysters (fourth generation) compared with wild types. Our findings indicate that the selective pressure for fast growth and resistance to QX disease over seven generations has altered the expression of genes involved in energy metabolism and antioxidant stress.

The differences evident in molecular responses between populations might also reflect distinct energy budget constraints due to higher maintenance costs under acidifying ocean conditions. In our study, CO_2 stress elicited higher expression levels of a suite of energy- and stress-related genes in the B2 line but lower expression of the same genes in wild type oysters. Some evidence shows that OA resilience in marine calcifiers comes at a metabolic cost, suggesting energy allocation trade-offs between CO_2 resilience and other physiological energy-demanding processes (Holcomb *et al.* 2010; Pedersen *et al.* 2014; Thomsen *et al.* 2013). Parker *et al.* (2015) recently reported such a trade-off by comparing the effects of transgenerational CO_2 exposure on different physiological parameters in wild type Sydney rock oysters. Wild type larvae, spat and adults that were exposed to elevated CO_2 over two consecutive generations were better able to cope with CO_2 stress (*i.e.* developed and grew at a faster rate, had a lower percentage abnormality and better regulated their extracellular pH) compared to oysters that were exposed to elevated CO_2 over a single generation only. In the current study, transgenerationally-conditioned wild type oysters exhibited reduced expression of a number of

stress-related genes following exposure to elevated CO_2, compared to non-conditioned wild type oysters. Such down-regulation of genes does not seem to be diminishing the physiological performance of these oysters, as shown by Parker *et al.* (2015). Similar physiological studies should be performed with transgenerationally-conditioned B2 oysters to examine whether the widespread increase in gene expression observed in response to CO_2 stress has implications on their overall fitness (*e.g.* growth, survival, calcification). The capacity of marine organisms to tolerate decreasing pH appears to be a result of differences in gene regulation, with energy being diverted from reproduction to systems involved in extracellular acid-base regulation (Parker *et al.* 2015; Somero 2010). If that is the case, the differential transcriptional responses of transgenerationally-conditioned B2 and wild type oysters are likely to produce distinct physiological outcomes. As such, the tolerance or susceptibility of oysters to OA could then be predicted based on the expression levels of key target genes, including those identified in the current study.

The long generation time of molluscs hinders the development of transgenerational experiments. Long-term exposures (more than 3 months) of molluscs to CO_2 stress are rare (Beniash *et al.* 2010; Hiebenthal *et al.* 2013; Michaelidis *et al.* 2005; Shirayama & Thornton 2005) and most studies conducted so far assess phenotypic responses in single-generation experiments. Multigenerational exposures to elevated CO_2 have been conducted predominantly in marine calcifiers with short generation times, such as the coccolithophores *Emiliania huxleyi* (Benner *et al.* 2013; Lohbeck *et al.* 2013; Lohbeck *et al.* 2012) and *Gephyrocapsa oceanica* (Jin *et al.* 2013). Only a few recent studies have assessed larval performance following transgenerational conditioning to OA in marine calcifiers with longer generation times, such as corals (Putnam & Gates 2015), sea urchins (Dupont *et al.* 2013), mussels (Sunday *et al.* 2011) and oysters (Parker *et al.* 2015; Parker *et al.* 2012). In most of these studies, parental conditioning to OA has ameliorated the typical impacts of CO_2 stress on the offspring and/or

following generations. This is in line with the transcriptional responses revealed in the current study.

In summary, this study assessed the molecular response of B2 and wild type oysters after three generations of exposure to CO_2 stress. We found that transgenerational exposure of oysters to elevated CO_2 results in substantial, but opposite changes in the regulation patterns of many stress-related genes between breeding lines. Overall effects of transgenerational conditioning were more pronounced in oysters derived from a selectively bred parental population. Resuls indicate that B2 oysters are more responsive to long-term, transgenerational exposure (conditioning) to OA, which could enable this population to better cope with climate change. Our findings begin to uncover the key intracellular processes responsible for heritable resilience and susceptibility to OA. Understanding the biological mechanisms associated with these processes will be critical for minimising future environmental and economic impacts, and for predicting future evolutionary responses of marine organisms to climate change.

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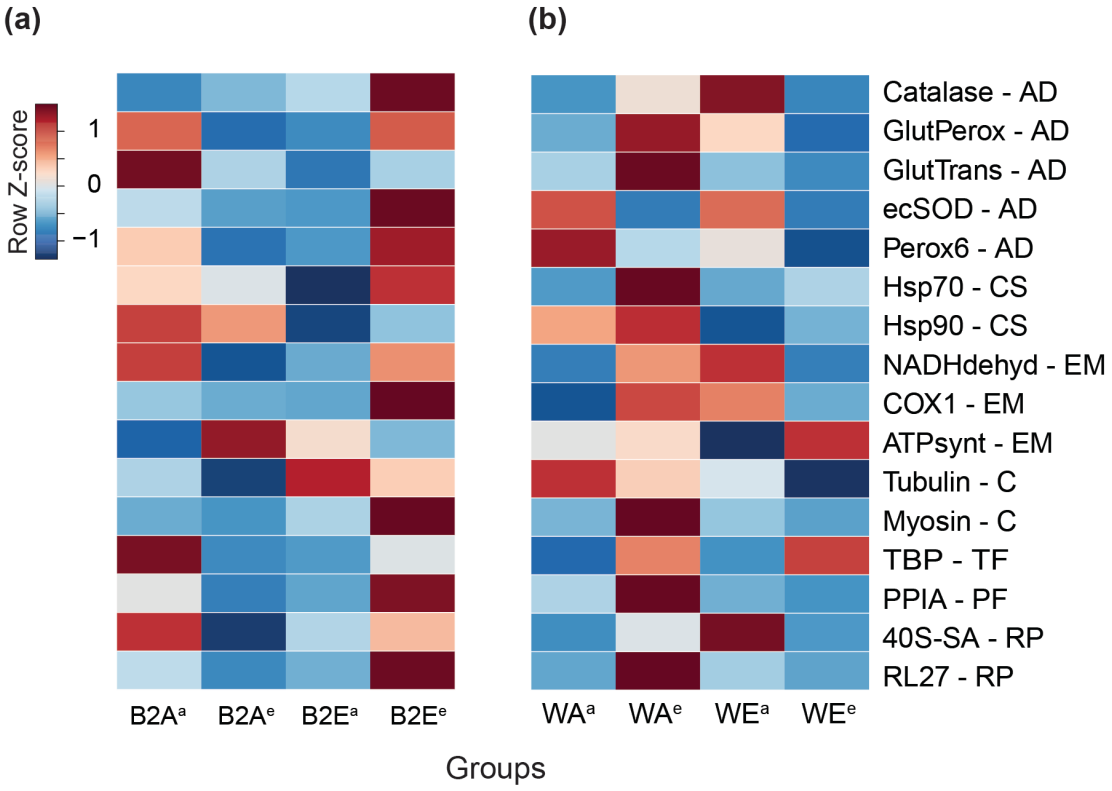
SUPPLEMENTARY INFORMATION



Supplementary Figure 3.1. Heat map of relative gene expression of Sydney rock oysters following transgenerational exposure to elevated CO_2. Transcriptional profiles of B2 **(a)** and wild type (W) **(b)** breeding lines that had been reared under elevated CO_2 (E) and then exposed to either ambient ($B2E^a$ and WE^a) or elevated CO_2 ($B2E^e$ and WE^e) immediately before analysis by qPCR. Also shown are data for B2 oysters reared under ambient (A) conditions and then exposed to either ambient ($B2A^a$ and WA^a) or elevated CO_2 ($B2A^e$ and WA^e) immediately before gene expression analysis. Gene names and their associated cellular functions are detailed in Table 3.2.

CHAPTER 4

CLIMATE CHANGE RESILIENT OYSTERS: TRANSCRIPTOMIC PROFILING OF TRANSGENERATIONAL RESPONSES TO OCEAN ACIDIFICATION

Prepared for submission to Global Change Biology as: Goncalves P, Jones DB, Thompson EL, Raftos DA. Climate change resilient oysters: Transcriptomic profiling of transgenerational responses to ocean acidification.

ABSTRACT

Some populations of marine organisms appear to have an inherent capacity for acclimation or adaptation to stressful environmental conditions, including those associated with climate change. Sydney rock oysters selectively bred for fast growth and disease resistance (the B2 line) exhibit coincidental resilience to ocean acidification (OA) at the physiological level. To understand the molecular basis of this physiological resilience, we performed a comprehensive transcriptomic analysis of B2 oysters that had been exposed to near-future projected ocean pH over two consecutive generations. Our results suggest that the distinctive performance of B2 oysters in the face of OA is mediated by the selective regulation of genes involved in a variety of cellular processes. Subsequent high-throughput qPCR revealed substantial differences in adaptive responses between B2 and wild type (non-selected) oysters that had been subjected to transgenerational CO_2 conditioning. These differential responses between populations uncovered a set of genes that was affected by CO_2 stress exclusively in B2 oysters, and so may be associated with their resilience to OA. The intracellular processes mediated by these genes primarily involve control of the cell cycle and maintenance of cellular homeostasis through damage mitigation. This is the first study to identify the broad set of molecular processes underlying transgenerational conditioning and potential resilience to OA in a marine calcifier. Our findings reveal the intracellular functions that may enable these organisms to cope with a rapidly changing ocean.

4.1 INTRODUCTION

Climate-induced environmental changes are threating marine organisms worldwide. Shifts in the fundamental chemistry and temperature of the oceans due to climate change are particularly damaging to marine calcifiers, including oysters, since the production of their shells is hampered by reducing seawater pH. Ocean acidification (OA) also affects the acid–base balance and energy metabolism of oysters (Fabry 2008; Pörtner *et al.* 2004), and has downstream impacts on immune responses, reproduction and development (Fabry 2008; Gazeau *et al.* 2013; Kurihara 2008; Miller *et al.* 2009; Parker *et al.* 2013).

Omics analyses have also revealed a range of inducible molecular responses in oysters to short-term CO_2 stress. Proteomic studies have shown that elevated CO_2 (pH 7.3 to 7.87) induces differential regulation of proteins involved in antioxidant defence, metabolism and the cytoskeleton in the oysters *Crassostrea virginica*, *Crassostrea hongkongensis*, *Crassostrea gigas* and *Saccostrea glomerata* (Dineshram *et al.* 2015; Dineshram *et al.* 2013; Dineshram *et al.* 2012; Thompson *et al.* 2015; Timmins-Schiffman *et al.* 2014; Tomanek *et al.* 2011; Wei *et al.* 2015). Other studies have used transcriptional approaches to understand the response of oysters to OA. *C. virginica* adults exhibited changes in the expression of genes involved in biomineralisation (carbonic anhydrase; up-regulation in mantle, down-regulation in gills), stress (down-regulation of Hsp70) and immune (down-regulation of integrin) responses under CO_2 stress (3,523 µatm pCO_2, pH 7.5 and 2,000 µatm pCO_2, pH 7.6, respectively) (Beniash *et al.* 2010; Ivanina *et al.* 2014). Comprehensive transcriptomic analyses have also assessed the response of *C. virginica* and *C. gigas* to a combination of low pH and elevated temperature. Decreasing pH has been shown to enhance immune functions and the production of antioxidants in *C. gigas*, while the combination of both pH and thermal stress induced the expression of protease inhibitors and cytoskeleton-related genes in *C. gigas* (Clark *et al.* 2013). Acidification

also enhances the expression of genes involved in antioxidant and metabolic processes in *C. virginica* (Chapman *et al.* 2011).

These data suggest that OA has substantial impacts on the proteomes and transcriptomes of oysters. However, previous studies have not addressed the potential for transgenerational change at the level of the transcriptome. The potential for transcriptional acclimation or adaptation to OA is implied by differential phenotypic responses observed in genetically distinct populations within species of oysters. Sydney rock oysters (*Saccostrea glomerata*) produced through selective breeding for fast growth and disease resistance (the B2 breeding line) are less affected by CO_2 stress than wild type (W, non-selected) oysters (Parker *et al.* 2012; Parker *et al.* 2011). B2 larvae have higher survival rates and grow faster than wild type larvae when exposed to elevated CO_2 (Parker *et al.* 2011). In addition, B2 adults have higher standard metabolic rates (SMR) than wild type oysters under ambient conditions, and their SMR is further increased by elevated CO_2 (Parker *et al.* 2012).

The proteomic and cytological responses of selectively bred and wild type Sydney rock oysters also differ under elevated CO_2 (Thompson *et al.* 2015 and Chapter 2 of this thesis). Proteomic analyses have shown that the majority of differentially regulated proteins are down-regulated in adult B2 oysters and up-regulated in wild type oysters after exposure to elevated CO_2 (856 µatm pCO_2, pH 7.84). Proteins involved in the mitochondrial electron transport chain and oxidative stress were the most affected by CO_2 stress (Thompson *et al.* 2015). This corresponds with cytological analyses. Substantial changes in mitochondrial integrity and ROS production were observed in CO_2-exposed selectively bred oysters, but not in wild type oysters (760 µatm pCO_2, pH 7.78) (Chapter 2). Such findings indicate that CO_2 stress alters the metabolism and resulting oxidative stress responses of B2 oysters in ways that differ from their wild type conspecifics, most likely due to increased intracellular energy production or adaptive energy reallocation in the B2 breeding line.

These differential transcriptional responses to OA in oysters were identified in populations that had been selectively bred for fast growth and disease resistance, not among oyster specifically bred for resilience to OA or conditioned to elevated CO_2. Despite the difficulty of performing experiments over successive generations of marine organisms with long generation times (months to years), there are some data on the physiological effects of transgenerational exposure to elevated CO_2. Assessment of larval or adult performance following transgenerational conditioning to OA has been conducted in fish (Allan *et al.* 2014; Donelson *et al.* 2012; Miller *et al.* 2012; Schade *et al.* 2014; Welch *et al.* 2014), corals (Putnam & Gates 2015), sea urchins (Dupont *et al.* 2013), mussels (Sunday *et al.* 2011) and oysters (Parker *et al.* 2015; Parker *et al.* 2012). Most of these studies show that the physiological impacts of OA are ameliorated by conditioning parents to CO_2 stress. For instance, larvae, spat and adults of wild type Sydney rock oysters exposed to elevated CO_2 over two consecutive generations are able to cope better with OA than oysters exposed to elevated CO_2 over a single generation (Parker *et al.* 2015). The CO_2-conditioned oysters showed faster growth, lower frequencies of abnormality during development and greater capacity to regulate their extracellular pH when exposed to elevated CO_2. A preliminary analysis of the molecular processes underlying such transgenerational conditioning of different Sydney rock oyster populations to OA is reported in Chapter 3. In that study, we analysed a suite of genes by qPCR to show that the capacity for heritable change varies between oyster breeding lines and is mediated by parental conditioning. B2 oysters exposed to three consecutive generations of CO_2 stress exhibited changes in both basal and inducible gene expression, and these responses depended upon the CO_2 environment experienced by their parents. Similar effects of transgenerational conditioning to OA were not evident in wild type oysters. These findings suggest that Sydney rock oysters might undergo rapid acclimation or adaptation in response to CO_2-driven OA, and that this capacity for heritable change depends on the genetic background of populations within the species.

Although it provided valuable preliminary information, our previous transcriptional analysis (Chapter 3) focused on a restricted suite of genes that are commonly affected by multiple environmental stressors, including temperature extremes, heavy-metal exposure and disease (Anderson *et al.* 2015; Chapman *et al.* 2011; Tomanek 2014; Zhang *et al.* 2012). Therefore, the complete set of molecular processes underlying transgenerational conditioning of oysters to OA remains unknown. The current study undertakes a more comprehensive transcriptomic analysis to address this deficit in our knowledge. We sequenced and assembled the complete transcriptome of B2 oysters that had been exposed to near-future projected ocean pH over two consecutive generations. By doing so, we were able to identify the molecular processes involved in the distinctive performance of this breeding line in response to CO_2 stress. In addition, we compared the gene expression profiles of CO_2-conditioned B2 and wild type oysters in order to determine whether adaptive responses to OA vary between breeding lines. This is the first study to identify the broad molecular basis of transgenerational conditioning to OA in a marine calcifier by exploring both the effects of transgenerational exposures and the differential adaptive potentials of genetically distinct populations.

4.2 Materials and Methods

4.2.1 Oysters

Adult B2 and wild type (W) Sydney rock oysters (8.04 ± 0.7 cm shell length), *Saccostrea glomerata* (Gould 1850), were the starting populations used for transgenerational conditioning to OA in the current study. They were provided by the Port Stephens Fisheries Institute of the New South Wales Department of Primary Industries (NSW DPI; Taylors Beach, NSW, Australia). Prior to this study, the B2 breeding line had been mass selected over seven generations for fast growth and resistance to QX disease and winter mortality. The scheme for transgenerational exposure of the starting populations to elevated CO_2 is shown in Figure 4.1.

Founding parents from the B2 and W populations were collected from the field at the beginning of their reproductive season and held in aquaria at either ambient or elevated CO_2 until they became gravid. Gametes were then collected by strip spawning and fertilized. The resulting F1 larvae were grown under the same conditions as their parents (ambient or elevated CO_2). Spat (approx. 5 mm retained) were then transferred into the field (Port Stephens, NSW, Australia) where they remained for 14 months until they reached reproductive maturity. F1 adults were transferred to the laboratory and held under the same CO_2 conditions experienced by their parents (ambient or elevated CO_2; see section 4.2.2). F1 adults were sampled for transcriptomic analysis after a one-month CO_2 exposure (see section 4.2.3).

4.2.2 Exposure to CO_2

Parents and F1 larvae were exposed to elevated CO_2 at the Port Stephens Fisheries Institute as described in Parker *et al.* (2015). F1 adults were exposed to elevated CO_2 at the Sydney Institute of Marine Science (Chowder Bay, NSW). Prior to CO_2 exposure, the F1 adults were acclimated to aquarium conditions in a flow-through seawater system (0.5 L per minute, seawater filtered at 20 µm). The nutritional supply from the flow-through seawater system was supplemented every three days with a concentrated blend of microalgae (Shellfish Diet® 1800, Reed Mariculture Inc., 4.3×10^8 algal cells per oyster). Following 10-days of acclimation, the F1 adults were exposed for one month to either ambient (297 µatm pCO_2, pH 8.12) or near-future elevated (775 µatm pCO_2, pH 7.77) CO_2 concentrations, according to their prior history of CO_2 exposure. Oysters whose parents and early-life stage were conditioned at ambient CO_2 were held under ambient conditions, while oysters whose parents and early-life stage were conditioned at elevated CO_2 were again exposed to elevated CO_2.

Seawater CO_2 concentrations were controlled by a custom-made system that included pneumatic components from Parker Hannifin (Castle Hill, NSW), CO_2 sensors from Vaisala (Hawthorn, VIC) and a control system from Greenstar Building Automation & Citywide

Electrical Services (Marrickville, NSW). CO_2-enriched air was bubbled into three header tanks, each of which supplied a single replicate tank. Three replicate tanks each were used for ambient and elevated CO_2 exposures. Each tank contained 7 oysters from different populations and CO_2 conditioning regimes. Temperature, salinity and carbonate chemistry (pH and total alkalinity) were monitored (Table 4.1) and no mortality was observed throughout the experiment. These transgenerational exposures produced a total of four treatments: B2 or W oysters exposed to either ambient (B2A or WA) or elevated (B2E or WE) CO_2 conditions over two generations (parental and F1) (Fig. 4.1).

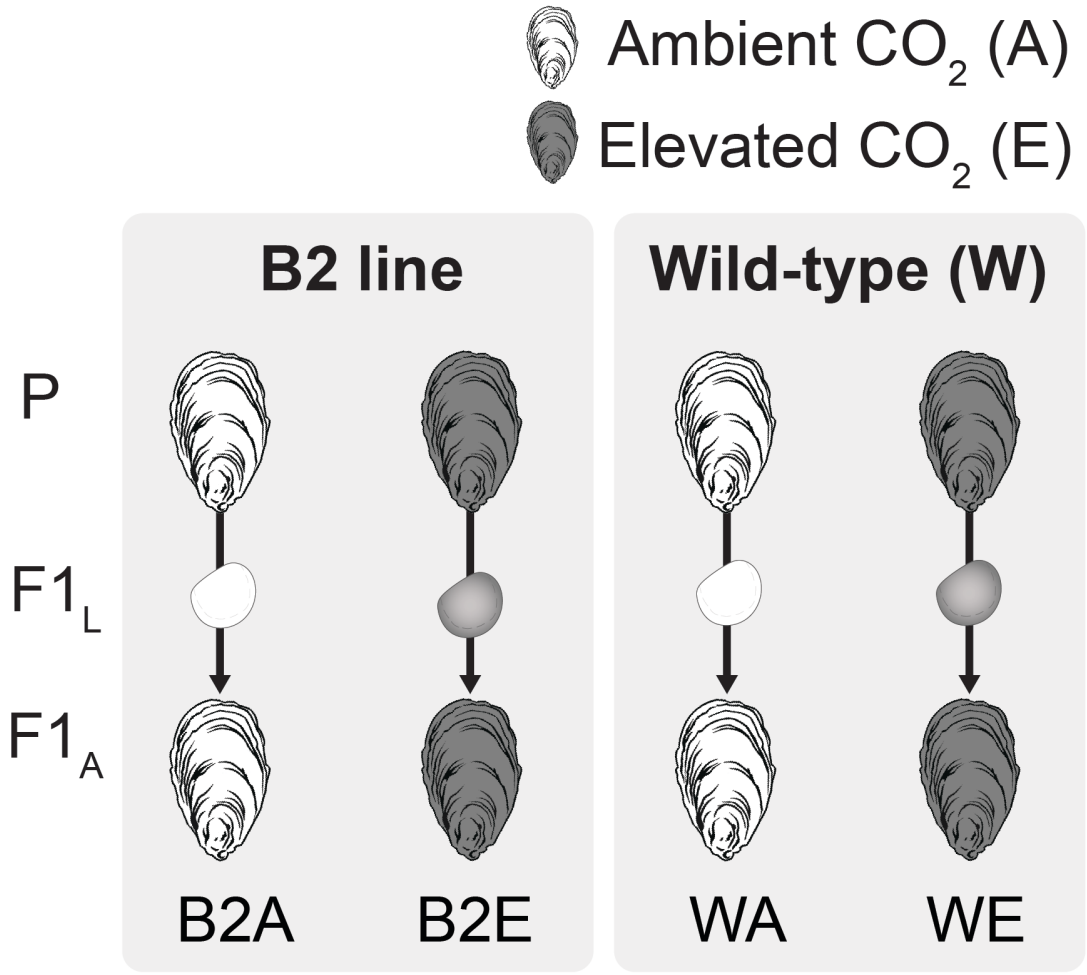


Figure 4.1. Experimental design for transgenerational CO_2 exposures. Selectively bred (B2) and wild type (W) parents (P) and their larval offspring (F1 larvae, $F1_L$) were both held under ambient (A) or elevated (E) CO_2 conditions (adult field grow-out at ambient conditions). Adults of the F1 generation ($F1_A$) were then exposed to the same conditions experienced by their parents immediately prior to transcriptomic analysis.

Table 4.1. Seawater chemistry during ocean acidification trial. Salinity, temperature and pH were determined once a day using a YSI 63 probe, total alkalinity was measured in an automatic titrator (Metrohm 888 Titrando) and pCO_2 was calculated using co2sys software (Lewis & Wallace 1998). Data are presented as mean ± SD (n = 22 days).

Parameter	Ambient CO_2 (A)	Elevated CO_2 (E)
pH	8.12 ± 0.03	7.77 ± 0.08
Temperature (°C)	18.44 ± 0.01	19.32 ± 0.44
Salinity (ppt)	33.94 ± 0.02	33.94 ± 0.66
Total alkalinity (mmol kg^{-1} SW)	2.20 ± 0.03	2.14 ± 0.02
pCO_2 (µatm)	297.30 ± 2.18	775.05 ± 138.31

4.2.3 RNA extraction

Following one-month of exposure to either ambient or elevated CO_2 conditions, gills from F1 adults were excised (n = 21 per treatment) and stored in RNA later (Ambion) at -20 °C. Total RNA was extracted from approximately 100 mg of gill tissue using TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. Resuspended RNA was treated with DNase I (Promega) and further precipitated with 0.3 M sodium acetate (pH 5.5) and isopropanol. Total RNA concentration and purity were checked with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000).

4.2.4 Transcriptome sequencing and *de novo* transcriptome assembly

Ten RNA samples from B2 oysters (5 oysters exposed to ambient CO_2, 5 · B2A; and 5 exposed to elevated CO_2, 5 · B2E) were selected for RNA sequencing (selected based on purity from the 21 samples collected per treatment; see section 4.2.3). Sequencing was performed by the Ramaciotti Centre for Gene Function Analysis, University of New South Wales. The TruSeq protocol (Illumina) was followed for cDNA synthesis and library preparation. A HiSeq2000 (Illumina) platform was used for transcriptome sequencing. Samples were barcoded and sequenced twice, using a single lane per run.

Raw sequencing paired-end reads of 100 bp from both runs were combined and quality-filtered using Trimmomatic (version 0.32) (Bolger *et al.* 2014). Reads were trimmed if the average quality within a window of 4 bp was below 25. Unpaired reads and reads smaller than 30 bp were discarded. The resulting quality of the trimmed reads was visualised using FastQC (version 0.10.1) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Following quality trimming, reads were also filtered for contamination with ribosomal RNA sequences by mapping to a Mollusca nucleotide (nt) database, which included the *Crassostrea gigas* genome (downloaded from NCBI in March 2015) (Table 4.2). Mapping was performed with Bowtie 2 (version 2.2.4) (Langmead & Salzberg 2012). The raw sequence reads will be available at the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) prior to manuscript submission for publication.

Table 4.2. Sequencing read statistics. Basic statistics of paired-end 100 bp reads for B2 line Sydney rock oysters transgenerationally exposed to ambient (B2A) and elevated (B2E) CO_2 conditions (n = 5 per treatment).

Treatment	Number of reads pre-trimming	Phred-score pre-trimming[a]	Number of reads post-trimming[b]	Phred-score post-trimming[a]
B2A	95,704,872	35.31	49,381,860	37.20
B2A	116,224,110	34.94	48,899,898	37.10
B2A	108,073,352	35.23	53,386,702	37.23
B2A	86,062,976	35.44	52,611,930	37.22
B2A	103,365,136	35.39	58,973,210	37.23
B2E	92,480,526	36.00	79,716,152	37.26
B2E	71,581,346	36.00	61,355,316	37.27
B2E	88,686,954	35.90	76,312,370	37.20
B2E	89,600,384	35.95	75,523,080	37.25
B2E	86,871,398	36.01	75,745,728	37.29
Total	938,651,054	35.62	631,906,246	37.22

[a] Mean quality score for R1 and R2 reads.

[b] Reads were removed mainly due to a large proportion of ribosomal RNA that was found in some libraries, not because of read quality issues.

Processed RNA reads were assembled into a reference transcriptome using three different algorithms; the CLC Genomics Workbench (version 7.5.2), Velvet (version 1.2.10) followed by Oases (version 0.2.08), and Trinity (version trinityrnaseq_r20140717). Oases and Trinity transcriptome assemblies were performed using processed reads that were normalised (k = 25, 30· coverage) by digital normalization (Brown *et al.* 2012) or Trinity's in silico read normalization, respectively. Different k-mer sizes were tested for CLC (k = 23, 24, 25) and Oases (k = 21, 23, 25, 27, 29), while Trinity assembly was conducted using the default parameters (k = 25). Only contigs longer than 400 bp were kept for downstream analysis. The performance of these tools and the effect of k-mer size were assessed according to N50 value, mean length, maximum length and number of contigs. The highest-quality assembly generated by each software was also assessed by mapping the processed reads back to each of the three selected transcriptomes (Bowtie 2, default parameters). The percentage of total aligned reads and uniquely aligned reads were compared among the different assemblies. Based on both assembly statistics and overall alignment rate, Trinity was chosen as the best transcriptome assembly and was used for all downstream analysis (Table 4.3).

Table 4.3. Transcriptome assembly statistics. Assessment of the highest-quality transcriptome assemblies for B2 line Sydney rock oysters generated by three different software packages (minimum contig length of 400 bp).

Statistics	CLC Genomics Workbench	Velvet + Oases	Trinity
k-mer size	24	27	25
N50 (bp)	1,064	1,902	1,303
Mean length (bp)	931	1,357	1,054
Maximum length (bp)	17,734	35,014	36,519
Number of contigs	149,755	405,522	263,740
Alignment rate	56.58%	65.77%	85.55%

4.2.5 Differential gene expression and contig annotation

To reduce contig redundancy and facilitate differential expression analysis, assembled contigs were combined into clusters using Corset (version 1.03) (Davidson & Oshlack 2014). Processed read pairs from each library were individually mapped back to the Trinity assembled transcriptome with Bowtie 2, using strict mapping parameters (--score-min L,-0.1,-0.1, --no-mixed, --no-discordant). Assembled contigs were then clustered based on the proportion of shared reads and expression patterns using default settings. The cluster-level count data generated by Corset were processed using the edgeR Bioconductor package (Robinson *et al.* 2010), testing for differences in gene expression between B2 oysters exposed to ambient (B2A) or elevated (B2E) CO_2. Clusters with no detectable reads in half of the analysed samples were discarded from statistical analyses. Contigs were considered to be differentially regulated (B2A vs. B2E) at $p < 0.05$ with FDR correction (FDR < 0.05).

The complete assembled transcriptome and the differentially expressed contigs were annotated using a Mollusca non-redundant (nr) database (downloaded from NCBI in November 2015) and the NCBI BLAST package (BLASTx, version 2.2.28+) (*e*-value cut-off 10^{-10}). The Mollusca nr database contained 378,914 sequences at the time of analysis, and included sequences derived from the *C. gigas* genome. Assignment of gene ontology (GO) terms to contigs with BLASTx hits was then performed with Blast2GO PRO (version 3.1.3), using default settings.

4.2.6 qPCR analysis

To validate the transcriptome expression data and to assess the transcriptional responses of wild type oysters to OA, we evaluated the relative expression profiles of 61 genes found to be differentially expressed (between the B2A and B2E treatments) by RNA sequencing. Contigs from multiple functional categories were selected based on their absolute fold-change

(highest up- or down-regulation). Only contigs with homologous sequence matches in BLASTx searches were considered for qPCR analysis. qPCR primers were designed using Primer3 software (Untergasser *et al.* 2012) and were based on sequences from the selected contigs (Supplementary Table 4.1).

qPCR was used to measure the relative expression of the 61 target genes in all F1 oysters (21 oysters each from the B2A, B2E, WA and WE treatments). cDNA for qPCR was synthesized from 1 µg of total RNA using ImProm-II™ Reverse Transcription System (Promega) and 0.5 µg of oligo$(dT)_{15}$ in a 20 µl reaction volume. qPCR assays were performed on a LightCycler® 480 II (Roche). Three microliter qPCR reactions were prepared in duplicate in 384-well plates using an epMotion® 5075 pipetting robot (Eppendorf) and an Echo® 550 Liquid Handler (Labcyte). Each reaction mixture contained 1.5 µl KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 300 nM each primer, 0.3 µl PCR grade water and 1 µl cDNA template (diluted 1:9). The cycling program consisted of 3 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 6 s. Melting curve analysis was performed at the end of the qPCR cycles to confirm primer specificity by collecting fluorescence data between 65 - 95 °C at 0.5 °C increments. Standard curves were generated in triplicate for each primer pair using five four-fold serial dilutions of a pool of cDNA samples, and reaction efficiencies were subsequently calculated (Supplementary Table 4.1). Amplification data were analysed using the LightCycler® 480 Real-Time PCR System (version 1.5.1.62) to obtain Cq values.

Reference gene stability was assessed using the web-based RefFinder platform, which integrates results from different tools (Xie *et al.* 2012). We tested the expression levels of three potential reference genes, which have been previously shown to be stable under CO_2 stress (Chapter 3): elongation factor 1 alpha (EF1α), β-actin and GAPDH. The geometric means of EF1α and β-actin were found to be the most stable combination and so these genes were used as references (geNorm stability value = 0.105; NormFinder stability value = 0.081; Stability value by ΔCt method / Average SD = 0.25). qPCR data are presented as changes in relative

expression normalized against the geometric mean of the Cq values of the two reference genes (Livak & Schmittgen 2001).

To visualise overall differences between populations and/or CO_2 conditions, we performed a principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) incorporating the expression levels of all genes analysed by qPCR or of contigs identified by RNA sequencing, respectively. Correspondence between RNA-seq and qPCR data was tested by Pearson correlation analysis using the log2 fold-change values of relative expression across the two platforms.

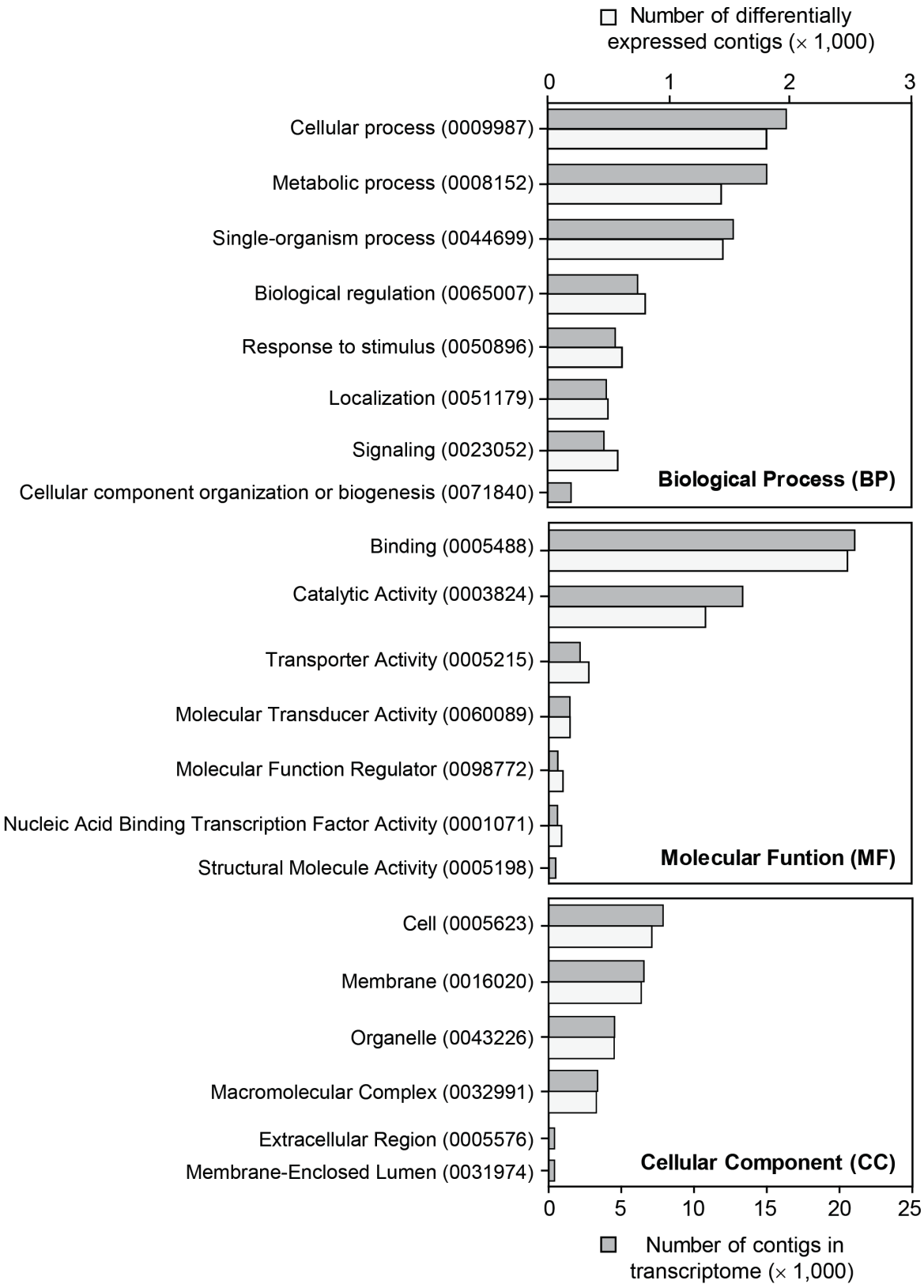
4.3 RESULTS

4.3.1 Sydney rock oyster transcriptome

We produced ten RNA-seq libraries, from five B2 oysters per CO_2 treatment (ambient or elevated), to generate a comprehensive reference transcriptome of Sydney rock oysters and to investigate the transgenerational effects of ocean acidification on their molecular processes. Library sequencing yielded 939 million paired-end reads (100 bp) that were quality-filtered and screened for ribosomal RNA contamination (Table 4.2). Sixty-seven percent of the reads (632 million) were retained and *de novo* assembled using three different software packages: CLC Genomics Workbench, Velvet followed by Oases, and Trinity. By testing the performance of these assemblers and the effect of different k-mer sizes for both CLC and Oases, we found that Trinity was able to produce the highest-quality transcriptome assembly. Trinity generated 263,740 assembled contigs (287 Mb) with a minimum length of 400 bp and an N50 value of 1,303 bp (Table 4.3). In addition to the best assembly metrics, the Trinity assembly also had the highest alignment rate (85.55%) to the processed reads, indicating a strong representation of the original short read sequences in the assembled contigs.

Once assembled, contigs were grouped into 150,130 gene clusters using Corset. The cluster-level count data were then processed using edgeR. Gene clusters with no detectable expression in half of the samples sequenced (5 out of 10 samples) were removed. 36,427 gene clusters (24%), corresponding to 85,055 contigs, were retained and used for gene annotation and differential gene expression analysis.

Gene annotation of assembled contigs was performed by BLAST analysis using the NCBI non-redundant (nr) protein database for Mollusca followed by Blast2GO. BLAST analysis showed that 61,127 contigs (72%; 25,077 gene clusters) from our filtered assembly had at least one match in the Mollusca database (*e*-value < 1^{-10}, word size = 4). The vast majority of the contigs (95%) were homologous to sequences from the Pacific oyster, *C. gigas*, the complete genome of which has been sequenced (Supplementary Figure 4.1) (Zhang *et al.* 2012). We were able to assign gene ontology (GO) terms using Blast2GO to 59% of the contigs with BLAST hits to known proteins in UniProtKB. Annotated contigs had 1 to 15 GO terms assigned. 5,161 (8%) additional contigs with BLAST hits had GO mapping results (preliminary GO annotation) (Supplementary Figure 4.2). The hits represented 9,897 discrete genes and 3,791 encoded uncharacterised proteins (Supplementary Table 4.2). This Sydney rock oyster transcriptome incorporated a comprehensive spread of functional systems within oyster cells, including numerous genes involved in energy metabolism, cellular signalling, intracellular stress and immune responses. The majority of the assembled contigs were found to be associated with cellular processes (25%; including cell cycle and cell communication), metabolic processes (23%; including biosynthetic and catabolic activity), single-organism processes (19%; including developmental and reproductive processes), biological regulation (9%; including regulation of biological process and quality) and response to stimulus (7%; including response to biotic and abiotic stress, and immune responses). The distribution of the most abundant GO terms for biological processes, molecular functions, and cellular components is presented in Figure 4.2.


Number of differentially expressed contigs (× 1,000)
0
1
2
3
Cellular process (0009987)
Metabolic process (0008152)
Single-organism process (0044699)
Biological regulation (0065007)
Response to stimulus (0050896)
Localization (0051179)
Signaling (0023052)
Cellular component organization or biogenesis (0071840)
Biological Process (BP)
Binding (0005488)
Catalytic Activity (0003824)
Transporter Activity (0005215)
Molecular Transducer Activity (0060089)
Molecular Function Regulator (0098772)
Nucleic Acid Binding Transcription Factor Activity (0001071)
Structural Molecule Activity (0005198)
Molecular Funtion (MF)
Cell (0005623)
Membrane (0016020)
Organelle (0043226)
Macromolecular Complex (0032991)
Extracellular Region (0005576)
Membrane-Enclosed Lumen (0031974)
Cellular Component (CC)
0
5
10
15
20
25
Number of contigs in transcriptome (× 1,000)

4.3.2 Effects of OA on B2 oysters: Analysis of differential gene expression

The relative abundance of sequencing reads from B2 oysters exposed to ambient (B2A) and elevated (B2E) CO_2 over two generations was compared in order to identify the molecular processes involved in transgenerational responses to OA. Supplementary Figure 4.3 shows an overview of the assembly, annotation and differential expression analysis workflow. Analysis of expression levels for the 36,427 gene clusters (85,055 contigs) revealed that B2 oysters exhibit substantially different gene expression profiles when reared under elevated CO_2 (B2E) compared to ambient conditions (B2A). As a result of these differences, NMDS plots showed a clear spatial separation between these two CO_2 conditions (Fig. 4.3a). Of 36,427 gene clusters, 2,909 were found to be differentially expressed (FDR-adjusted $p < 0.05$) in response to CO_2 stress (corresponding to 7,621 contigs). The majority of the differentially expressed contigs (2,356 clusters; 81%) were down-regulated by transgenerational exposure to elevated CO_2 (Fig. 4.3b). However, the highest absolute fold-changes (up to 138-fold) were observed among the up-regulated contigs.

Figure 4.2. (opposite page) Characterisation of the Sydney rock oyster transcriptome and contigs affected by ocean acidification. Distribution of the most abundant gene ontology (GO) terms assigned to the transcriptome (dark grey; shown in bottom x-axis) and to contigs that were differentially expressed under elevated CO_2 conditions relative to ambient conditions (light grey; shown in top x-axis). Transcriptome *de novo* assembly was performed using RNA sequencing reads from oysters exposed to both ambient and elevated CO_2 conditions. Differential expression analysis was performed using edgeR (FDR-adjusted $p < 0.05$). Nearly 10% of the contigs identified in the oyster transcriptome were found to be differentially expressed under elevated CO_2 conditions. Gene and gene ontology (GO) annotation was conducted using BLASTx and Blast2GO. Only GO terms containing at least 400 contigs for the transcriptome analysis and 100 contigs for the differential expression analysis are presented. Corresponding GO IDs are shown in parentheses.

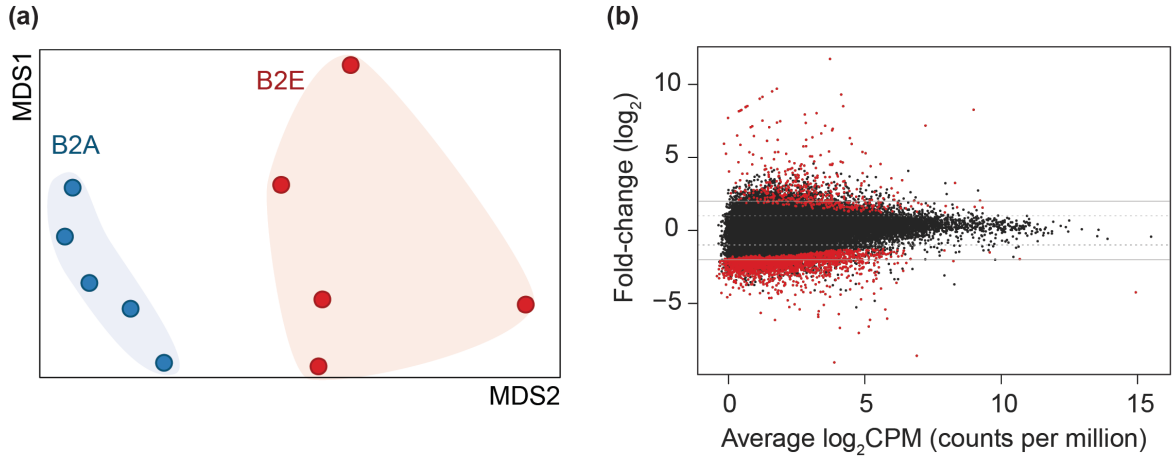


Figure 4.3. Effects of transgenerational exposure to ocean acidification on oyster transcriptomes. **(a)** Multidimensional scaling (MDS) plot summarising the expression levels of 36,427 gene clusters identified by Trinity and Corset in B2 oysters exposed to ambient (B2A, in blue) or elevated (B2E, in red) CO_2 conditions for two consecutive generations. Each data point represents an individual oyster. **(b)** MA plot displaying contigs identified in B2 Sydney rock oysters exposed to elevated CO_2 over two consecutive generations (fold-change calculated as B2E/B2A). Differential expression analysis was performed using edgeR. Contigs that were significantly differentially regulated at $p < 0.05$, FDR < 0.05 are displayed in red. Dashed grey lines delimit the 2-fold interval and continuous grey lines indicates 4-fold changes.

BLAST analysis showed that 73% of the differential contigs (1,983 clusters or 5,515 contigs) had at least one positive hit when searched against the Mollusca database (*e*-value < 1^{-10}) (Supplementary Table 4.3). Of the contigs with BLAST hits, 71% were successfully annotated and 7% had GO mapping results (preliminary GO annotation) (Supplementary Figure 4.2). Differentially expressed contigs encompassed genes associated with cellular processes (24%; including cell cycle and cell communication), single-organism processes (19%; including developmental and reproductive processes), metabolism (19%; including biosynthetic and catabolic activity), biological regulation (11%; including regulation of biological process and quality) and response to stimulus (8%; including response to biotic and abiotic stress, and immune responses) (Fig. 4.2). These functions represent 25%, 19%, 23%, 9% and 7% of the total Sydney rock oyster transcriptome, respectively.

The contigs with the highest fold differences between B2A and B2E oysters were further assigned more inclusive biological functions based on GO and InterProScan annotations. Eight broad categories of biological function (stress response, immune response, metabolism, cytoskeleton, cell communication, cell cycle, protein regulation and nucleic acid regulation) were used to characterize these contigs. These functional categories were based on the classification scheme reported in Anderson *et al.* (2015). Differentially expressed contigs were shown to be involved mainly in the cell cycle (cell division, cell growth and differentiation, cell migration, cell death and maintenance of cellular homeostasis), metabolism (metabolic and catabolic processes, oxidative and reductive reactions, glycolysis and lipid metabolism) and cell communication (cell signalling, cell adhesion and cell-cell interactions) (Fig. 4.4 and Table 4.4). They included uracil-DNA glycosylase (UDG) (138-fold higher in B2E), Hsp70 (87-fold higher in B2E), multiple epidermal growth factor-like domain proteins 10 and 11 (MEGF10 and 11, 81- and 67-fold higher in B2E, respectively), mitochondrial serine/threonine-protein kinase (PINK1) (72-fold higher in B2E), pentraxin fusion protein (52-fold higher in B2E), mannose-6-phosphate isomerase (MPI) (48-fold higher in B2E), hemagglutinin/amebocyte aggregation factor (HAAF) (36-fold lower in B2E), perlucin (36-fold higher in B2E), dynactin subunit 1 (DCTN1) (34-fold higher in B2E), and inhibitors of apoptosis 1 and 2 (IAP1 and 2, 22- and 33-fold lower in B2E) (Table 4.4). The complete list of contigs most affected by CO_2 stress, their functional categories and normalised read counts can be found in Supplementary Table 4.4.

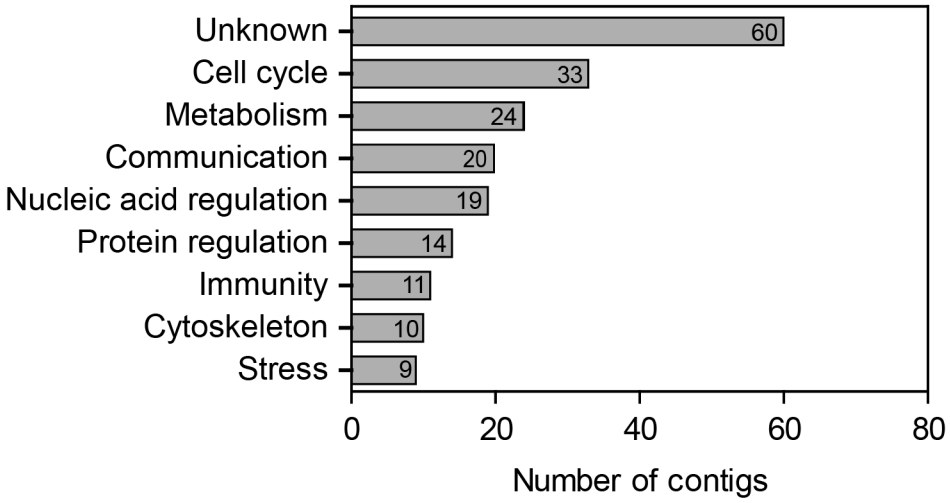


Figure 4.4. Biological functions associated with the contigs most affected by CO_2 stress. Contigs with the highest fold differences between B2A and B2E oysters (top 200) were assigned broad functional categories based on Blast2GO analysis. The number of contigs in each category is shown. Cell cycle includes regulation of cell division, cell growth and differentiation, cell migration, cell death (apoptosis and autophagy) and maintenance of cellular homeostasis. Metabolism includes metabolic and catabolic processes, oxidative and reductive reactions, glycolysis and lipid metabolism. Communication includes cell signalling, cell adhesion and cell-cell interactions. Nucleic acid regulation includes transcription, DNA replication and other DNA- and RNA-specific processes. Protein regulation includes translation, protein processing, modification and trafficking.

Table 4.4. Contigs most affected by CO_2 stress in B2 oysters. Subset (top 50) of the list of contigs with the highest fold differences between oysters exposed to ambient (B2A) and elevated (B2E) CO_2 conditions (fold-change calculated as B2E/B2A). Assigned broad functional categories were based on Blast2GO analysis. Blue reflects low expression (low average normalised read counts) and red reflects high expression (high average normalised read counts).

Sequence information				Average normalised read counts	
Sequence description	**Assigned functions**	**Response**	**Fold-change (log)**	**Ambient CO_2**	**Elevated CO_2**
Uracil-DNA glycosylase	Nucleic acid regulation	up-regulation	11.744819	0	518.4
Heat shock 70 kDa protein 12b	Stress	up-regulation	9.307071788	0.6	686.4
Multiple epidermal growth factor 10	Cell cycle	up-regulation	9.021693779	0	78.6
Serine threonine-protein kinase mitochondrial	Cell cycle	up-regulation	8.506490318	1.2	754.8
Multiple epidermal growth factor 11	Cell cycle	up-regulation	8.204831127	0	45.8
Tripartite motif-containing protein 3	Immunity	up-regulation	7.706717215	0	31.2
Pentraxin fusion protein	Immunity	up-regulation	7.181512076	28.6	4853.2
Sco-spondin	Communication	up-regulation	7.167381454	2	457
Protein kinase c delta type	Cell cycle	down-regulation	-7.021996222	794.6	8.6
Mannose-6-phosphate isomerase partial	Metabolism	up-regulation	6.959014187	2.8	544.8
Hemicentin- partial	Communication	up-regulation	6.498008457	0.2	41
Hemagglutinin amebocyte aggregation factor	Immunity	down-regulation	-6.038620982	1651.2	34.4
Tripartite motif-containing protein 2	Immunity	up-regulation	5.922820943	10	967
Dynactin subunit 1	Cytoskeleton	up-regulation	5.859856941	0.4	44.8
Perlucin	Immunity	up-regulation	5.770902268	3.2	259.4
Apoptosis 2 inhibitor	Cell cycle	down-regulation	-5.761174674	242.8	5.8
Protein mab-21	Cell cycle	up-regulation	5.753971274	2.8	243.2
Fibroblast growth factor receptor 2	Cell cycle	down-regulation	-5.727106944	404.4	13.8
Short-chain collagen c4	Cell cycle	down-regulation	-5.534541071	206.4	9
Peripheral myelin protein 22	Cell cycle	up-regulation	5.409170028	7.8	493.6
Arginine kinase	Metabolism	up-regulation	5.374886893	18.8	1093.4
Fucolectin-1	Immunity	up-regulation	5.286605088	22.8	1039.2
Poly [ADP-ribose] polymerase 14	Nucleic acid regulation	up-regulation	5.249884682	2.6	157.6
Leucine-rich repeats and immunoglobulin protein 1	Communication	down-regulation	-5.248412537	119	4
Methylcrotonoyl- carboxylase beta mitochondrial	Metabolism	up-regulation	5.071146636	6.2	286.8

Table 4.4 continued

Sequence information				Average normalised read counts	
Sequence description	**Assigned functions**	**Response**	**Fold-change (log)**	**Ambient CO_2**	**Elevated CO_2**
Endothelin-converting enzyme 1	Protein regulation	up-regulation	4.878529151	2.4	110.2
Apoptosis 1 inhibitor	Cell cycle	down-regulation	-4.788531866	119.6	6.4
Leucine-rich repeat-containing protein 74b	Communication	up-regulation	4.723869251	8.4	320.8
Cytosolic carboxypeptidase 2	Protein regulation	up-regulation	4.655179582	18.2	730.8
Probable serine threonine-protein kinase pats1	Cell cycle	down-regulation	-4.629505876	76.4	4.8
CD63 antigen	Cell cycle	up-regulation	4.570183531	14.8	519.6
Heat shock 70 kDa protein 12a	Stress	up-regulation	4.532812989	6.2	227.4
Tho complex subunit 3	Nucleic acid regulation	up-regulation	4.519450225	1.6	61
Ficolin-partial	Immunity	up-regulation	4.484448761	14.2	508.4
Serine arginine repetitive matrix protein 1	Nucleic acid regulation	down-regulation	-4.403030452	25.6	1.8
Histone H3	Nucleic acid regulation	down-regulation	-4.288134705	68	5.6
Cytochrome P450 1a1	Metabolism	up-regulation	4.281779113	3.2	103
Transient receptor potential cation channel subfamily m member 2	Stress	up-regulation	4.223111457	7.2	240
BTB/POZ domain-containing protein 2	Cytoskeleton	up-regulation	4.199778814	5.8	172
Leucine-rich repeat-containing protein 15	Communication	up-regulation	4.178577091	5.6	165.4
Epidermal retinol dehydrogenase 2	Metabolism	up-regulation	4.147908356	4.6	163.2
E3 ubiquitin-protein ligase HERC2	Nucleic acid regulation	up-regulation	4.077126379	106.8	3657.2
Vacuolar protein sorting-associated protein 13a	Protein regulation	down-regulation	-4.053425442	85.8	7.4
Receptor-type tyrosine-protein phosphatase kappa	Cell cycle	up-regulation	4.039365551	1.2	31
Bactericidal permeability increasing protein	Immunity	down-regulation	-3.980866503	944.6	119.4
Heavy metal-binding protein hip	Stress	up-regulation	3.959213858	5.6	120
Protein jagged-2	Cell cycle	down-regulation	-3.91391197	180.6	18.4
IgGFc-binding protein	Communication	up-regulation	3.836595744	25.6	600.4
Histone H4	Nucleic acid regulation	down-regulation	-3.831698977	145.2	17
Probable ATP-dependent RNA helicase DDX58	Nucleic acid regulation	up-regulation	3.807212456	4	97.2

4.3.3 Differential responses of wild type and B2 oysters to OA

qPCR analysis was performed both to validate the transcriptome expression data and to assess the transcriptional response of wild type, as well as B2, oysters to OA. We measured the relative expression of 61 contig sequences that were found to be differentially expressed by RNA sequencing in B2 oysters. Among the contigs selected, 28 were found by RNA sequencing to be up-regulated and 32 down-regulated in B2 oysters responding to OA. Expression patterns measured by qPCR were equivalent to those detected by RNA sequencing for 72% of the contigs analysed. Three contigs (perlucin, IAP1 and fatty acid-binding protein) could not be detected by qPCR (mean Cq $\geq$ 35) and so were excluded from the analysis. Pearson correlation analysis of RNA-seq vs. qPCR expression fold-change (log_2) data showed a strong concordance between the expression data generated by both platforms (r = 0.72, p = 3.16e-10) (Supplementary Figure 4.4). Interestingly, out of the 16 contigs (28%) for which RNA-seq and qPCR identified different regulation patterns, only four (7%) had opposite responses between platforms. Mannose-6-phosphate isomerase (MPI), endothelin-converting enzyme 1 (ECE1), mitochondrial-like glutaredoxin-2 and BTB/POZ domain-containing protein 2 (BTBD2) were found to be up-regulated by RNA-seq but down-regulated by qPCR. The remaining 12 genes (21%) with distinct responses between methods were found not to be differentially regulated using qPCR (fold-changes between 0.71 and 1.36).

The expression levels of the 61 selected contigs were also analysed in wild type oysters exposed to ambient or elevated CO_2 over two consecutive generations. The transcriptional response of this population was investigated in order to test whether adaptive responses to OA vary between oyster breeding lines. Eight genes were excluded from this analysis because they could not be detected by qPCR in B2 and/or W oysters (perlucin, IAP1, fatty acid-binding protein, UDG, dual oxidase, pentraxin fusion protein, ECE1 and structural maintenance of chromosomes protein 3; mean Cq $\geq$ 35). qPCR of the remaining set of target genes showed that B2 and wild type oysters exhibit different gene expression profiles under both ambient and

elevated CO_2 conditions. 34 genes (65%) differed significantly in expression between B2 and W oysters that had not been exposed to elevated CO_2 (B2A vs. WA; absolute fold-change between 1.73 and 11.06). The expression of most of these genes (28 out of 34) was lower in B2 oysters (B2A) than in wild type oysters (WA). Genes with lower expression in B2A oysters included the IgGFc-binding protein (0.23-fold), complement C1q/tumor necrosis factor-related protein 7 (C1q; 0.24-fold), sentrin-specific protease 8 (0.25-fold), serine arginine repetitive matrix protein 1 (0.33-fold) and titin (0.33-fold) (Fig. 4.5a). Conversely, transcript levels of 6 genes were higher in B2A oysters relative to WA oysters (absolute fold-change between 1.99 and 11.06). These included the von Willebrand factor D (VWF) and EGF domain-containing protein (4.43-fold), two pore calcium channel protein (4.19-fold) and HAAF (2.65-fold) (Fig. 4.5a). As a result of these differences, principal component analysis (PCA) showed a clear spatial separation of the gene expression profiles for the B2A and WA treatments (Fig. 4.5b).

Even though the transgenerational exposure to elevated CO_2 resulted in substantial differences in the gene expression profiles between breeding lines, several genes showed equivalent regulation patterns in B2 and wild type oysters. As previously observed via RNA sequencing, CO_2 stress reduced the relative expression of numerous genes in B2 oysters (B2E). Decreases in transcript levels were also found in W oysters responding to elevated CO_2 (WE). A total of 25 genes (47%) were found at lower levels in both populations after exposure to CO_2 stress (Fig. 4.5a). This lower transcript abundance was observed for genes from multiple functional groups, including the tricarboxylate transport protein mitochondrial-like (0.57-fold in B2E and 0.58-fold in WE), histone H4 (0.51-fold in B2E and 0.20-fold in WE) and BTBD2 (0.49-fold in B2E and 0.39-fold in WE). Despite this similar pattern of down-regulation for many transcripts in response to elevated CO_2, the overall gene expression profiles of B2E and WE oysters were clearly distinct (Fig. 4.5b). This is a reflection of the genes that exhibited contrasting responses between populations and, most importantly, genes that were affected by CO_2 stress in only one of the two populations. Overall, PCA showed that CO_2 conditioning had

the most substantial effect on B2 oysters compared to wild type (W) oysters (Fig. 4.5b). There was a clear spatial separation between the B2A and B2E clusters, while the WE group overlapped with WA oysters.

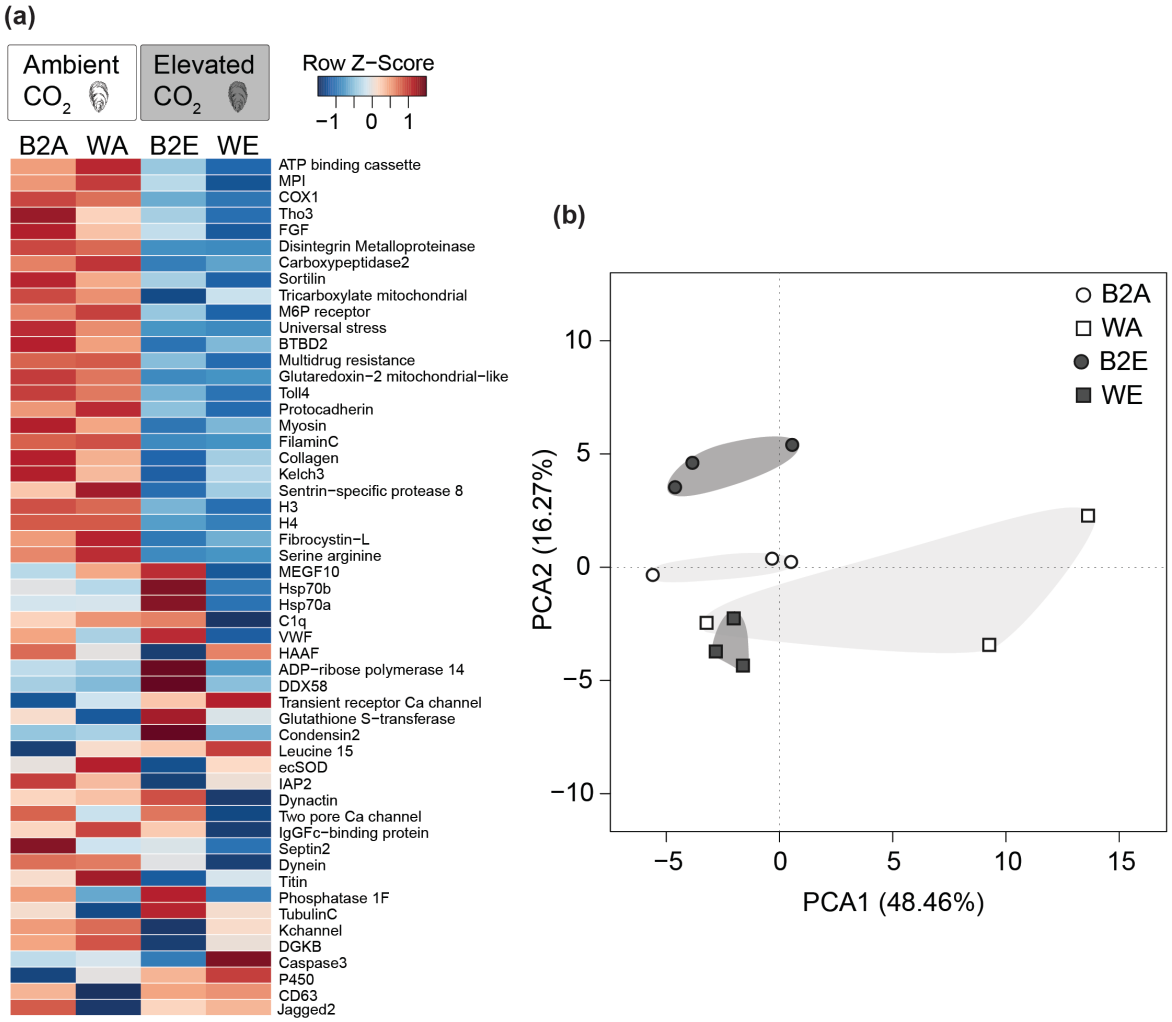


Figure 4.5. Transcriptional responses of B2 and W oysters following transgenerational exposure to elevated CO_2. **(a)** Heat map of relative gene expression. The gene expression profiles of B2 and wild type (W) breeding lines that had been exposed either ambient (A) or elevated CO_2 (E) over two consecutive generations were assessed by qPCR. Red reflects up-regulation and blue reflects down-regulation. Gene selection was based on contigs found to be differentially expressed in B2E oysters by RNA sequencing (compared to B2A). Gene names and their associated cellular functions are detailed in Supplementary Table 4.1. **(b)** Principal component analysis (PCA) of gene expression in both oyster breeding lines and CO_2 conditions. PCA combines the expression levels of the 53 genes displayed in (a). Each point represents the relative mean expression levels of oysters from the same exposure tank (3 tanks containing 7 oysters each per condition).

Contrasting responses between B2E and WE oysters were observed for MEGF10 (3.92-fold in B2E and 0.27-fold in WE), Hsp70a (2.11-fold in B2E and 0.41-fold in WE), C1q (1.77-fold in B2E and 0.21-fold in WE) and VWF (2.65-fold in B2E and 0.12-fold in WE). Transcript levels for these genes were up-regulated in B2E and down-regulated in WE oysters, relative to their respective ambient controls (B2E vs. B2A and WE vs. WA). In contrast, coagulation factor HAAF was down-regulated in B2E oysters (0.35-fold), but up-regulated in WE (2.18-fold) in response to CO_2 stress (Fig. 4.5a).

In addition to the genes that showed contrasting responses between populations, we also identified a suite of genes whose responsiveness was population-specific. These genes were shown to be affected by CO_2 stress either in B2E or WE oysters. For instance, elevated CO_2 increased the expression of glutathione S-transferase omega (3.47-fold), poly (ADP-ribose) polymerase 14 (3.26-fold) and condensin-2 (3.12-fold) in B2 oysters (B2E), but these genes were not affected by the same stress in wild type oysters (WE). Conversely, while B2E oysters exhibited lower levels of the extracellular superoxide dismutase (ecSOD; 0.55-fold) and IAP2 (0.27-fold) in response to elevated CO_2, no change in their relative expression was observed in WE. Similar population-specific responses were observed in wild type oysters. The transcript levels of dynein heavy chain axonemal (0.23-fold), septin-2 (0.48-fold), two pore calcium channel protein 1 (0.53-fold) and titin (0.58-fold) were down-regulated in WE upon CO_2 exposure, but were not differentially regulated by this stress in B2E. No genes were found to be simultaneously up-regulated in WE and not differentially regulated in B2E.

4.4 DISCUSSION

Predicting the impacts of climate change on marine ecosystems depends on understanding how individuals and populations respond to environmental change. In this study, we evaluated the effects of transgenerational exposure to elevated CO_2 on the transcriptome of

Sydney rock oysters. RNA sequencing was used to identify the molecular processes involved in transgenerational conditioning of these oysters to ocean acidification (OA). Our findings suggest that the transcriptome of Sydney rock oysters undergoes rapid change in response to decrease pH resulting from selective regulation of genes associated with a variety of cellular processes, primarily related to mitigation of cellular damage. The current study also shows that these extensive transcriptional responses differ between oyster breeding lines. High-throughput qPCR revealed a set of genes that are differentially regulated only in selectively bred B2 oysters and so may be responsible for their reported resilience to OA.

Some marine organisms have a notable capacity for acclimation or adaptation to stressful environmental conditions, including those associated with climate change (Donelson *et al.* 2012; Dupont *et al.* 2013; Parker *et al.* 2015; Sanford & Kelly 2011; Schoville *et al.* 2012). This capacity is often mediated by phenotypic plasticity within species, but it can also be enhanced through selective breeding over multiple generations. Hence, selective breeding has been identified as one of the most promising strategies to future proof natural populations and aquaculture industries by helping marine species to persist in a rapidly changing ocean (Doubleday *et al.* 2013; van Oppen *et al.* 2015). Sydney rock oysters have been subjected to artificial selection for fast growth and disease resistance for more than twenty years (seven generations) (Dove *et al.* 2013). The selection pressure for those traits has coincidentally resulted in resilience against CO_2-driven OA in the B2 breeding line (Parker *et al.* 2012; Parker *et al.* 2011). In the current study, we exploited this unique genetic resource by undertaking a comprehensive whole-transcriptome analysis to decipher the molecular basis of the improved performance of this breeding line. Whole transcriptome sequencing is an effective tool for revealing the molecular processes responsible for differential responses between populations and environmental conditions. Here, the whole transcriptome of B2 oysters was found to comprise genes involved in a variety of biological functions, including energy metabolism, cellular signalling, intracellular stress and immune responses. We were able to reconstruct high

quality contigs through *de novo* assembly and identify 9,897 discrete genes. This sequencing effort represents the most comprehensive transcriptome resource available for Sydney rock oysters. It provides valuable information for future studies investigating the response of this species to disease, as well as to biotic and abiotic environmental stressors. Contigs from Sydney rock oysters (*S. glomerata*) showed substantial similarity with characterised proteins from the Pacific oyster (*C. gigas*), which suggests a high degree of conservation in the protein coding mRNAs of these two species.

The transgenerational exposure of B2 oysters to elevated CO_2 resulted in changes in the expression levels of 2,909 genes clusters, when compared to B2 oysters reared under ambient conditions. We were able to identify 1,984 sequence clusters, which corresponded to 1,590 unique differentially expressed genes. The differential genes were predicted to be involved in a variety of cellular processes such as the cell cycle and cell communication, metabolism, response to stress and the immune system. The vast majority of differentially expressed transcripts were found at lower levels in oysters that had been transgenerationally exposed to OA. Similarly, reduced expression in response to OA has been previously reported in oysters using global proteome analyses. Proteomic analyses of *C. gigas* and *S. glomerata* from the B2 breeding line identified lower concentrations for the majority of proteins differentially regulated by CO_2 stress (pH 7.5 to 7.84) relative to ambient conditions (Dineshram *et al.* 2013; Dineshram *et al.* 2012; Thompson *et al.* 2015). The differentially regulated proteins identified in these previous studies included a number associated with oxidative stress, protein synthesis and energy metabolism. Such a widespread reduction in protein and transcript concentrations might be associated with cellular dysfunction and physiological suppression typically experienced by marine organisms under OA. Following exposure to CO_2 stress, marine calcifiers often exhibit a decrease in metabolic activity, which in turn leads to lower growth and calcification rates (Hofmann & Todgham 2010; Lannig *et al.* 2010; Pörtner *et al.* 2004).

Alternatively, the down-regulation of many genes/proteins may result in adaptive trade-offs in energy allocation. Such trade-offs often appear to be involved in resilience to OA in marine calcifiers. It seems that a variety of energy-demanding physiological processes are compromised to provide energy for adaptive processes that allow organisms to withstand the usually deleterious effects of elevated CO_2 (Holcomb *et al.* 2010; Pedersen *et al.* 2014; Thomsen *et al.* 2013). Parker *et al.* (2015) recently identified such a trade-off by analysing the effects of transgenerational exposure to CO_2 stress on a range of physiological parameters in wild type Sydney rock oysters. Their findings suggest that the resilience of oysters to OA may be mediated by adaptive differential gene expression, whereby energy is diverted from reproduction to extracellular acid-base regulation. In a similar context, the molecular mechanisms associated with transgenerational conditioning of a reef fish (*Acanthochromis polyacanthus*) to ocean warming have been recently described (Veilleux *et al.* 2015). Following transgenerational exposure to thermal stress, the expression of genes involved in metabolism, stress and immune responses increased, suggesting that elevated temperature alters energy production and allocation in this species as well. The down-regulation observed in the current study for a substantial number of genes indicates that some biological processes are affected and potentially compromised following transgenerational conditioning to OA. The down-regulation of these processes may allow B2 oysters to better cope with the impacts of OA through altered resource partitioning.

Decreased expression of numerous genes was also evident among wild type oysters responding to elevated CO_2. High-throughput qPCR revealed that transgenerational exposure to CO_2 stress resulted in down-regulation of 47% of the genes tested in both B2 and wild type oysters. However, some genes exhibited contrasting responses between populations, while the responsiveness of others was shown to be population-specific. Contrasting responses between B2 and wild type oysters under CO_2 stress were observed for numerous biological processes, and PCA indicated that B2 oysters were more responsive to elevated CO_2 than wild type

oysters. Genes involved in immune responses (multiple epidermal growth factor-like domains proteins 10 - MEGF10, and complement C1q/tumor necrosis factor-related protein 7 - C1q), stress responses (Hsp70), and the regulation of cellular homeostasis (von Willebrand factor D and EGF domain-containing protein) were up-regulated in B2 oysters but down-regulated in wild types (relative to their respective ambient controls). These data suggest that at least one aspect of the transgenerational response of B2 oysters to elevated CO_2 involves the induction of genes involved in cellular stress responses and the maintenance of cellular homeostasis in order to mitigate the effects of cell damage resulting from reducing pH. Moreover, while the data could be interpreted to suggest that trade-offs in other systems may result from the up-regulation of immune response and homeostasis genes in the B2 line, the resultant physiological changes are impossible to be accurately predicted.

MEGF10 is of particular interest in this context. It encodes a membrane protein involved in the phagocytosis of apoptotic cells, as well as in cell adhesion and motility. RNA-seq analysis of corals has previously revealed higher levels of MEG10 infected with white band disease (Libro *et al.* 2013), while MEG11 in the marine snail, *Chlorostoma funebralis,* was up-regulated by thermal stress (Gleason & Burton 2015). In contrast, the expression of MEGF10 was found to be down-regulated by hypoxia and hypo-osmotic stress in the Pacific oyster *C. gigas* (Sussarellu *et al.* 2010). Among the other differential genes identified in the current study, C1q domain-containing proteins include a wide range of molecules that participate in the control of inflammation, innate immunity (pathogen recognition) and energy homeostasis (Gerdol *et al.* 2011; Wang *et al.* 2012). Increased expression of C1q domain-containing proteins has been observed in a population of *C. gigas* that shows resistance to acute heat stress (higher survival rates during experimentally-induced summer mortality events) (Fleury & Huvet 2012). Expression of C1q domain-containing proteins was also shown to be affected by bacterial challenge in *C. virginica* and *S. glomerata* (Green & Barnes 2010; McDowell *et al.* 2014). The high levels of immune-related proteins found in the current study suggests that, under CO_2

stress, B2 oysters are able to enhance the mechanisms associated with immune responses, including pathogen recognition, cellular communication and clearance of apoptotic cells.

Heat shock proteins (Hsp) were also among the most substantially affected genes identified in B2 oysters. Throughout numerous animal taxa, Hsp is one of the most responsive multigene families to a variety of stressful environmental conditions, including (but not restricted to) acute heat stress (Feder & Hofmann 1999). Hsp70s act as molecular chaperones, promoting protein folding, membrane translocation, degradation of misfolded proteins and other regulatory processes (Liu & Chen 2013). The Hsp protein family plays a key role in cells during stress by maintaining cellular homeostasis. Exposure of oysters to temperature extremes (12 °C to 19 °C increases relative to current ambient conditions) has previously been reported to result in dramatic increases (up to 2,000-fold) in Hsp expression (Farcy *et al.* 2009; Meistertzheim *et al.* 2007; Zhang *et al.* 2012; Zhang *et al.* 2015). Elevated CO_2 conditions have also been shown to affect the expression of Hsp in oysters. Thompson *et al.* (2015) recently found higher concentrations of Hsp70 proteins in wild type Sydney rock oysters, but lower levels in the B2 breeding line following a single exposure to CO_2 stress.

Genes encoding proteins that contain von Willebrand (VWF) factor D and EGF-like domains were also prominent in the differential transcriptome of B2 oysters. Proteins containing these domains participate in a variety of cellular processes, playing important roles in protein-protein interactions and the assembly of protein subunits, as well as in the maintenance of cellular homeostasis (Whittaker & Hynes 2002). Although the biological functions of VWF domain-containing proteins are not fully described in molluscs, they are likely to be involved in such fundamental regulatory processes. Proteomic analysis recently revealed that VWF factor D domain-containing proteins are up-regulated by thermal stress and down-regulated by exposure to air in *C. gigas* (Zhang *et al.* 2015).

High-throughput gene expression profiling of B2 and wild type oysters also revealed population-specific gene responses (*i.e.* genes that are differentially expressed in a single

population, not responsive in the other). Genes shown to be affected by CO_2 stress in B2 oysters only are of particular interest because of their potential involvement in the physiological resilience to OA previously reported for this population. Transgenerational conditioning of B2 oysters to elevated CO_2 resulted in increased expression of uracil-DNA glycosylase (UDG) and condensin-2, which are involved in nucleotide regulation, as well as enhanced transcription of the immune-related genes, pentraxin fusion protein and perlucin, and the stress-related proteins glutathione S-transferase omega and serine/threonine-protein kinase mitochondrial (PINK1). All of these genes that were up-regulated in B2 oysters remained unaffected by CO_2 stress in wild type oysters. In the context of the up-regulated genes involved in nucleotide regulation, a number of studies have shown that environmental stress can induce genomic instability. Intracellular stress responses triggered by changes in environmental conditions can culminate in random mutations particularly when cells are poorly adapted to these environments (Galhardo *et al.* 2007). Base-excision repair is one of the most important mechanisms protecting cells from endogenous (*e.g.* reactive oxygen species) and external (*e.g.* thermal stress, pH, toxic chemicals) damage and preventing mutagenesis (David *et al.* 2007; Krokan *et al.* 2000). Uracil-DNA glycosylases (UDG) are key components involved in DNA repair and prevention of DNA damage. They hinder the propagation of base mismatches to downstream transcription and translation processes. The increased levels of UDG observed in CO_2-conditioned oysters might suggest a compensatory response of their cells to control mutations triggered by CO_2 stress. By inducing the expression of UDG under elevated CO_2 conditions, B2 oysters could ultimately prevent cell death due to stress-induced DNA damage. In this context, condensin is a protein complex responsible for the structural reorganisation of chromatin into condensed chromosomes during cell division (Hirota *et al.* 2004). The differential regulation of condensin in response to stress has not previously been demonstrated in molluscs and further research is required to understand its role under elevated CO_2 environments.

Genes encoding pentraxins were also found in high levels in B2 oysters following transgenerational conditioning to elevated CO_2 but were not detected in wild type oysters. Pentraxins are lectins, and so play important roles in the innate immune response by recognizing and binding to specific carbohydrate moieties on the surface of microorganisms. Carbohydrate binding facilitates agglutination, complement-mediated opsonisation, cell lysis and a range of other cellular processes (Armstrong 2015). However, only a few studies have reported the response of pentraxin to environmental stress, thus its participation in oyster stress responses is poorly understood (Armstrong 2015; Olafsen 1995). Another differentially expressed lectin identified in the current study, perlucin, is involved in nucleation of calcium carbonate ions during shell formation (Blank *et al.* 2003; Mann *et al.* 2000; Weiss *et al.* 2000). Its expression levels might be expected to be altered by CO_2-driven OA, since the availability of calcium carbonate ions is reduced as seawater pH decreases. However, no changes in perlucin mRNA levels were observed in the mussel *Mytilus edulis* after exposure to elevated CO_2 (pH 7.8) (Hüning *et al.* 2013; Li *et al.* 2015). The identification of differential genes such as perlucin in B2 oysters suggests that they may be able to better maintain carbonate homeostasis and shell calcification under CO_2 stress. This is supported by previous studies showing that B2 oysters deposit shell faster and have fewer shell abnormalities under elevated CO_2 environments when compared to wild type Sydney rock oysters (Parker *et al.* 2012; Parker *et al.* 2011).

Intracellular stress response proteins also featured significantly in our differential transcriptome for B2 oysters. Elevated CO_2 is known to induce oxidative stress in numerous mollusc species (Matoo *et al.* 2013; Thompson *et al.* 2015; Tomanek *et al.* 2011). In the current study, we found elevated expression of the stress-related proteins glutathione S-transferase omega and mitochondrial serine/threonine-protein kinase (PINK1). Glutathione S-transferases are involved in the mitigation of lipid peroxidation resulting from oxidative stress. This gene was previously found to be up-regulated by hypoxia in *C. gigas* (David *et al.* 2005), while lower expression levels were observed in B2 juveniles exposed to CO_2 stress over three consecutive

generations (Chapter 3). Higher levels of the stress-related gene PINK1 were also found in CO_2-exposed B2 oysters (relative to ambient control conditions). PINK1 protects cells from stress-induced mitochondrial dysfunction by phosphorylating mitochondrial proteins (Mizushima & Komatsu 2011; Narendra *et al.* 2010). It participates in the clearance of damaged mitochondria via autophagy and is associated with oxidative stress. The up-regulation of both glutathione S-transferase omega and PINK1 indicates that processes related to cellular stress are induced in response to elevated CO_2 in B2 oysters, but not in wild type individuals. The activation of such mechanisms could prevent oxidative and mitochondrial damage that might otherwise result in cell death.

In summary, this study investigated the molecular processes underlying transgenerational conditioning of oysters to OA. By exploring differences in the adaptive responses between CO_2-conditioned B2 and wild type oysters, we identified a suite of genes that may contribute to the distinctive performance of B2 oysters to CO_2 stress. These genes range across broad levels of the whole transcriptome and are mostly related to the mitigation of cellular damage. However, more precise characterisation of these genes suggests that some specific biological functions are being preferentially affected, primarily those involved in the cell cycle and cellular homeostasis. This may reflect inducible responses in B2 oysters to control the cell cycle and maintain cellular homeostasis under elevated CO_2 conditions. Such changes have the potential to prevent apoptosis resulting from oxidative damage or to mitigate the effects of apoptosis through regulation of the cell cycle. This is the first comprehensive study to identify the broad set of molecular processes involved in responses to OA in a CO_2-resilient organism. Our findings reveal the biological functions that may enable marine calcifiers to cope with acidifying environments in the face of climate change. Identifying the mechanisms of stress tolerance in marine calcifiers can provide fundamental insights into their potential for adaptation to a wide range of environmental challenges. This understanding will be critical to minimise future environmental and economic impacts of a rapidly changing ocean.

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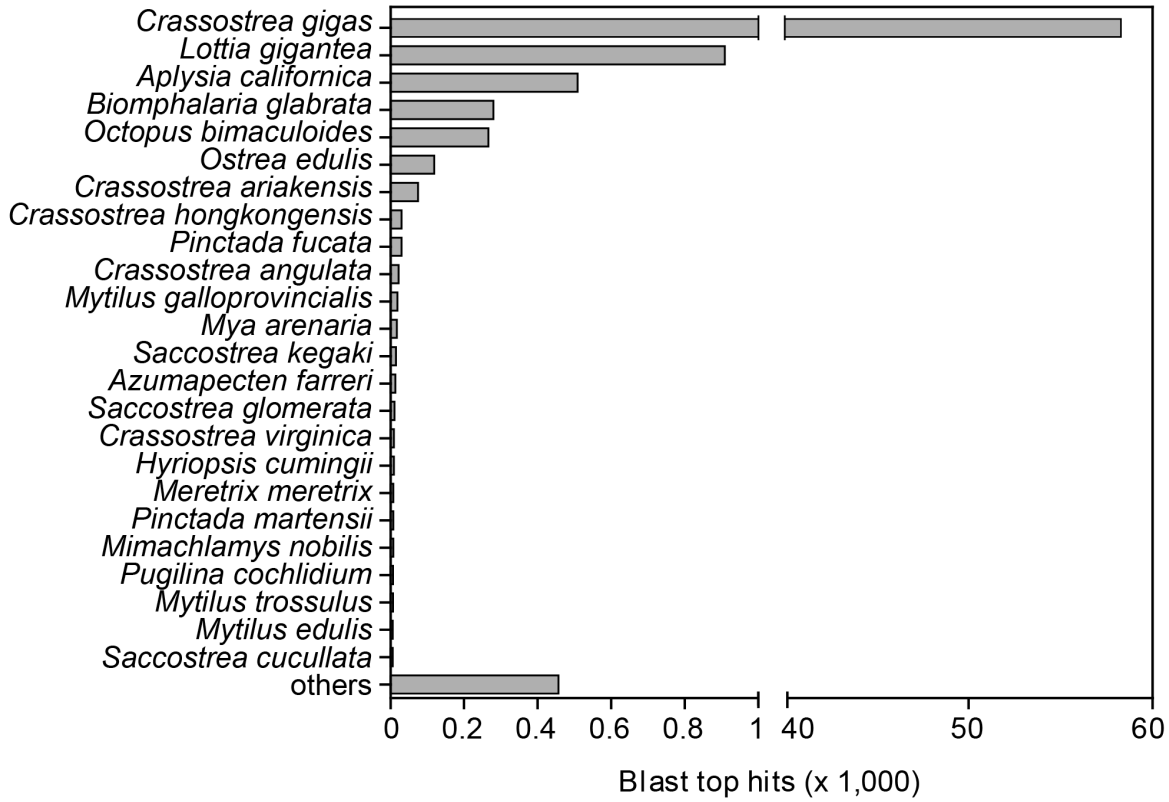
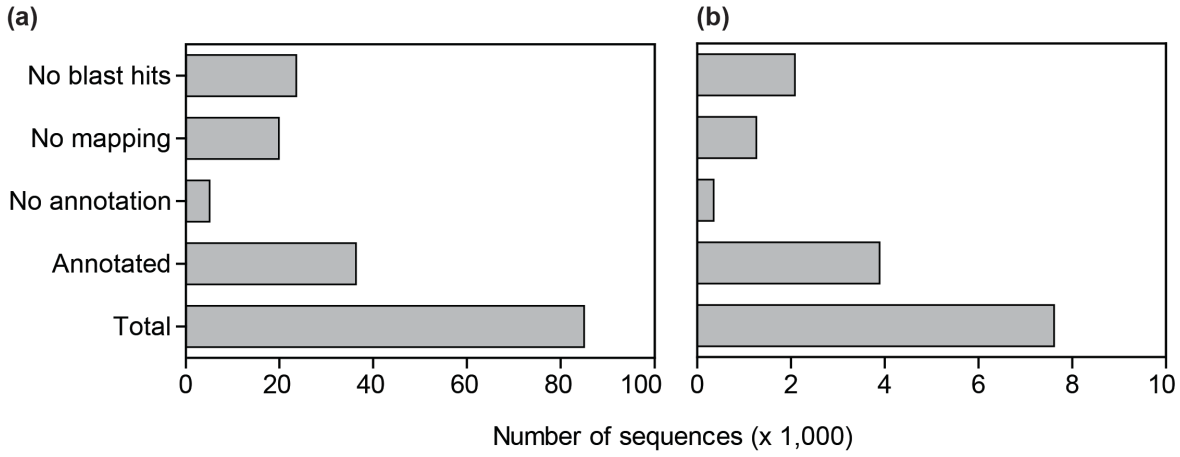
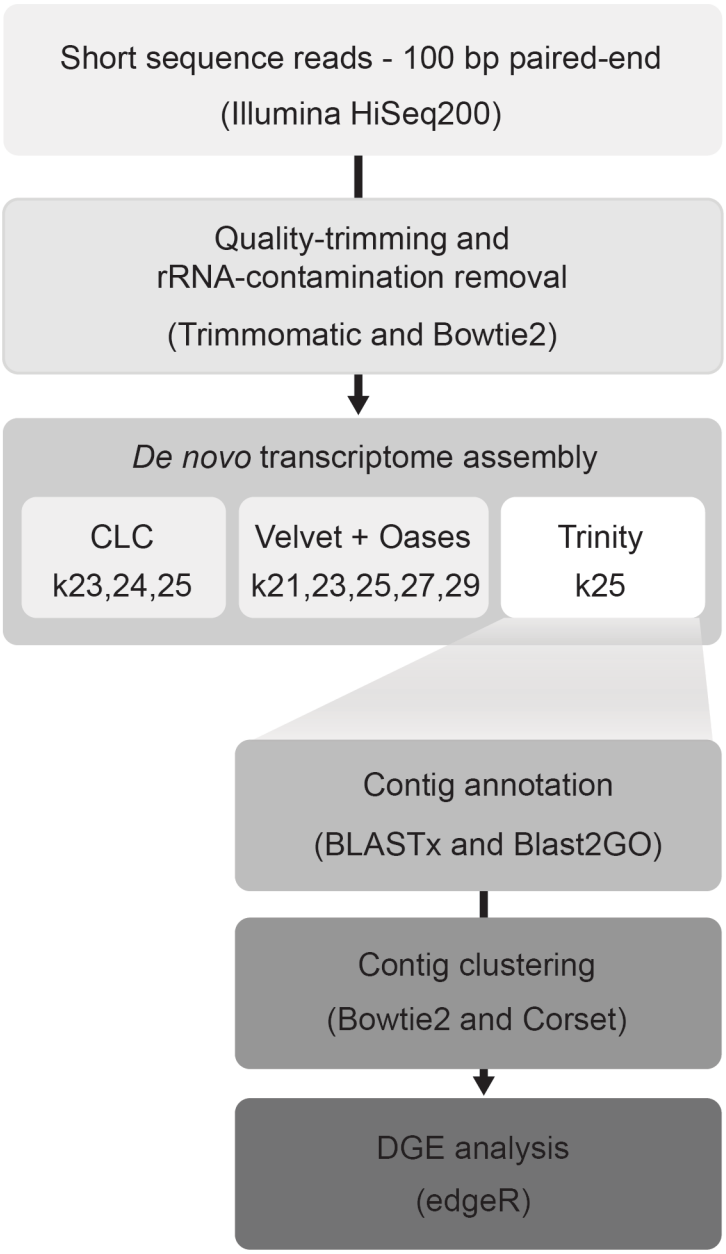
SUPPLEMENTARY INFORMATION



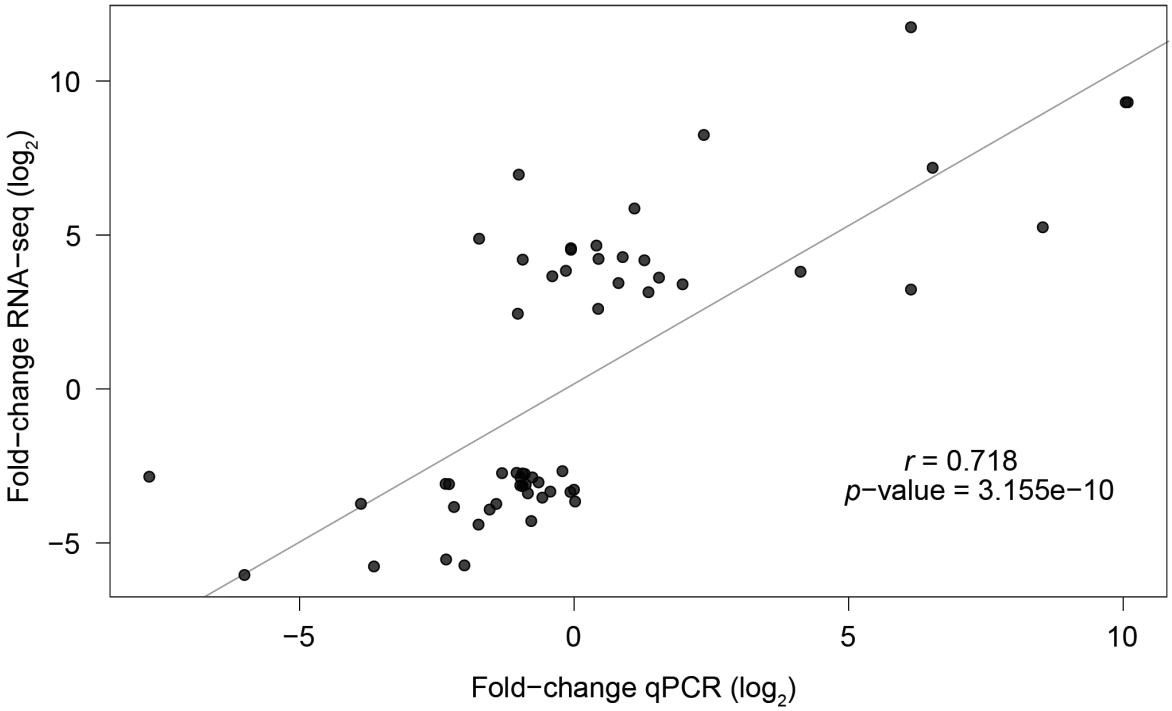
Supplementary Figure 4.1. Top hit species obtained from BLASTx analysis, using a Mollusca non-redundant (nr) database.



Supplementary Figure 4.2. Distribution of data from annotation analyses. Results from Blast2GO three-step analysis including BLASTx, mapping and annotation of Sydney rock oyster transcriptome **(a)** and contigs differentially expressed under CO_2 stress **(b)**. 'No blast hits' represents contigs with no homologous sequence matches to a Mollusca non-redundant (nr) database; 'No mapping' represents contigs with homologous sequence matches to a Mollusca non-redundant (nr) database, but no gene ontology (GO) annotation; 'No annotation' corresponds to contigs with a preliminary GO annotation (mapping), but that failed to meet the minimal annotation threefold; 'Annotated' represents contigs that were successfully blasted and annotated.



Supplementary Figure 4.3. Overview of the assembly, annotation and differential expression analysis workflow.



Supplementary Figure 4.4. Comparison of the expression data generated by RNA sequencing and qPCR. Pearson correlation analysis was carried out based on log_2 fold-change values for 57 genes. Fold-change was calculated as the ratio of the relative expression in oysters exposed to elevated and ambient CO_2 (B2E / B2A). Results show a high correlation ($r = 0.72$, $p = 3.155e\text{-}10$) between the two quantitative gene expression platforms.

Supplementary Table 4.1. Primers used for qPCR analysis. Also available in: https://drive.google.com/file/d/0BwlanLU1MxH5bUY4MEh2TXR4eUU/view?usp=sharing

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
DCTN 1	dynactin subunit 1	GCCACGTCTGAAACC TTTGCT	CGGTCTCTTCCTGCC TGCTT	112	2.07	Cell cycle, Cytoskeleton, Protein regulation	Cluster-88976.1--c109359_g1_i12
DGKB	diacylglycerol kinase beta	GCAGGTAACGTCGTA CTGTCC	GCATGGGTTCATCCT CATCCC	132	2.01	Cell cycle, Communication	Cluster-76827.0--c95993_g2_i1
Struct Maint	structural maintenance of chromosomes protein 3	CCAACATGCAGCTAC AGTGTAC	ACTGAGTGACACTGA AGATGGC	75	1.99	Cell cycle, Communication, Immune response	Cluster-109736.2--c112509_g2_i5
Septin2	septin-2	TGCCGTAGTGTACCC ATCATTC	TGTCCCAGACCTTAG ACCAGAG	111	2.11	Cell cycle, Cytoskeleton	Cluster-36759.0--c105265_g2_i1
Caspas e3	caspase 3	CTTGCGTGATGCGTT CTATTGG	GCTGCATTGTAGGTG CCGTTT	90	2.04	Cell cycle, Immune response	Cluster-114230.2--c97346_g1_i5
MEGF 10	multiple epidermal growth factor-like domains protein 10	ATCCGTCATCGCAAC TCCCA	CGGATGGGATATGAC TGCAACG	130	2.08	Cell cycle, Metabolism, Protein regulation	Cluster-3690.0--c104186_g3_i1
IAP2	inhibitor of apoptosis 2	GTATTCCTGCCCTGTG GTC	GGTCCCTTTGATGTT CTTCC	93	1.98	Cell cycle, Stress, Immune response	Cluster-90512.1--c94920_g1_i2
IAP1	inhibitor of apoptosis 1	TGCGATACGTGTGCT GCTGA	CAGGCAGACACAAA CAGGACAA	135	1.96	Cell cycle, Stress, Immune response	Cluster-91211.0--c114749_g1_i1
CD63	CD63 antigen	CGCCGACACAACCAA GAACA	TCAACATCGCTGACT TCGTCG	93	2.04	Communication	Cluster-37586.1--c104081_g1_i3
Conde nsin-2	condensin-2 complex subunit g2	TCATCAGACGACGGA CCACC	GAGAAGGTCAGGGT CGCCAT	142	2.02	Communication, Cell cycle, Nucleic acid regulation	Cluster-82138.1--c110378_g1_i2
Protoca dherin	protocadherin fat 4	ACTCCCGACGCTGGA AATCA	CCATCACCGTCCAAA GGAATG	150	2.06	Communication, Membrane transport	Cluster-54355.0--c112409_g1_i2
FGF	fibroblast growth factor receptor 2	TGGTCCTGCTTGGTTT GATG	CTGCTTGCTCTCAGC CTTTATG	98	2.00	Communication, Metabolism, Protein regulation	Cluster-108314.0--c113307_g2_i2
Jagged 2	protein jagged-2	ATGCCAGCCCTACCA ATGGG	TCCACCCGTACACAG CAGTC	130	1.98	Communication, Nucleic acid regulation, Metabolism	Cluster-70268.0--c109332_g1_i1
BTBD 2	BTB/POZ domain-containing protein 2	GGAACCGTCCACTCC ACATCT	CCAGCACTTGGTCGC TCATC	99	2.14	Communication, Stress	Cluster-87330.1--c106425_g1_i2
Filami nC	filamin-c	CCAATCCCGATGGTA CTGTTGC	CGTGGGTCGGACTCT ACATTTG	125	2.08	Cytoskeleton	Cluster-93520.0--c97957_g1_i2
Titin	titin	AACGGTTATCCTTGC GGACG	GCACGTCACCACAGT GCTAG	81	1.90	Cytoskeleton	Cluster-44863.0--c80283_g1_i1

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
Dynein	dynein heavy chain axonemal	GGACTGAGCGCCA CATTGTC	TTGACCTGCCTGTG CTTTCC	115	1.97	Cytoskeleton, Cell cycle, Metabolism	Cluster-78817.0--c83373_g1_i1
Collagen	short-chain collagen c4	GGACGCCATAACG GAAGAAC	GGTCGTGAAGATG GGAATGG	97	2.03	Cytoskeleton, Metabolism	Cluster-77089.1--c107854_g1_i4
Myosin	myosin-IIIb	CCCTGCAGTTTGTC GTTAAGCA	TGGACATACCTGG GTTTGAGGA	82	1.99	Cytoskeleton, Nucleic acid regulation, Metabolism	Cluster-87569.2--c114302_g1_i3
β-actin	β-actin	GCACCTGAATCGCT CGTTG	CAGCAGCATCGTC ATCATCC	86	1.95	Cytoskeleton, Reference gene	Chapter 3
VWF	von Willebrand factor D and EGF domain-containing protein	CCCAGACAACAAG AGCGTGG	GGCAGATCCCGTC TCATGGA	123	1.98	Homeostasis, Cell cycle, Protein regulation	Cluster-97446.0--c116588_g1_i4
HAAF	hemagglutinin/amebocyte aggregation factor	CGAGCTTCTTGGTC CACAGC	TTCCAGTGTCCAGG AGCAGG	105	2.16	Immune response	Cluster-30855.1--c104174_g2_i3
IgGFc-binding protein	IgGFc-binding protein	GCTGCATCCTTTCC CAAAGTCA	CCAGCATCGTTAG CCGACAA	71	2.01	Immune response, Communication	Cluster-99578.3--c110214_g3_i1
Pentraxin	pentraxin fusion protein	GGCCATTTCTCGGG ACCTAC	GGTCAAGGTAATT CGGCGTTC	118	1.95	Immune response, Communication	Cluster-14923.1--c100539_g2_i4
C1q	complement C1q tumor necrosis factor-related protein 7	GCCTTGGTGATTGT TCAAGTTC	CACTTCATGCCCA GTACCACAG	75	1.92	Immune response, Communication	Cluster-11830.0--c114049_g3_i4
Toll4	toll-like receptor 4	ACAGGAAACAGAG GCTGGCTAC	GGAACCACTCTCA GCAGGTGTC	93	1.98	Immune response, Communication	Cluster-44310.0--c96407_g1_i2
Perlucin	perlucin	ATGACCAACGACA GCACCATTC	TCCGCGTCCAGCA TTTATAGC	148	2.06	Immune response, Metabolism, Communication, Calcification	Cluster-73087.0--c90325_g1_i5
Kelch3	kelch-like protein 3	TTGTGCCGCAGAGC TGAATG	CATCTGTCAGTGCT TGGCTGG	117	1.95	Immune response, Protein regulation, Communication	Cluster-72323.0--c105029_g1_i2
Fibrocystin-L	fibrocystin-L	AGGCTGGTCACCTC CTTTCC	TTCTACACTCCGCC CTTGCC	74	1.99	Immune response, Translation	Cluster-112582.0--c116838_g2_i1
MPI	mannose-6-phosphate isomerase	GCAAGGCCACTTCA CTTTCAC	CAGAAGGCGATCT TGTGTTTCC	87	1.99	Metabolism	Cluster-3673.0--c94904_g1_i4
ATP-binding cassette	ATP-binding cassette sub-family a member 3	GGGCTGTCCATCCA ACAAGG	CGCTTCATGCCACC AGACAG	111	1.96	Metabolism	Cluster-14516.3--c109076_g1_i5
COX1	cytochrome oxidase subunit I	TTTCCTACCACGGG ATGTG	TGAGCTAATACCA GCCAAGTGA	65	2.04	Metabolism	Chapter 3
M6P receptor	Cation-independent mannose-6-phosphate receptor	GGAATGGTGGCAA GGGTTGG	ACAGGGTGATCCA GACAGGC	97	2.01	Metabolism, Communication, Membrane transport, Cytoskeleton	Cluster-87912.1--c96611_g2_i5

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
Fatty acid-binding protein	fatty acid-binding protein	TGTGAATGTTAGT GGCGGATCA	TCAGATAACGTGG CACCCAGTA	81	1.97	Metabolism, Membrane transport	Cluster-88308.1--c98275_g1_i2
Kchannel	potassium channel subfamily t member 2	AGGATGGACACTC ACAAGACCT	GCCAGTGTTAGGG ACAGTAAGC	125	2.08	Metabolism, Membrane transport	Cluster-61933.1--c98546_g1_i2
Sortilin	sortilin-related receptor	AGGGAGAGACCTG GAACACC	CCCTGGTTCTGTCA GTACACC	74	1.97	Metabolism, Membrane transport	Cluster-79968.0--c115575_g1_i1
Two pore Ca channel	two pore calcium channel protein 1	TCCCAGCTCCTCCT TCTCCA	GAAGTTGTGGTCCC GCCCTA	151	2.03	Metabolism, Membrane transport	Cluster-95715.1--c113835_g1_i4
Tricarboxylate mitochondrial	tricarboxylate transport protein mitochondrial-like	CATCCACAGGCCC GTTAACTG	GGTAATGTGCGTCT GACAGGC	124	1.99	Metabolism, Membrane transport	Cluster-92964.1--c101868_g1_i5
UDG	uracil-DNA glycosylase	ACAGATGCCGTCA TATCGCA	GGCTGACAATGGA GAAGGATGG	147	2.08	Metabolism, Nucleic acid regulation	Cluster-4271.1--c86269_g1_i2
Poly (ADP-ribose) polymerase 14	poly (ADP-ribose) polymerase 14	TCCACCCTGGTATC CGTTATGT	CCCAGCAGGTTGTA AATGGAAG	120	1.98	Metabolism, Nucleic acid regulation	Cluster-53829.1--c108134_g2_i5
Tho3	tho complex subunit 3	GTTTACGGTTGCCT GGCACC	CCTGCGTCCCGATT TCTGTC	87	1.96	Metabolism, Nucleic acid regulation, Protein regulation	Cluster-103303.0--c108074_g2_i1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	ACCGCGCCAGTCT TTGTTG	GGCATTGTTGAGG GTCTGATG	90	1.94	Metabolism, Potential reference gene	Chapter 3
Carboxypeptidase 2	cytosolic carboxypeptidase 2	GCTTGATGCTTGCA GAGGCT	CGTCTTCTCCTCTT GATGGTCC	74	2.03	Metabolism, Protein regulation	Cluster-112465.0--c103102_g1_i3
Transient receptor Ca channel	transient receptor potential cation channel subfamily m member 2	GAGCAACGGTCAA GAACAGG	AAGCACGTATGGT ATGGAGGAG	105	1.95	Metabolism, Protein regulation	Cluster-3796.2--c103010_g1_i7
Phosphatase 1F	protein phosphatase 1F	GCTGCACTGGATT AACGGTGT	ACCTGCCTCTCTGG TTTGTGG	133	2.06	Metabolism, Protein regulation	Cluster-87959.2--c109034_g1_i5
ECE1	endothelin-converting enzyme 1	GGCTTGGGACTGA CTAATGACC	GCACTGCGTTGTAA TAAGCGAC	97	2.03	Metabolism, Protein regulation	Cluster-75158.0--c87919_g1_i3
Disintegrin Metalloproteinase	disintegrin and metalloproteinase domain-containing protein 10	TATCTCACAGACG CCGCCAG	CTACATGTTCGCGG ACCGTG	91	1.99	Metabolism, Protein regulation, Communication	Cluster-114448.0--c104827_g1_i2
Serine arginine	serine arginine repetitive matrix protein 1	CGCTGAACCGATT GTCCTGG	AAATGGTGAGAGT GGGTGTTGG	86	2.01	Nucleic acid regulation	Cluster-96489.0--c85937_g1_i1
H3	histone H3	CTCTTACGGGCTGC CTTGGT	ACACTCTGCCCTCG TCATTCA	128	2.03	Nucleic acid regulation, Cell cycle	Cluster-110131.0--c78549_g1_i1
H4	histone H4	AAACCCGTGGTGT CCTGAAAG	TTGGCGTGCTCTGT GTAGGTG	73	1.99	Nucleic acid regulation, Cell cycle	Cluster-93517.1--c90347_g2_i2

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
DDX58	ATP-dependent RNA helicase DDX58	CGCTTCTCACTCGCCCTTTC	GCCTCGATCTCCCAAACTGC	93	2.09	Nucleic acid regulation, Metabolism	Cluster-82669.1--c111146_g2_i3
EF1α	elongation factor 1 alpha	CCATAGCGGCATCTCCACTC	CCTTGATTGCCACACTGCTC	125	1.97	Nucleic acid regulation, Reference gene	Chapter 3
Sentrin	sentrin-specific protease 8	AGCAGAGGGTCATTTGTCCTTG	TCCAAGAGCTGCTCAACATGC	97	2.04	Protein regulation	Cluster-96379.2--c107078_g1_i5
Leucine 15	leucine-rich repeat-containing protein 15	TGTACCGCGACTGCTTCATACA	TCTGTGAGCGGATTACCCAACT	126	1.98	Protein regulation, Communication	Cluster-75395.0--c110209_g1_i1
TubulinC	tubulin-specific chaperone C	GTAGCTCCCACGTATGCCAC	GCTTCAGCGTCCTCTTCTCC	95	1.97	Protein regulation, Cytoskeleton	Cluster-93882.0--c101601_g1_i2
Universal stress	universal stress protein A	TAGATCCGCCTCCACCTCCT	GACGCCAAGATAAACGGCACT	93	2.02	Stress	Cluster-87337.1--c105847_g1_i4
ecSOD	extracellular superoxide dismutase	TAGCCAATGAACGTCCCAGA	GATCTCCATGACGACGACCA	100	1.99	Stress	Cluster-107002.1--c103921_g3_i2
Multidrug resistance	multidrug resistance protein 1	GTATTGGCCGGGTGCTGAAG	AGATGATGGCGAAGGCTGGT	108	2.01	Stress	Cluster-78870.1--c116398_g1_i2
Dual oxidase	dual oxidase	TGTGTCTTTCCACTGGCTGTC	ACAGGAAACGCCGAACCTTG	107	2.04	Stress, Metabolism	Cluster-78095.0--c90549_g1_i1
Glutaredoxin-2 mitochondrial-like	glutaredoxin-2 mitochondrial-like	TGGAACCTGTATTGGTGGAGCA	TCCACTTGAGGGTTATGGCACT	96	2.06	Stress, Metabolism	Cluster-116115.3--c99186_g1_i4
Glutathione S-transferase	glutathione S-transferase omega	CCTGTGACGTAGCAAAGCCAGT	CCGAGGTAGGGAAACAGCTTGA	126	1.97	Stress, Metabolism	Cluster-113541.2--c110195_g2_i3
P450	cytochrome P450 1A1	AAACGAAATCCAGGCACGGC	GATGCACATGGGAGGATGCG	87	1.94	Stress, Metabolism, Protein regulation	Cluster-78884.0--c102501_g2_i4
Hsp70b	heat shock 70 kDa protein 12b	CGTGACTGAGAAAGGGTGTTTG	CATACAAGTGGCTTTGGCTTGG	136	2.00	Stress, Protein regulation	Cluster-79384.0--c74600_g1_i2
Hsp70a	heat shock 70 kDa protein 12a	CGTGACTGAGAAAGGGTGTTTG	CAAGTGGCTTTGGCTTGGA	132	2.04	Stress, Protein regulation	Cluster-79384.0--c74600_g1_i3

Note: The following Supplementary Tables are too large for printing and can be found online in their respective links:

Supplementary Table 4.2. List of gene clusters and contigs identified in the transcriptome of Sydney rock oysters. Available in: https://drive.google.com/file/d/0BwlanLU1MxH5RWZydkRnbHM4R2c/view?usp=sharing

Supplementary Table 4.3. List of gene clusters and contigs differentially expressed (FDR-adjusted $p < 0.05$) in B2-line oysters in response to ocean acidification. Available in: https://drive.google.com/file/d/0BwlanLU1MxH5SlBLSEpKazc3RGc/view?usp=sharing

Supplementary Table 4.4. Top 200 most affected contigs following exposure to CO_2 stress. Available in: https://drive.google.com/file/d/0BwlanLU1MxH5OW51b3E3YUFySWc/view?usp=sharing

CHAPTER 5

CONTRASTING IMPACTS OF OCEAN ACIDIFICATION AND WARMING ON THE MOLECULAR RESPONSES OF CO_2-RESILIENT OYSTERS

Prepared for submission to BMC Genomics as: Goncalves P, Thompson EL, Raftos DA. Contrasting impacts of ocean acidification and warming on the molecular responses of CO_2-resilient oysters.

ABSTRACT

This study provides an integrative characterisation of the molecular processes altered by both elevated CO_2 and increasing temperature in oysters. Differences in resilience of marine organisms against the environmental stressors associated with climate change will have significant implications for the sustainability of coastal ecosystems worldwide. Some evidence suggests that climate change resilience can differ between populations within a species. Sydney rock oysters from the B2 breeding line are able to better withstand the negative effects of CO_2 stress at the physiological level. Here, we used proteomics and transcriptomics to evaluate whether the differential response of B2 oysters to elevated CO_2 also extends to increasing temperature. Substantial and distinctive effects on protein concentrations and gene expression were evident among B2 oysters responding to elevated CO_2 or elevated temperature. The combination of both stressors also altered both proteomes and gene expression. However, the impacts of elevated CO_2 and temperature were not additive or synergistic. The data suggest that the simultaneous exposure of CO_2-resilient oysters to near-future projected ocean pH and temperature results in complex changes in molecular processes in order to prevent stress-induced cellular damage. The differential response of B2 oysters to the combined stressors also indicates that the addition of thermal stress may affect their resilience to decreased pH. Overall, this study reveals some intracellular mechanisms that might enable marine calcifiers to endure the emergent, adverse seawater conditions resulting from climate change.

5.1 INTRODUCTION

Environmental change in marine ecosystems resulting from global climate change represents one of the most serious threats to marine organisms. Oceans are becoming warmer and more acidic as atmospheric concentrations of CO_2 increase (IPCC 2013). Marine organisms will need to acclimate or adapt in the face of these shifts in seawater chemistry and temperature. Many studies have described the negative impacts of ocean acidification (OA) and rising temperatures on the calcification, energy metabolism, reproduction, development and growth of marine calcifiers, such as oysters (Fabry 2008; Gazeau *et al.* 2013; Miller *et al.* 2009; Parker *et al.* 2013; Pörtner *et al.* 2004). However, the combined, potentially synergistic effects of OA and increasing water temperature are largely unknown in marine calcifiers, particularly at the intracellular level.

Proteomic studies have shown that CO_2-driven OA (pH 7.3 to 7.87) alters the concentrations of proteins involved in antioxidant defence, stress responses, energy metabolism and the cytoskeleton in the oysters *Crassostrea virginica*, *Crassostrea hongkongensis*, *Crassostrea gigas* and *Saccostrea glomerata* (Dineshram *et al.* 2013; Dineshram *et al.* 2012; Thompson *et al.* 2015; Timmins-Schiffman *et al.* 2014; Tomanek *et al.* 2011; Wei *et al.* 2015). Similarly, transcriptional analysis of oysters exposed to elevated CO_2 (*C. virginica*; pH 7.5 to 7.6) have identified changes in the expression of genes associated with calcification, stress and immune responses (Beniash *et al.* 2010; Ivanina *et al.* 2014). Increasing temperature also causes substantial changes in the molecular processes of marine organisms (Tomanek 2014). Heat shock proteins (Hsp) are one of the protein families most affected by thermal stress in oysters. Exposure to temperature extremes (15 °C to 19 °C increases relative to current ambient conditions) have been shown to substantially increase (up to 2,000-fold) Hsp expression in the Pacific oyster *C. gigas* (Farcy *et al.* 2009; Zhang *et al.* 2012). Acute heat stress (12 °C increase) also induced changes in the transcriptional levels of other genes involved in cellular

homeostasis and protein synthesis (Meistertzheim *et al.* 2007). At the protein level, increased concentrations of Hsp, as well as changes in the concentrations of proteins involved in energy metabolism, calcium binding and immune responses, were observed in *C. gigas* following exposure to temperature extremes (20 °C increase relative to ambient) (Zhang *et al.* 2015). Although relevant, these studies have focused on acute heat stress and so do not reflect near-future climate change scenarios where projected temperature increases range between 1.5 °C and 3.2 °C by the end of this century (IPCC 2013).

The transcriptomic response of oysters to a combination of low pH and increasing temperature was recently assessed using next-generation sequencing (NGS) (Chapman *et al.* 2011; Clark *et al.* 2013). The authors found that OA and the combination of both OA and increasing temperature have distinct effects on the intracellular systems of *C. virginica* and *C. gigas*. OA enhanced the expression of genes involved in antioxidant and metabolic processes in *C. virginica*. Both OA and increasing temperature affected protein synthesis and processes involved in cell growth, as well as changed the expression of genes related to metabolism (Chapman *et al.* 2011). In the Pacific oyster, *C. gigas*, low pH enhanced the expression of immune response genes and the production of antioxidants, while the combination of both pH and thermal stresses induced the expression of protease inhibitors and cytoskeleton-related genes (Clark *et al.* 2013).

Recent evidence also suggests that oysters may be able to acclimate or adapt to such stressful conditions (Miller *et al.* 2009; Parker *et al.* 2012; Thomsen & Melzner 2010). Molluscs from naturally CO_2-enriched environments do not show the negative physiological effects typically induced by OA when they are experimentally exposed to elevated CO_2 (Miller *et al.* 2009; Thomsen & Melzner 2010). Moreover, environmental stressors associated with climate change often elicit different phenotypic responses in distinct populations within a species. Populations of *C. gigas* selectively bred for tolerance to acute heat stress (higher survival rates

during experimentally-induced summer mortality events) show distinctive transcriptional profiles relative to populations that are sensitive to rising temperatures (Lang *et al.* 2009). These heat-tolerant oysters exhibited lower expression levels of Hsp27, collagen, peroxinectin and S-crystallin, and higher expression of cystatin B. Elevated CO_2 concentrations have also been shown to induce different physiological and intracellular responses in two genetically distinct populations of Sydney rock oysters (*S. glomerata*). Larvae from oysters produced through selective breeding for fast growth and disease resistance (the B2 line) have higher survival rates and grow faster than wild type (non-selected) larvae following exposure to elevated CO_2 (Parker *et al.* 2011). B2 adults have higher standard metabolic rates (SMR) than wild type oysters under ambient conditions, and their SMR is further increased by CO_2 stress (Parker *et al.* 2012). Differential responses between populations of Sydney rock oysters are also evident at the intracellular level. Distinctive changes in protein and gene regulation were found in B2 oysters after exposure to elevated CO_2 concentrations (pH 7.78 to 7.84). Molecules involved in metabolism, oxidative stress, transcription, protein modification and signal transduction were the most affected by CO_2-driven OA (Thompson *et al.* 2015; Chapters 3 and 4 of this thesis). Such shifts in expression patterns were associated with changes in the functional characteristics of oyster cells. Mitochondrial integrity and the production of reactive oxygen species (ROS) were altered by CO_2 exposure in selectively bred oysters, but not in wild type individuals (Chapter 2). These findings suggest that the distinctive performance of B2 oysters might result from their capacity to regulate metabolic activity and control oxidative stress in face of elevated CO_2 through differential regulation of molecules involved in these processes.

Despite the comprehensive analyses of responses to individual stressors, few studies have evaluated the response of oysters to the combined effects of heat stress and CO_2-driven OA. Hence, the interactive effects of elevated CO_2 and temperature on the intracellular processes of different oyster populations are not well understood and require further

investigation. In the current study, we investigated the combined effects of CO_2-induced OA and increasing temperature on the proteomic and transcriptomic responses of oysters that are potentially resilient to OA (B2 breeding line). This work is the first to explore the heritable potential of oysters for acclimation or adaptation to both climate change variables at the molecular level. The molecular responses of marine organisms to environmental stressors include numerous genes and proteins involved in multiple biological functions. Thus, by assessing the differential regulation of both proteins and genes, we were able to provide a more comprehensive, integrative characterisation of the molecular processes affected by near-future projected ocean pH and temperature. This understanding will be crucial for assessing the performance of marine calcifiers and the sustainability of their populations in a changing ocean. It will also aid the development of marker-assisted breeding programs designed to safeguard oyster aquaculture industries as climate change progresses.

5.2 Materials and Methods

5.2.1 Oysters and exposure to CO_2 and temperature

Adult Sydney rock oysters (*Saccostrea glomerata*; Gould 1850) from the B2 breeding line were provided by the Port Stephens Fisheries Institute (PSFI, Taylors Beach, NSW, Australia) of the New South Wales (NSW) Department of Primary Industries (DPI). This oyster population has been mass selected for fast growth and resistance to QX disease (causative agent: *Marteilia sydneyi*) and winter mortality syndrome (causative agent currently under review) for seven generations.

Oysters (6.63 ± 0.71 cm shell length) were transferred to the Sydney Institute of Marine Science (Chowder Bay, NSW) and acclimated to aquarium conditions in a flow-through seawater system (0.5 L per minute, seawater filtered at 20 μm). The nutritional supply from the flow-through seawater was supplemented every three days with a concentrated blend of

microalgae (Shellfish Diet® 1800, Reed Mariculture Inc., 4.3×10^8 algal cells per oyster). Following the 10-day acclimation, B2 oysters were exposed for one month to combinations of ambient (330 µatm pCO_2, pH 8.09) or near-future elevated (886 µatm pCO_2, pH 7.72) CO_2 concentrations with ambient (22.11 °C) or elevated (25.38 °C) temperatures (Table 5.1). The elevated CO_2 (mean decrease of 0.37 pH units) and temperature (mean increase of 3.26 °C) conditions are based on projected ocean surface increases by the year 2100 (IPCC 2013). Seawater CO_2 concentrations and temperature were controlled by a custom-made system that included pneumatic components from Parker Hannifin (Castle Hill, NSW), CO_2 sensors from Vaisala (Hawthorn, VIC) and a control system from Greenstar Building Automation & Citywide Electrical Services (Marrickville, NSW). CO_2-enriched air and heated water were added into two header tanks, which supplied the treatments with reduced pH (elevated CO_2). Each treatment comprised three replicate tanks (45 L), each of which contained 7 oysters (n = 21 oysters per treatment). Temperature, salinity and carbonate parameters (pH and total alkalinity) of seawater were monitored throughout the experiment.

The experimental exposures produced a total of four treatments: aCO_2 + aT (ambient CO_2 and ambient temperature), aCO_2 + eT (ambient CO_2 and elevated temperature), eCO_2 + aT (elevated CO_2 and ambient temperature), and eCO_2 + eT (elevated CO_2 and elevated temperature). Following the one-month exposure to CO_2 and/or thermal stress, oyster gills were collected and used for both proteomics and transcriptomics. For proteomics, oyster gills were immediately frozen at -80 °C (n = 15 per treatment), while gill samples for transcriptional analysis were stored in RNA later (Ambion) at -20 °C (n = 21 per treatment; including same oysters used for proteomics).

5.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 100 mg of gill tissue (n = 21 oysters per treatment; individually sampled and processed) using TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. RNA was treated with DNase I (Promega) and further precipitated with 0.3 M sodium acetate (pH 5.5) and isopropanol. Concentration and quality of total RNA were checked with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using ImProm-II™ Reverse Transcription System (Promega) and 0.5 µg of oligo(dT)$_{15}$ in a 20 µl reaction volume.

5.2.3 qPCR analysis

The transcriptional responses of B2 oysters to ocean acidification and warming were investigated by assessing the expression profiles of 60 genes involved in multiple biological processes. These genes were selected based on a previous study that explored the transcriptome of B2 oysters exposed to elevated CO_2 (Chapter 4). Primers for quantitative (q) PCR analysis were based on sequences of the contigs most affected by ocean acidification (highest up- or down-regulation) (Supplementary Table 5.1).

Three microliter qPCR reactions were prepared in duplicate in 384-well plates using an epMotion® 5075 pipetting robot (Eppendorf) and an Echo® 550 Liquid Handler (Labcyte). Each reaction contained 1.5 µl KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems), 300 nM each primer, 0.3 µl PCR grade water and 1 µl cDNA template (diluted 1:9). qPCR assays were carried out on a LightCycler® 480 II (Roche). The cycling program used consisted of 3 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 6 s. At the end of the qPCR cycles, melting curve analysis was performed by collecting fluorescence data between 65 - 95 °C at 0.5 °C increments. Reaction efficiencies were calculated from standard

curves generated in triplicate for each primer pair using five four-fold serial dilutions of a pool of cDNA samples (Supplementary Table 5.1). Cq values were obtained using the LightCycler® 480 Real-Time PCR System (version 1.5.1.62).

Gene expression stability of three potential reference genes was evaluated using the web-based RefFinder platform, which integrates results from different software tools (Xie *et al.* 2012). Elongation factor 1 alpha (EF1α), β-actin and GAPDH were previously found to be stable under CO_2 stress (Chapter 3) and thus were tested in the current study. The geometric mean of these three genes combined were found to be the most stable combination (compared to each gene used individually or in pairs) and so all three genes were used as references (geNorm stability value = 0.102; NormFinder stability value = 0.051; Stability value by ΔCt method / Average SD = 0.17; BestKeeper stability value = 0.251).

qPCR data are presented as changes in relative expression normalized with the geometric mean of the Cq values of EF1α, β-actin and GAPDH (Livak & Schmittgen 2001). To visualise overall differences between CO_2 and temperature conditions, we performed a non-metric multi-dimensional scaling (NMDS) analysis including the expression levels of differentially expressed genes (absolute fold-change ≥ 1.5). NMDS was based on Bray-Curtis similarity coefficients calculated from gene expression levels of oysters from the same exposure tank (3 tanks with 7 oysters each per condition).

5.2.4 Protein extraction

Proteins were isolated from approximately 100 mg of gill tissue (n = 15 per treatment; individually sampled and processed) using 1 ml of TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. After removal of RNA and DNA fractions, proteins were precipitated with acetone (3· volume) for 10 min followed by centrifugation for 10 min at 12,000 · g (4 °C). The resulting protein pellet was purified by multiple washes, following the

protocol previously described by Thompson *et al.* (2015), with slight modifications. Briefly, protein pellets were washed by adding 1 ml of 0.3 M guanidine hydrochloride in 95% ethanol (3 · 10 min, room temperature) before centrifugation at 8,000 · g (4 °C). A final wash was carried out with 95% ethanol for 10 min followed by centrifugation as above. Protein pellets were air dried at room temperature and resuspended in 50 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT and bromophenol blue).

Once purified, protein samples were quantified using Bradford reagent (Sigma-Aldrich) with BSA as standard (Bradford 1976). Equivalent amounts of proteins (30 µg) were pooled from five oysters taken from the same exposure tank, resulting in three biological replicates per treatment (3 · 5 oysters per treatment) and twelve 2D-gels across the four treatments (150 µg protein per gel).

5.2.5 2D gel electrophoresis

The proteomic responses of B2 oysters to ocean acidification and warming were assessed by two-dimensional (2D) gel electrophoresis (2DE). A total of three gels were run per treatment (representing each of the three replicate tanks per treatment). Proteins (150 µg in 125 µl of rehydration buffer containing 0.2% pharmalytes) were immobilised in pH linear gradient gel strips (7 cm, pH 4-7; ReadyStrip™ IPG Strips, Bio-Rad) by overnight passive rehydration. Isoelectric focusing (IEF) was performed using an IPGphor IEF System (GE Healthcare) at 100 V for 2 h, 250 V for 20 min, a gradient up to 5,000 V for 2 hours and then 5,000 V for 2 h. Following IEF, gel strips were equilibrated for 20 min in equilibration buffer I (1% DTT, 75 mM of 1.5 M Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) and then for 20 min in equilibration buffer II (2.5% iodoacetamide instead of 1% DTT in equilibration buffer I). Second dimension separation was conducted using 12% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) in a Mini-PROTEAN® Tetra Vertical Electrophoresis System

(Bio-Rad). After electrophoresis, gels were stained with blue silver (Candiano *et al.* 2004) and visualised using a ChemiDoc XRS+ (Bio-Rad). Quantitative image analysis of protein spots was performed by PDQuest 2-D Analysis Software (Bio-Rad).

5.2.6 Proteomic data analysis

Statistical analysis was performed on the normalised intensities of protein spots from 2D gels. Fold differences for each differentially expressed protein spot were calculated from the mean normalised intensities relative to the control condition (aCO_2 + aT). The statistical significance of differences between treatments was assessed using a linear model, considering the ambient control condition as the reference ($p < 0.05$), and by the significance analysis of microarrays (SAM; FDR < 10%). Differences between CO_2 and temperature conditions were also visualised in non-metric multi-dimensional scaling (NMDS) plots. NMDS was based on Bray-Curtis similarity coefficients calculated from normalised intensities of all protein spots identified in 2D gels or from normalised intensities of differentially regulated proteins.

5.2.7 In-gel digestion of differentially regulated proteins

The protein spots found to be differentially regulated by elevated CO_2 and/or elevated temperature were excised manually and subjected to in-gel digestion. A fresh set of 2D gels was used for spot picking. Gel pieces were washed with 100 mM ammonium bicarbonate and then destained with 50% acetonitrile in 50 mM ammonium bicarbonate (multiple washes for 10 min each). Following destaining, gel pieces were dehydrated in 100% acetonitrile for 5 min before being air dried. They were then reduced with 10 mM DTT in 100 mM ammonium bicarbonate at 56 °C for 1 h, alkylated with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (45 min at room temperature) and further washed and dehydrated as above. Trypsin (12.5 ng/µl in 50 mM ammonium bicarbonate; Promega) was added to each gel piece and the

mixture was incubated first for 30 min at 4 °C and then overnight at 37 °C. After overnight digestion, gel pieces were washed twice in 50% acetonitrile and 2% formic acid for 30 min to recover the peptide-containing supernatant (50 to 60 µl final volume). Supernatants were concentrated to 12 µl in a vacuum concentrator (Concentrator Plus, Eppendorf) and then centrifuged at 12,000 · g for 10 min to remove microparticles.

5.2.8 Protein characterisation by mass spectrometry

Trypsin-digested peptides were analysed by nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Finnigan Surveyor MS Pump Plus coupled to a Finnigan LTQ XL™ Linear Ion Trap Mass Spectrometer (Thermo Scientific). Chromatography was performed in reversed-phase peptide trap columns packed to approximately 9 cm (100 µm ID) with Magic C18AQ (5 µm, 200 Å, Michrom Bioresources), in a fused silica capillary with an integrated electrospray tip, coupled to pre-columns packed with PS-DVB resin (3 cm, 100 µm ID, Agilent Technologies). A 1.8 kV electrospray voltage was applied through a liquid junction up-stream of the C18 column. Sample injection was carried out using an EASY-nLC II (Thermo Scientific). Peptides were washed with buffer A (2% acetonitrile, 0.1% formic acid) for 2 min at 140 nl/min and then eluted from the column with 0-20% gradient of buffer B (95% acetonitrile, 0.1% formic acid) at 140 nl/min for 38 min. Following peptide elution, the column was washed with 95% buffer B at 140 nl/min for 10 min. Spectra were acquired for 60 min in positive ion mode for the scan range of 400 m/z to 1,500 m/z. Automated peak recognition, dynamic exclusion and MS/MS of the top nine most intense precursor ions at 35% normalisation collision energy were performed using Xcalibur™ software (version 2.06, Thermo Scientific).

Mass spectrometry data files were analysed using Global Proteome Machine (GPM) software (version 2012.05.01) and the X!Tandem algorithm (Craig & Beavis 2003, 2004). Files

were searched against a non-redundant (nr) protein database for Mollusca (downloaded from NCBI in March 2015), using default settings. The Mollusca nr database contained 378,914 sequences, including those derived from the *C. gigas* genome, in addition to common human and trypsin peptide contaminants. Spectra were also searched against a reversed sequence database for estimation of false discovery rates (FDR). Peptides that yielded log(e) values ≤ -10 and at least five spectral counts were retained for protein characterisation.

5.3 RESULTS

5.3.1 Experimental conditions for exposure to OA and warming

Seawater chemical and physical parameters were assessed throughout the experimental exposures (Table 5.1). Tanks for elevated CO_2 and for elevated temperature treatments were supplied by independent seawater reservoirs, which resulted in differences in pH and temperature among treatments. Such variation in pH and temperature was also reflected by differences in seawater carbonate chemistry, since pCO_2 concentration was calculated based upon measurements of these two variables (in addition to salinity and total alkalinity, which did not show significant variability). Seawater pH was 0.04 units lower in elevated CO_2 + ambient temperature (eCO_2 + aT) relative to the elevated CO_2 + elevated temperature treatment (eCO_2 + eT) ($p = 0.04$, two-tailed t-test). As a result, pCO_2 concentration was higher (+94.20 µatm) in elevated CO_2 + ambient temperature (eCO_2 + aT) tanks relative to the elevated CO_2 + elevated temperature condition (eCO_2 + eT) ($p = 0.03$, two-tailed t-test) (Table 5.1).

Similar variation was also evident between the elevated temperature treatments. Tanks for ambient CO_2 + elevated temperature (aCO_2 + eT) and elevated CO_2 + elevated temperature (eCO_2 + eT) were also supplied by independent flow-through seawater systems, which led to differences in temperature between treatments. Tanks for ambient CO_2 + elevated temperature

(aCO_2 + eT) treatment were 0.96 °C warmer than those for elevated CO_2 + elevated temperature (eCO_2 + eT) (p = 3.884e-11, two-tailed t-test) (Table 5.1).

Table 5.1. Seawater chemistry during ocean acidification and warming trial. Salinity, temperature and pH (NBS scale) were determined using a YSI 63 probe. Total alkalinity was measured in an automatic titrator (Metrohm 888 Titrando) and pCO_2 was calculated using co2sys software (Lewis & Wallace 1998). Data are presented as mean ± SD (n = 22 days). Abbreviations: a, ambient; e, elevated; T, temperature.

Parameter	Treatments			
	aCO_2 + aT	**aCO_2 + eT**	**eCO_2 + aT**	**eCO_2 + eT**
pH	8.10 ± 0.02	8.08 ± 0.02	7.70 ± 0.06	7.74 ± 0.08
Temperature (°C)	22.23 ± 0.16	25.86 ± 0.17	21.99 ± 0.30	24.90 ± 0.57
Salinity (ppt)	33.84 ± 0.02	33.84 ± 0.13	33.83 ± 0.45	33.83 ± 0.52
Total alkalinity (mmol kg^{-1} SW)	2.20 ± 0.02	2.20 ± 0.03	2.15 ± 0.03	2.14 ± 0.04
pCO_2 (µatm)	319.6 ± 14.6	339.6 ± 15.6	933.2 ± 130.1	838.0 ± 149.2

5.3.2 Proteomic and transcriptional profiles of oysters in response to ocean acidification and warming

We evaluated the effects of ocean acidification (OA) and warming on the proteomes and gene expression of oysters. The differential proteomes of oysters in response to elevated CO_2 and/or elevated temperature were resolved by 2D gel electrophoresis. A total of 345 protein spots were detected across the 2D gels, with molecular weights (MW) ranging from 12 to 200 kDa and isoelectric points (pI) ranging from 4 to 7. A representative proteome map of B2 oysters exposed to both reduced pH and elevated temperature (eCO_2 + eT) is shown in Figure 5.1a. The exposure of B2 oysters to elevated CO_2 (eCO_2 + aT), elevated temperature (aCO_2 + eT) or to a combination of both pH and thermal stresses (eCO_2 + eT) resulted in distinct proteomic profiles relative to control (ambient) conditions (aCO_2 + aT) (Fig. 5.1b). However, the spatial distribution of replicates from oysters exposed to elevated CO_2 only (eCO_2 + aT)

overlapped with those of oysters exposed to elevated temperature only (aCO_2 + eT) and with those exposed to a combination of elevated CO_2 and elevated temperature (eCO_2 + eT) (Fig. 5.1b). Oysters exposed to elevated temperature only (aCO_2 + eT) were the most spatially distinct from ambient controls (aCO_2 + aT) in the NMDS analysis.

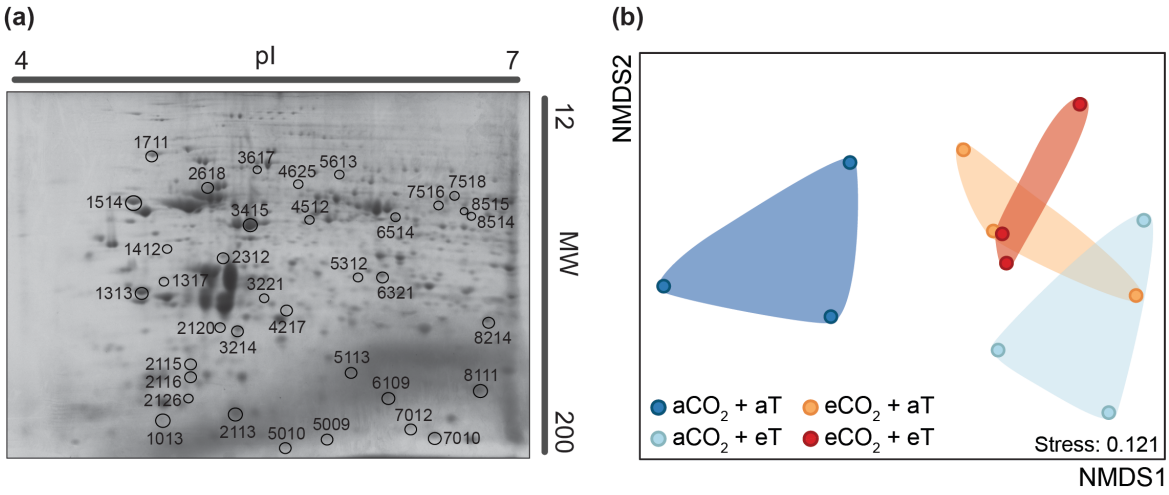


Figure 5.1. Proteomic responses of Sydney rock oysters to ocean acidification and warming. **(a)** Proteome map of B2 line oysters following exposure to elevated CO_2 and elevated temperature (eCO_2 + eT). Proteins were isolated from gills and separated by 2D gel electrophoresis (150 µg, pool of 5 oysters). Resolved proteins were visualized by staining gels with blue silver stain. Protein spots differentially regulated due to pH and/or temperature treatments are highlighted (linear model, $p < 0.05$; or SAM, FDR < 10%). Numbers associated with each protein spot correspond to arbitrary identifiers generated by PDQuest during image analysis. pI, isoelectric point; MW, molecular weight in kDa. **(b)** Non-metric multidimensional scaling (NMDS) plot showing the effects of elevated CO_2 and/or elevated temperature on oyster proteomes. NMDS combines the normalised intensities of 345 protein spots from 3 pools (replicates) of 5 oysters each per condition. aCO_2 + aT: ambient CO_2 and ambient temperature; aCO_2 + eT: ambient CO_2 and elevated temperature; eCO_2 + aT: elevated CO_2 and ambient temperature; and eCO_2 + eT: elevated CO_2 and elevated temperature.

Of the 345 protein spots identified, 39 were found to differ significantly in intensity ($p < 0.05$ or FDR < 10%) in at least one of the elevated CO_2 and/or elevated temperature treatments relative to control conditions (aCO_2 + aT) (Fig. 5.2a). Of these, three differential proteins were

common to all three elevated treatments (aCO_2 + eT, eCO_2 + aT and eCO_2 + eT). Nine of the proteins were differentially expressed in at least two of these treatments, while 27 of the differential proteins were unique to particular treatments. Of these, most (19; 70%) were unique to the elevated CO_2 + ambient temperature treatment (eCO_2 + aT).

Fold differences were determined by comparing the mean normalised protein spot intensities of each treatment where at least one parameter (CO_2 or temperature) was elevated relative to those of ambient control conditions. Absolute fold changes (up and down-regulation) ranged from 1.50 (spot no. 3214; Rho GDP dissociation inhibitor 1) to 16.07 (spot no. 4010; unidentified protein). Mass spectrometry was performed on 36 differentially regulated proteins (proteins spots are highlighted in Fig. 5.1a). Twenty-three proteins (64%) were successfully identified based on homology to a Mollusca non-redundant (nr) database. The identities and functions of differential proteins are listed in Table 5.2 and described in the following sections (5.3.3 to 5.3.5).

The transcriptional responses of B2 oysters to elevated CO_2 and/or elevated temperature were analysed by qPCR. We evaluated the relative expression profiles of 60 genes that have been previously shown to be differentially regulated following transgenerational exposure of B2 oysters to CO_2 stress (Chapter 4). Twenty-one genes (35%) were found to be affected by one or both stresses (elevated CO_2 and/or elevated temperature). Figure 5.2b shows the number of differentially expressed genes that were unique to particular treatments and those that were affected by one or more treatments. Five differentially regulated genes were common to all three elevated treatments (aCO_2 + eT, eCO_2 + aT and eCO_2 + eT). Seven of the differentially regulated genes were differentially expressed in at least two treatments, while nine were unique to particular treatments. Of these, most (4; 44%) were unique to the ambient CO_2 + elevated temperature treatment (aCO_2 + eT). The identities and functions of the differentially expressed genes are described in the following sections (5.3.3 to 5.3.5).

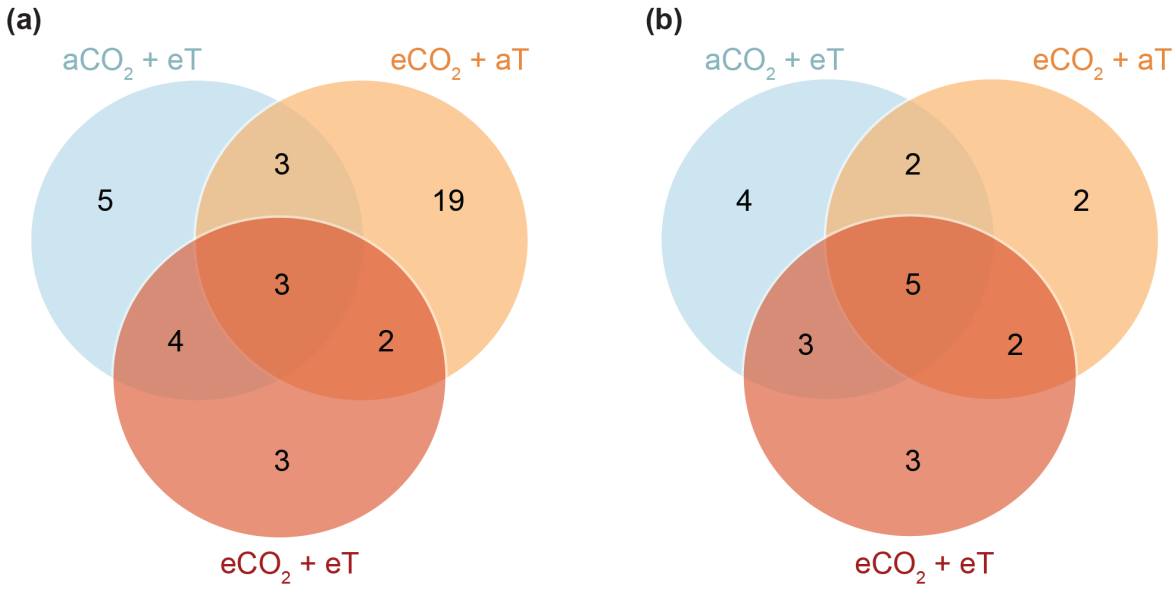


Figure 5.2. Effects of ocean acidification and warming on the molecular responses of oysters. Venn diagrams showing the number of proteins **(a)** and genes **(b)** that were differentially regulated in response to elevated CO_2 and/or elevated temperature in Sydney rock oysters. Differential regulation was determined by comparing spot normalised intensities or relative gene expression between each treatment and the control, ambient condition. aCO_2 + eT: ambient CO_2 and elevated temperature; eCO_2 + aT: elevated CO_2 and ambient temperature; and eCO_2 + eT: elevated CO_2 and elevated temperature.

5.3.3 Effects of ocean acidification

The exposure of oysters to elevated CO_2 altered the expression of numerous individual proteins and genes. This is reflected by the clear spatial discrimination of cumulative protein and gene expression profiles between CO_2-exposed oysters (eCO_2 + aT) and the other treatments (Fig. 5.3a and Fig. 5.4a). Elevated CO_2 significantly affected the expression of 27 proteins and 11 transcripts (relative to aCO_2 + aT) (Fig. 5.2). This represented the highest number of differentially regulated proteins among all of the CO_2 and temperature treatments. NMDS plots incorporating only data for differential proteins (rather than the entire proteome) reveal that the eCO_2 + aT treatment, in which only CO_2 was elevated, had the most substantial response relative to ambient controls (aCO_2 + aT) (Fig. 5.3a). The majority of differentially regulated proteins (17; 63%) and genes (7; 64%) were found at lower concentrations in eCO_2

+ aT oysters relative to the control treatment (aCO_2 + aT). In this comparison, the proteins found in lower abundance included actin (0.23-fold), ferritin (0.33-fold), cytochrome b5 (0.40-fold), vacuolar protein sorting-associated protein 13A (VPS13A; 0.49-fold) and Rho GDP dissociation inhibitor 1 (Rho-GDI; 0.50-fold) (Fig. 5.3b). Similarly, genes encoding von Willebrand factor D and EGF domain-containing protein (VWF; 0.17-fold), structural maintenance of chromosomes protein 3 (0.27-fold), multiple epidermal growth factor-like domains protein 10 (MEGF10; 0.36-fold), dual oxidase (0.54-fold) and heat shock 70 kDa protein 12a (Hsp70a; 0.57-fold) were down-regulated by CO_2 stress (Fig. 5.4b).

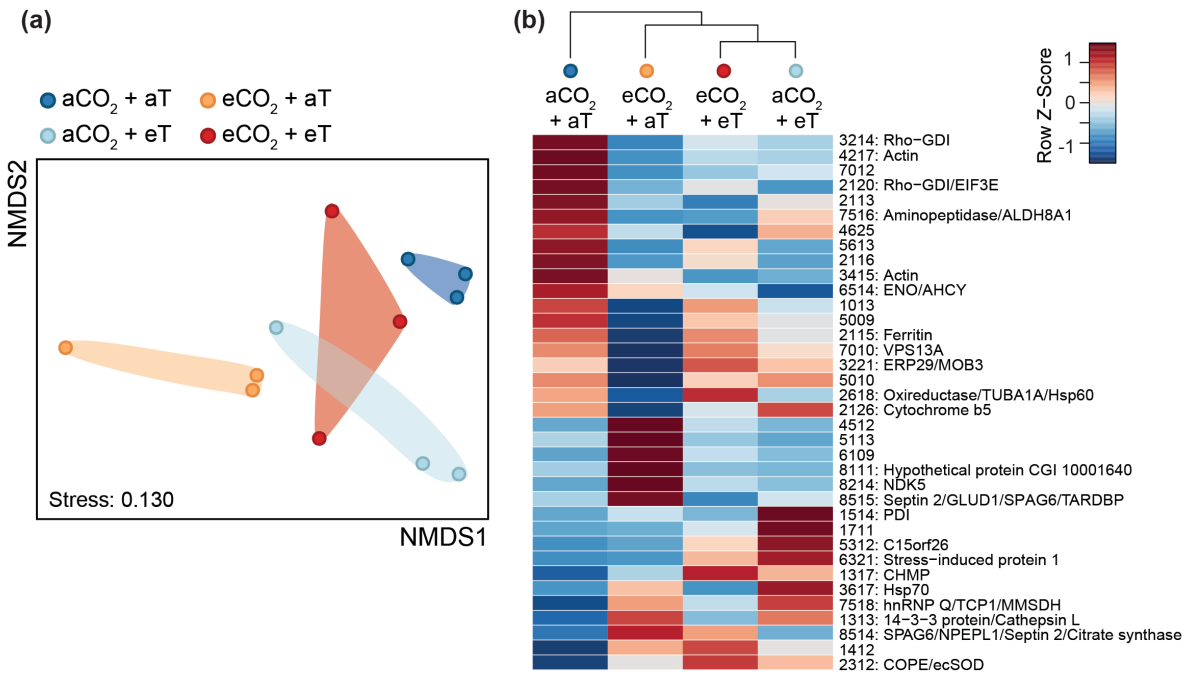


Figure 5.3. Proteomic responses of oysters to ocean acidification and warming. **(a)** Non-metric multidimensional scaling (NMDS) plot summarising the cumulative normalised intensities of differentially regulated protein spots among B2 oysters exposed to elevated CO_2 and/or elevated temperature (T). Each point represents the spot intensity levels of pools containing 5 oysters each per condition. **(b)** Heat map of mean normalised intensities of differentially regulated proteins. Proteins are identified by their spot numbers followed by their putative identifications obtained using mass spectrometry. Protein names and their associated cellular functions are detailed in Table 5.2. aCO_2 + aT: ambient CO_2 and ambient temperature; aCO_2 + eT: ambient CO_2 and elevated temperature; eCO_2 + aT: elevated CO_2 and ambient temperature; and eCO_2 + eT: elevated CO_2 and elevated temperature.

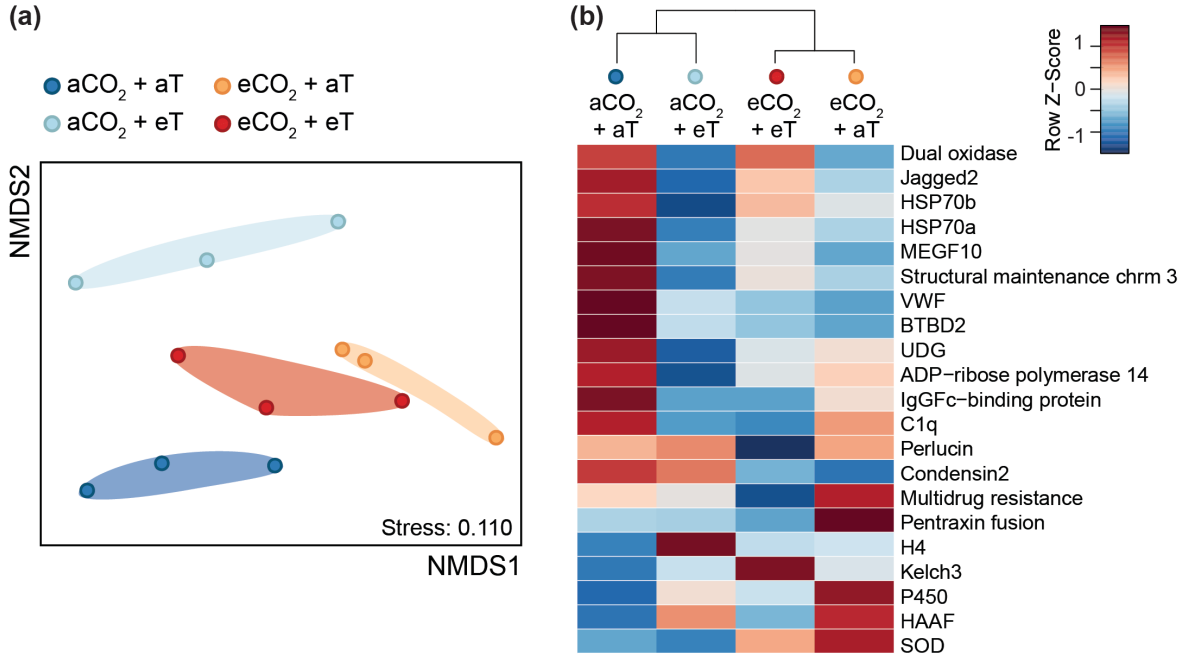


Figure 5.4. Transcriptional responses of oysters to ocean acidification and warming. **(a)** Non-metric multidimensional scaling (NMDS) plot showing the cumulative expression profiles of differentially regulated genes among B2 oysters exposed to elevated CO_2 and/or elevated temperature (T). Each point represents the relative mean expression levels of oysters from the same exposure tank (3 tanks containing 7 oysters each per condition). **(b)** Heat map of mean relative expression of differentially regulated genes assessed by qPCR. Gene names and their associated cellular functions are detailed in Supplementary Table 5.1. aCO_2 + aT: ambient CO_2 and ambient temperature; aCO_2 + eT: ambient CO_2 and elevated temperature; eCO_2 + aT: elevated CO_2 and ambient temperature; and eCO_2 + eT: elevated CO_2 and elevated temperature.

Few proteins and genes were up-regulated by elevated CO_2 alone. Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q; 2.66-fold), nucleoside diphosphate kinase-like protein 5 (NDK5; 2.51-fold), 14-3-3 protein (2.26-fold), septin 2 (2.15-fold) and sperm associated antigen 6 (SPAG6; 2.00-fold) were found at higher concentrations in eCO_2 + aT oysters when compared to the aCO_2 + aT treatment (Fig. 5.3b). At the transcriptional level, extracellular superoxide dismutase (ecSOD; 2.46-fold), cytochrome P450 (6.88-fold), pentraxin fusion protein (2.44-fold) and hemagglutinin/amebocyte aggregation factor (HAAF; 1.83-fold) had higher expression levels in CO_2-exposed oysters relative to controls (Fig. 5.4b).

5.3.4 Effects of ocean warming

Thermal stress also affected the proteomes and transcriptional responses of B2 oysters, albeit to a lesser extent than elevated CO_2 at the protein level. Oysters exposed to elevated temperature alone (aCO_2 + eT) exhibited protein and gene expression profiles that were clearly different from those of oysters held under ambient conditions (aCO_2 + aT) and oysters exposed to elevated CO_2 alone (eCO_2 + aT) (Fig. 5.3a and Fig. 5.4a). The transcriptional response of aCO_2 + eT oysters was also distinct from the combined eCO_2 + eT treatment (Fig. 5.4a). However, this distinction was less apparent in their proteomic responses. NMDS of differentially regulated proteins showed substantial overlap between the aCO_2 + eT and eCO_2 + eT treatments (Fig. 5.3a). Elevated temperature altered the regulation of 15 proteins and 14 genes (Fig. 5.2), and affected a greater number of genes compared to elevated CO_2 alone. Most of differentially regulated proteins (9; 60%) and genes (11; 79%) were found at lower concentrations in oysters exposed to elevated temperature (aCO_2 + eT) relative to control conditions (aCO_2 + aT). In this comparison, down-regulated proteins included enolase (0.42-fold), Rho-GDI (0.49-fold) and actin (0.51-fold) (Fig. 5.3b), while uracil-DNA glycosylase (UDG; 0.42-fold), dual oxidase (0.44-fold) and Hsp70 (0.42-fold for Hsp70a; 0.49-fold for Hsp70b) were among the down-regulated transcripts (Fig. 5.4b). Conversely, protein disulphide isomerase (PDI; 3.85-fold) and stress-induced protein 1 (2.50-fold) were found in higher concentrations following heat stress (Fig. 5.3b), as well as the genes encoding cytochrome P450 (3.93-fold), histone H4 (2.20-fold) and HAAF (1.64-fold) (Fig. 5.4b).

5.3.5 Combined effects of ocean acidification and warming

Oysters exposed to both elevated CO_2 and elevated temperature (eCO_2 + eT) exhibited a unique gene expression profile that was different from all other treatments and more closely resembled the transcriptional profile of ambient controls (Fig. 5.4a). Similarly, the differential

proteomes of oysters exposed to both CO_2 and heat stresses were intermediate between those of oysters exposed to CO_2 stress alone (eCO_2 + aT) and controls (aCO_2 + aT), and overlapped with the proteomes of oysters exposed to heat stress only (aCO_2 + eT) (Fig. 5.3a).

The combination of elevated CO_2 and elevated temperature changed the expression patterns of 12 proteins and 13 genes (Fig. 5.2). The majority of differentially regulated proteins (7; 58%) and genes (10; 77%) were found at lower concentrations in eCO_2 + eT relative to ambient conditions (aCO_2 + aT). Proteins found in lower abundance in exposed oysters included actin (0.41 to 0.47-fold), Rho-GDI (0.67-fold) and protein spot 7516, which contained putative aminopeptidase W07G4.4 and/or aldehyde dehydrogenase family 8 member A1 (ALDH8A1; 0.47-fold) (Fig. 5.3b). Genes down-regulated by elevated CO_2 and elevated temperature included VWF (0.26-fold), perlucin (0.60-fold), multidrug resistance protein 1 (0.63-fold) and IgGFc-binding protein (0.63-fold) (Fig. 5.4b). In contrast, charged multivesicular body protein 4c (2.02-fold), stress-induced protein 1 (1.88-fold) and protein spot 2312 (containing coatomer subunit epsilon and/or ecSOD; 2.85-fold), were found at higher concentrations in eCO_2 + eT oysters (Fig. 5.3b). At the transcriptional level, ecSOD (1.90-fold), cytochrome P450 (3.28-fold) and kelch 3 (1.55-fold) showed higher expression levels in exposed oysters relative to controls (Fig. 5.4b).

Only 3 proteins and 5 genes were affected by all stress treatments (elevated CO_2 alone, elevated temperature alone, and the combination of elevated CO_2 and elevated temperature) (Fig. 5.2). Rho-GDI, actin and an unidentified protein (spot no. 2311) were found in lower concentrations in oysters exposed to all three treatments (0.22 to 0.67-fold) relative to oysters held under ambient conditions (Fig. 5.3b). At the transcriptional level, MEG10, Hsp70a, structural maintenance of chromosomes 3 and VWF were down-regulated by all of the treatments (0.01 to 0.65-fold), while cytochrome P450 transcripts were more abundant (3.28 to 6.88-fold) (Fig. 5.4b).

Table 5.2. List of proteins identified by mass spectrometry that were differentially regulated by CO_2 and/or thermal stress. Fold changes (FC) were calculated from the mean normalised intensities of protein spots relative to the control, ambient condition (aCO_2 + aT). FC values > 1 reflect up-regulation in the elevated treatment relative to ambient controls. Values < 1 reflect down-regulation in the elevated treatment. The table also shows arbitrarily assigned protein spot numbers, putative identifications (description and species) and their respective accession numbers based on homology to a Mollusca nr database, spectral counts, log(e) values and annotated biological processes (UniProtKB). aCO_2 + eT: ambient CO_2 and elevated temperature; eCO_2 + aT: elevated CO_2 and ambient temperature; and eCO_2 + eT: elevated CO_2 and elevated temperature.

Treatment	FC	Spot no.	Description	Species	Accession number	Spectral counts	log(e)	Biological function[1]
aCO_2 + eT	2.33	5312	Uncharacterized protein C15orf26-like	*Crassostrea gigas*	gi|405971678|gb|EKC36501.1|	17	-61.1	Probable CC
aCO_2 + eT	0.49	2120	Rho GDP dissociation inhibitor 1 (Rho-GDI)	*Crassostrea gigas*	gi|405966436|gb|EKC31723.1|	49	-75.7	CC, E
		2120	Eukaryotic translation initiation factor 3 subunit K (EIF3E)	*Crassostrea gigas*	gi|405952580|gb|EKC20375.1|	7	-37.7	T
aCO_2 + eT	0.42	6514	PREDICTED: enolase like isoform X2 (ENO)	*Crassostrea gigas*	gi|762107263|ref|XP_011436228.1|	8	-65.5	E
		6514	S adenosylhomocysteine hydrolase (AHCY)	*Crassostrea ariakensis*	gi|253769244|gb|ACT35639.1|	7	-38.8	E
		6514	Adenosylhomocysteinase A	*Crassostrea gigas*	gi|405974443|gb|EKC39086.1|	6	-32.9	E
aCO_2 + eT	3.85	1514	Protein disulfide isomerase (PDI)	*Crassostrea gigas*	gi|405964146|gb|EKC29663.1|	44	-117.4	S, A, PM
eCO_2 + aT	0.33	2115	Ferritin	*Ostrea edulis*	gi|388571224|gb|AFK73708.1|	13	-16.6	S, A, I, CC
eCO_2 + aT	2.51	8214	Nucleoside diphosphate kinase like protein 5 (NDK5)	*Crassostrea gigas*	gi|405978785|gb|EKC43147.1|	11	-42.1	CC, A, S

Table 5.2 continued

Treatment	FC	Spot no.	Description	Species	Accession number	Spectral counts	log(e)	Biological function[1]
eCO_2 + aT	2.26	1313	14-3-3 protein	*Crassostrea gigas*	gi|405963584|gb|EKC29146.1|	219	-338.4	CS, S, PM
eCO_2 + aT	0.40	2126	Cytochrome b5	*Crassostrea gigas*	gi|405966177|gb|EKC31489.1|	19	-74.2	A, CC, S
eCO_2 + aT	0.33	2618	PREDICTED: Putative uncharacterized oxidoreductase YDR541C	*Crassostrea gigas*	gi|762130447|ref|XP_011448359.1|	44	-191	Probable A
		2618	PREDICTED: tubulin alpha 1A chain (TUBA1A)	*Aplysia californica*	gi|871213161|ref|XP_012946898.1|	35	-194.1	C, CC, PM
		2618	60 kDa heat shock protein, mitochondrial (Hsp60)	*Crassostrea gigas*	gi|405966599|gb|EKC31862.1|	16	-89.6	PM, S, CC
eCO_2 + aT	0.47	3221	Endoplasmic reticulum protein (ERP29)	*Crassostrea gigas*	gi|405975720|gb|EKC40268.1|	9	-28.9	PM, T
		3221	Mps One Binder kinase activator-like 3 (MOB3)	*Crassostrea gigas*	gi|405967824|gb|EKC32951.1|	8	-41.9	IT
eCO_2 + aT	2.29	3617	Stress-70 protein, mitochondrial	*Crassostrea gigas*	gi|405950030|gb|EKC18038.1|	84	-313.6	PM, S, CC
		3617	AF144646 1 heat shock protein 70 (Hsp70)	*Crassostrea gigas*	gi|4838561|gb|AAD31042.1|	40	-156.7	PM, S, CC
		3617	HSC70 protein	*Ostrea edulis*	gi|18076565|emb|CAC83684.1|	38	-131.1	PM, S

Table 5.2 continued

Treatment	FC	Spot no.	Description	Species	Accession number	Spectral counts	log(e)	Biological function[1]
eCO_2 + aT	0.49	7010	PREDICTED: vacuolar protein sorting-associated protein 13A like partial (VPS13A)	*Aplysia californica*	gi|871271078|ref|XP_012945076.1|	5	-12	PM, IT
eCO_2 + aT	2.66	7518	Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q)	*Crassostrea gigas*	gi|405972362|gb|EKC37135.1|	34	-197.3	T
		7518	T-complex protein 1 subunit zeta (TCP1)	*Crassostrea gigas*	gi|405953549|gb|EKC21190.1|	25	-81.4	PM, T
		7518	Putative methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial (MMSDH)	*Crassostrea gigas*	gi|405970294|gb|EKC35210.1|	6	-29.8	E, T
eCO_2 + aT	2.42	8111	Hypothetical protein CGI 10001640	*Crassostrea gigas*	gi|405955698|gb|EKC22710.1|	57	-232	Probable S
eCO_2 + aT	2.00	8514	Sperm associated antigen 6 (SPAG6)	*Crassostrea gigas*	gi|405950834|gb|EKC18795.1|	48	-134.3	CC, C
		8514	Putative aminopeptidase NPEPL1	*Crassostrea gigas*	gi|405949954|gb|EKC17964.1|	22	-79.7	Probable PM
		8514	Septin 2	*Crassostrea gigas*	gi|405970532|gb|EKC35428.1|	8	-40.7	C, CC
		8514	Citrate synthase, mitochondrial	*Crassostrea gigas*	gi|405970598|gb|EKC35491.1|	7	-37.4	E

Table 5.2 continued

Treatment	FC	Spot no.	Description	Species	Accession number	Spectral counts	log(e)	Biological function[1]
eCO_2 + aT	2.15	8515	Septin 2	*Crassostrea gigas*	gi|405970532|gb|EKC35428.1|	32	-163.3	C, CC
		8515	Glutamate dehydrogenase 1, mitochondrial (GLUD1)	*Crassostrea gigas*	gi|405974984|gb|EKC39587.1|	14	-98.4	E
		8515	Sperm associated antigen 6 (SPAG6)	*Crassostrea gigas*	gi|405950834|gb|EKC18795.1|	11	-68.9	CC, C
		8515	TAR DNA-binding protein 43 (TARDBP)	*Crassostrea gigas*	gi|405957161|gb|EKC23392.1|	5	-20.3	T, CC
eCO_2 + eT	2.02	1317	PREDICTED: charged multivesicular body protein 4c-like (CHMP4C)	*Aplysia californica*	gi|524911700|ref|XP_005110710.1|	6	-20.8	IT, CC
		1317	Charged multivesicular body protein 4b (CHMP4B)	*Crassostrea gigas*	gi|405946542|gb|EKC17665.1|	6	-18.9	IT, CC
aCO_2 + eT eCO_2 + eT	2.50 1.88	6321	Stress-induced protein 1	*Crassostrea gigas*	gi|405961170|gb|EKC27013.1|	6	-13.1	S, PM
aCO_2 + eT	2.33	2312	PREDICTED: coatomer subunit epsilon-like (COPE)	*Crassostrea gigas*	gi|762107581|ref|XP_011436400.1|	10	-30.3	IT
eCO_2 + eT	2.85	2312	Extracellular superoxide dismutase (ecSOD)	*Saccostrea glomerata*	gi|229485195|gb|ACQ73551.1|	6	-18.2	S, A
aCO_2 + eT eCO_2 + eT	0.51 0.47	3415	Actin	*Crassostrea gigas*	gi|405973339|gb|EKC38058.1|	255	-577	C, CC

Table 5.2 continued

Treatment	FC	Spot no.	Description	Species	Accession number	Spectral counts	log(e)	Biological function[1]
eCO_2 + aT	0.46	7516	Putative aminopeptidase W07G4.4	*Crassostrea gigas*	gi|405956520|gb|EKC23074.1|	16	-82.9	Probable PM
eCO_2 + eT	0.47	7516	Aldehyde dehydrogenase family 8 member A1 (ALDH8A1)	*Crassostrea gigas*	gi|405965667|gb|EKC31029.1|	11	-55.8	E
aCO_2 + eT	0.62	3214	Rho GDP dissociation inhibitor 1 (Rho-GDI)	*Crassostrea gigas*	gi|405966436|gb|EKC31723.1|	10	-37.1	CC, E
eCO_2 + aT	0.50							
eCO_2 + eT	0.67							
aCO_2 + eT	0.38	4217	Actin	*Crassostrea ariakensis*	gi|333449498|gb|AEF33434.1|	34	-138	C, CC
eCO_2 + aT	0.23							
eCO_2 + eT	0.41							

[1]A = antioxidant response (including regulation of oxidative stress, cell redox homeostasis); C = cytoskeleton; CC = cell cycle (including cell movement, proliferation, differentiation, regulation of apoptotic processes); CS = cell signalling; E = energy metabolism (including glycolysis, metabolic and catabolic processes); I = immune response; IT = intracellular transport; PM = protein modifications (including protein folding, phosphorylation, dephosphorylation, polymerisation, targeting); S = response to stress; T = transcription and translation (including protein synthesis).

5.4 DISCUSSION

Assessment of molecular processes can provide significant insights into the intracellular basis of changes resulting from genetic evolution or phenotypic plasticity. Given that molecular responses to environmental stressors involve a complex array of genes and biological functions, more comprehensive and integrative approaches are required to understand the regulatory patterns of molecular processes. In this study, we have used a combination of molecular techniques to investigate the effects of ocean acidification (OA) and warming on CO_2-resilient oysters. We identified substantial changes in protein concentrations and gene expression in response to elevated CO_2 or elevated temperature. Although the combination of stressors also altered the expression patterns of proteins and genes, the concurrent effects of elevated CO_2 and temperature were not shown to be synergistic or additive.

B2 oysters represent a unique genetic resource because of their capacity to better withstand the impacts of elevated CO_2 at the physiological level, compared to non-selected oysters from the same species (*S. glomerata*). This breeding line has been produced through mass selection for faster growth and resistance to the most significant oyster diseases in Australia (QX disease and winter mortality) (Dove *et al.* 2013). Surprisingly, artificial selection for these characteristics appears to have coincidentally resulted in resilience against CO_2-driven OA (Parker *et al.* 2012; Parker *et al.* 2011). Recent studies started investigating the molecular processes driving the distinctive performance of B2 oysters under elevated CO_2 conditions. Thompson *et al.* (2015) found that the majority of proteins differentially regulated by CO_2 stress (856 µatm pCO_2, pH 7.84) were found at lower concentrations in B2 oysters, but at higher concentrations in wild type oysters, relative to their respective controls (ambient CO_2). The differentially regulated proteins included a number involved in mitochondrial electron transport chain, oxidative stress and protein synthesis.

The transgenerational responses of the B2 breeding line to CO_2 stress have also been investigated at the level of gene expression. Exposure of B2 oysters to elevated CO_2 over three consecutive generations (F2 juveniles) resulted in the up-regulation of genes associated with antioxidant defence, metabolism and the cytoskeleton (Chapter 3). Whole-transcriptome sequencing of B2 oysters exposed to elevated CO_2 over two consecutive generations (F1 adults) revealed a complex array of genes and biological functions affected by OA. In addition to those genes implicated in metabolism and stress response, B2 oysters exhibited altered expression patterns for genes involved in the cell cycle, maintenance of cellular homeostasis, growth and development, and immune responses (Chapter 4). The previous studies also suggested that B2 oysters might be able to undergo rapid acclimation or adaptation, responding faster than wild types in the face of OA. All of these data indicate that B2 oysters may be a valuable resource for climate-proofing Sydney rock oyster aquaculture and the restoration of natural oyster beds. However, there is little information on how B2 oysters will respond to more realistic future scenarios that combine OA with elevated temperature. The current study addresses this deficit by characterising the molecular processes of B2 oysters that are affected by the combination of OA and increasing temperature at both protein and transcriptional levels.

5.4.1 Overlapping responses to OA, increasing temperature and the combined stressors

A suite of proteins and genes showed substantial changes in expression in response to all stress treatments - elevated CO_2 alone, elevated temperature alone, and combination of elevated CO_2 and elevated temperature. This overlap in stress responses among treatments suggests that a similar set of molecular processes is recruited in the face of OA and warming in B2 oysters. Intracellular processes simultaneously affected by OA and/or increasing temperature included control of redox balance, maintenance of cellular homeostasis, stress

responses and the cytoskeleton. Differentially regulated molecules included Rho GDP dissociation inhibitor 1 (Rho-GDI), actin, Hsp70a, von Willebrand factor (VWF) D and EGF domain-containing protein, multiple epidermal growth factor-like domains proteins 10 (MEG10), structural maintenance of chromosomes 3 (SMC3) and cytochrome P450. Rho-GDI and actin are of particular interest in this study. Both proteins were found in lower concentrations in stress-exposed oysters relative to control conditions. Rho-GDI is an inhibitor of the small GTPase Rho, and is involved in oxidative stress and cytoskeleton organisation. Following interaction of Rho-GDI with membrane proteins, this complex activates NADPH oxidase, which in turn initiates the production of reactive oxygen species (ROS) (Di-Poi *et al.* 2001). Lower abundance of Rho-GDI has been observed in the mussel *Mytilus galloprovincialis* recovering from hyposaline stress (Tomanek *et al.* 2012). The down-regulation of Rho-GDI found in our study may be associated with the control of oxidative damage typically induced by OA and rising temperature. The low abundance of Rho-GDI may prevent the formation of ROS in response to CO_2 and/or thermal stresses in B2 oysters by reducing the activation of NADPH. Alternatively, the down-regulation of this protein could increase the activity of Rho GTPase, leading to cytoskeletal modifications. In this context, it is relevant that the major cytoskeletal protein, actin, was also found at lower concentrations in oysters exposed to any stress treatment. Actins are highly abundant structural proteins that play a fundamental role in the division, shape and mobility of eukaryotic cells. The changes observed in actin abundance could be related to cytoskeletal remodelling due to intracellular stress (McDonagh & Sheehan 2008; Tomanek 2014).

At the transcriptional level, a gene encoding the stress-response protein Hsp70 was also down-regulated in oysters exposed to any stress treatment (OA, increased temperature and the combination of these stresses). Heat shock proteins, including Hsp70, are among the most abundant intracellular proteins protecting cells from stress-induced damage. Hsp70s act as

molecular chaperones, promoting protein folding, membrane translocation, degradation of misfolded proteins and maintenance of cellular homeostasis (Liu & Chen 2013). Although exposure to stress typically results in an increase in Hsp70 expression, other transcriptional and proteomic analyses also showed down-regulation of this protein in the oysters *C. virginica* and *S. glomerata* (B2 line) exposed to elevated CO_2 (pH 7.59 to 7.88; one-month exposure) (Ivanina *et al.* 2014; Thompson *et al.* 2015). In the pearl oysters, *Pinctada fucata*, Hsp70 mRNA levels increased immediately after exposure to elevated CO_2 alone, elevated temperature alone and the combined stressors. However, Hsp70 expression decreased after 4 days of stress exposure (relative to non-exposed oysters) (Liu *et al.* 2012). In line with these studies, our findings suggest that longer exposures to stressful seawater conditions cause a decrease in the concentration of Hsp70. Such reductions in the expression of Hsp70, as well as of Rho-GDI and actin, may indicate that B2 oysters are prone to protein denaturation and cytoskeleton remodelling caused by CO_2 and thermal stresses.

Exposure to OA, increased temperature and the combination of both stresses also altered expression of genes involved in the maintenance of cellular homeostasis and the cell cycle. Genes encoding proteins that contain von Willebrand factor D (VWF) and EGF-like domains were substantially down-regulated in CO_2-exposed B2 oysters. Von Willebrand factor D (VWF) and EGF domain-containing protein is involved in the maintenance of cellular homeostasis, as well as in protein-protein interactions and the assembly of protein subunits (Whittaker & Hynes 2002). MEGF10, another gene down-regulated by all three elevated treatments, encodes a membrane protein involved in phagocytosis of apoptotic cells, cell adhesion and motility. The expression of MEGF10 has also been found to be down-regulated by hypoxia and hypo-osmotic stress in the Pacific oyster *C. gigas* (Sussarellu *et al.* 2010). A gene encoding the structural maintenance of chromosomes protein 3 (SMC3) was also down-regulated by CO_2 and/or thermal stresses in the current study. SMC3 is a central component

responsible for maintaining chromosome cohesion during cell division. In addition to its involvement in DNA replication and cell cycle, SMC3 is also associated with DNA repair (Brinch *et al.* 2012). To our knowledge, this is the first report showing differential regulation of SMC3 in response to stress in oysters. Due to this lack of information about the role that SMC3 plays in oysters, it is difficult to explain the implication of its low expression in stressed oysters. In contrast, cytochrome P450 was the only molecule that showed increased expression in response to all three stress treatments. Members of the cytochrome P450 superfamily are involved in a variety of detoxification and endogenous processes. Induction of cytochrome P450 transcription, protein concentration or enzyme activity has been frequently reported after exposure to environmental stress, particularly chemical contaminants (Luchmann *et al.* 2015; Torres *et al.* 2008). Besides its role on xenobiotic biotransformation, cytochrome P450 is associated with hormone biosynthesis and degradation, and oxidative stress (Kubota *et al.* 2011). The down-regulation of genes involved in the cell cycle and maintenance of cellular homeostasis, along with the increased expression of cytochrome P450, indicate that changes in seawater chemical and/or physical characteristics may have substantial effects on oyster growth and development, and their ability to respond to stress. Cytological analyses evaluating cellular replication, ROS production and cytoskeleton organisation in response to CO_2 and thermal stresses would help to elucidate the implications of these findings.

5.4.2 Molecular responses induced by OA

Despite the similarities in response to OA, increasing temperature and the combined stressors, these treatments produced additional unique impacts on different proteins and genes. For instance, CO_2 stress induced the highest changes at the protein level among all of the CO_2 and temperature treatments, both in terms of number of proteins affected and magnitude of those changes. Previous studies have shown that molluscs are particularly susceptible to OA.

Changes in seawater chemistry have negative effects on growth, development, survival, calcification and acid-base regulation in different species of molluscs (Gazeau *et al.* 2013; Harvey *et al.* 2013; Parker *et al.* 2013). These changes are likely to be a reflection of complex rearrangements at the molecular level that ultimately result in physiological impacts (Somero 2010). Increasing temperature, in contrast, has not been shown to produce as strong impacts on the performance of molluscs (Harvey *et al.* 2013). Among the genes/proteins affected by elevated CO_2 alone in the current study, a number are associated with responses to stress, redox balance and cytoskeleton organisation. In the context of the molecules involved in redox balance, cytochrome b5 and ferritin were found at lower concentrations following exposure to elevated CO_2. Both proteins are involved in reduction-oxidation reactions and iron homeostasis. Given that these processes are linked to energy metabolism (through expression of Krebs cycle enzymes, for example), lower concentrations of cytochrome b5 and ferritin may be associated with shifts in energy requirements induced by CO_2 stress in B2 oysters (Tomanek 2011).

Other stress-related genes affected by elevated CO_2 alone included extracellular superoxide dismutase (ecSOD) and septin 2 (both up-regulated). EcSOD participates in ROS scavenging while septin is required for normal organization of the actin cytoskeleton (Kremer *et al.* 2007; Tomanek *et al.* 2011). Increased concentrations of ecSOD and septin 2 may be related to the capacity of B2 oysters to cope with oxidative damage typically induced by OA. They may mitigate damage to the actin cytoskeleton and other fundamental cellular components, minimising cell death through apoptosis. Overall, these findings suggest that B2 oysters may be able to withstand and limit the adverse impacts of CO_2 stress on their physiological responses (*i.e.* growth, survival and metabolism) by hindering disturbance of the cellular redox balance and controlling cellular homeostasis.

5.4.3 Molecular responses to increasing temperature

Thermal stress alone also induced unique, substantial changes in the regulation of proteins and genes in B2 oysters. While elevated CO_2 was the stress that altered the largest number of proteins, elevated temperature was responsible for the greatest changes at the transcriptional level. Thermal stress reduced the concentration of enolase and uracil-DNA glycosylase transcript levels, but increased the abundance of protein disulphide isomerase and stress-induced protein 1. Enolase participates in carbohydrate metabolism, specifically glycolysis, and may also be involved in stress responses (Gerlt *et al.* 2005). Uracil-DNA glycosylase (UDG) is a major component of the cellular machinery responsible for DNA repair and prevention of DNA damage (David *et al.* 2007; Krokan *et al.* 2000). In contrast to the findings of the current study, high transcript levels of UDG were observed in B2 oysters following transgenerational conditioning to OA (Chapter 4). Very little information is available on the biological role and transcriptional regulation of enolase and UDG in molluscs responding to stress. However, our data might suggest that thermal stress reduces the ability of cells to control oxidative damage and resulting mutation, with consequence for the prevention of heat-induced cell death.

Protein disulphide isomerase (PDI) and stress-induced protein 1 were among the molecules whose expression was increased by thermal stress. PDI is a molecular chaperone found in the endoplasmic reticulum. It is involved in protein folding, assembly and post-translation modifications, including those induced by ROS (Tu & Weissman 2004). Higher levels of PDI have also been observed in thermally-stressed sea cucumbers (Zhang *et al.* 2013) and in Pacific oysters (*C. gigas*) (Harney *et al.* 2016). In line with our results, stress-induced protein 1, another molecular chaperone, was also found to be up-regulated in *C. gigas* following exposure to elevated temperature (Zhang *et al.* 2015). It has been extensively reported that elevated temperature triggers oxidative stress in different species of marine invertebrates

(Meistertzheim *et al.* 2007; Tomanek 2014; Zhang *et al.* 2012). This response is often elicited by an imbalance between production and neutralisation of ROS due to heat-induced metabolic shift and increased oxygen requirements. Our findings suggest that the increased expression of molecular chaperones may preserve correct conformation and stability of a number of proteins, in an attempt to prevent damage from thermally-driven stress.

5.4.4 Molecular responses to the combination of OA and warming

The combination of OA and warming induced specific changes that were not evident when oysters were exposed to elevated CO_2 or temperature alone. The combined stressors caused down-regulation of perlucin and multidrug resistance protein 1. Perlucin is a C-type lectin involved in nucleation of calcium carbonate ions during shell formation (Blank *et al.* 2003; Mann *et al.* 2000; Weiss *et al.* 2000). Although the expression of perlucin may be expected to be altered by CO_2-driven OA, no changes in its mRNA levels were observed in the mussel *Mytilus edulis* after exposure to elevated CO_2 (pH 7.8) (Hüning *et al.* 2013; Li *et al.* 2015). In contrast, higher levels of perlucin was observed in B2 oysters following transgenerational exposure to CO_2 stress alone (pH 7.77) (Chapter 4). Therefore, it appears that B2 oysters may be able to maintain carbonate homeostasis and shell deposition under CO_2 stress alone, but their calcification process may be hampered when increased temperature is combined to CO_2 stress. Multidrug resistance proteins are also involved in immune defence, preventing drugs from entry into the cytoplasm or nucleus. These proteins play a role in detoxifying various toxic agents in marine organisms due to their ability to transport xenobiotic conjugates and metabolites (Kingtong *et al.* 2007). Our findings indicate that multidrug resistance protein 1 may also participate in oyster responses to OA and warming. Combined, the substantial changes in protein and gene expression observed in this study suggest that inducible responses of oysters to near-future changes in seawater temperature and pH may depend upon energy reallocation.

Such reallocation may regulate fundamental cellular processes, including control of oxidative damages and maintenance of cellular homeostasis.

5.4.5 Concurrent effects of OA and warming are not synergistic or additive

Numerous studies suggest that elevated CO_2 enhances the sensitivity of marine organisms to thermal stress and vice versa (Albright & Mason 2013; Feidantsis *et al.* 2015; Gunderson *et al.* 2015). However, our data did not reveal such a synergistic or additive interaction at the molecular level. In the context of this study, additive or synergistic effect is taken to be a disproportionately enhanced response (number of differentially expressed genes/proteins and/or fold-change) when the effects of combined stressors are compared to those stressors in isolation. Here, responses to the combined stressors did not resemble an additive or synergistic effect of the individual stressors. Instead, they more closely resembled the proteomic and transcriptional profiles of oysters held under fully ambient conditions. This lack of synergy may be due to restrictions in our experimental design or to potential antagonistic effects that the combination of OA and increasing temperature produce in B2 oysters. In our study, significant differences in pH, temperature and pCO_2 concentration were observed among the elevated treatments. Tanks for elevated CO_2 and for elevated temperature treatments were supplied by independent seawater reservoirs, which resulted in differences in seawater chemistry and temperature among treatments. These differences may have prevented the production of synergistic or additive effects by CO_2 and thermal stresses.

The lack of a synergistic or additive response may also have been due to the times at which the different stressors were applied (simultaneously rather than sequentially). Timing of stress exposure is particularly important when organisms respond to different stressors using the same mechanisms of cellular protection (Todgham & Stillman 2013). It has been demonstrated that both CO_2 and thermal stresses typically affect molecular processes associated

with energy metabolism and oxidative stress in oysters (Tomanek *et al.* 2011; Zhang *et al.* 2012). As such, the simultaneous exposure to elevated CO_2 and elevated temperature performed in this study might have masked the potential additive or synergistic effects of OA and ocean warming.

Despite these qualifications, it remains possible that synergistic or additive responses to OA and increased temperature do not occur in B2 oysters. Previous studies have reported that thermal stress ameliorates some impacts of elevated CO_2. Increasing temperature (2 °C to 3.2 °C increase relative to control conditions) minimised the negative effects of elevated CO_2 (pH 7.79 to 7.90) on growth and calcification rate of *C. gigas* larvae (Harney *et al.* 2016), and on calcification of coral reefs (McNeil *et al.* 2004) and the sea star *Pisaster ochraceus* (Gooding *et al.* 2009). In addition, a recent meta-analysis assessing the physiological responses of marine organisms to OA and warming revealed positive effects of the combined stressors on growth of echinoderms and calcifying phytoplankton, and on photosynthesis of calcifying and non-calcifying marine autotrophs. Similarly, OA alone has been shown to negatively affect both calcification and photosynthesis of corals, while these processes were not disturbed by the combined stressors (Harvey *et al.* 2013). In line with these studies, our findings could suggest that the addition of warming may alleviate the adverse effects of acidification on the molecular responses of oysters. Alternatively, the data may suggest that the combination of elevated CO_2 and temperature cumulatively overwhelms the stress response system of B2 oysters. Further analysis should be performed to test these hypotheses by evaluating the response of B2 oysters to both thermal and CO_2 stresses at the physiological level (*i.e.* growth, development, survival) or following transgenerational exposures to these conditions. In addition, we acknowledge that the transcriptional and proteomic analyses reported here investigated different levels of biological complexity (selected genes vs. whole proteomics). Therefore, given the restricted nature of our transcriptional analysis (selected genes), it is also possible that additional genes

might be differentially expressed due to CO_2 and/or thermal stresses that have not been captured among the 60 genes selected for analysis.

5.4.6 Conclusions

The current study has investigated the impacts of ocean acidification and warming on the proteomes and transcriptional responses of oysters. Our findings suggest that low seawater pH and elevated temperature affect a variety of molecular processes at the protein and transcriptional levels. These effects are not additive or synergistic, even though many of the same intracellular processes are affected by elevated CO_2 and temperature when the stressors are applied separately. The differential responses observed in oysters exposed to a combination of stressors indicate that the inherent capacity of B2 oysters to better cope with elevated CO_2 may not be substantially relevant once thermal stress is added in marine environments. Further studies are required to clarify the complex biological outcomes of the concurrent exposure to ocean acidification and warming, as well as to elucidate the potential of B2 oysters to further acclimate or adapt to such stressful conditions.

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SUPPLEMENTARY INFORMATION

Supplementary Table 5.1. Primers used for qPCR analysis. Also available in: https://drive.google.com/file/d/0BwlanLU1MxH5TlZqYmVGdkR6bGs/view?usp=sharing

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
DGKB	diacylglycerol kinase beta	GCAGGTAACGTCGTACTGTCC	GCATGGGTTCATCCTCATCCC	132	2.01	Cell cycle, Communication	Chapter 4
Septin2	septin-2	TGCCGTAGTGTACCCATCATTC	TGTCCCAGACCTTAGACCAGAG	111	2.11	Cell cycle, Cytoskeleton	Chapter 4
Caspase3	caspase 3	CTTGCGTGATGCGTTCTATTGG	GCTGCATTGTAGGTGCCGTTT	90	2.04	Cell cycle, Immune response	Chapter 4
Condensin-2	condensin-2 complex subunit g2	TCATCAGACGACGGACCACC	GAGAAGGTCAGGGTCGCCAT	142	2.02	Communication, Cell cycle, Nucleic acid regulation	Chapter 4
CD63	CD63 antigen	CGCCGACACAACCAAGAACA	TCAACATCGCTGACTTCGTCG	93	2.04	Communication	Chapter 4
IgGFc-binding protein	IgGFc-binding protein	GCTGCATCCTTTCCCAAAGTCA	CCAGCATCGTTAGCCGACAA	71	2.01	Immune response, Communication	Chapter 4
Pentraxin	pentraxin fusion protein	GGCCATTTCTCGGGACCTAC	GGTCAAGGTAATTCGGCGTTC	118	1.95	Immune response, Communication	Chapter 4
C1q	complement C1q tumor necrosis factor-related protein 7	GCCTTGGTGATTGTTCAAGTTC	CACTTCATGCCCAGTACCACAG	75	1.92	Immune response, Communication	Chapter 4
Structural maintenance chrm 3	structural maintenance of chromosomes protein 3	CCAACATGCAGCTACAGTGTAC	ACTGAGTGACACTGAAGATGGC	75	1.99	Cell cycle, Communication, Immune response	Chapter 4
Protocadherin	protocadherin fat 4	ACTCCCGACGCTGGAAATCA	CCATCACCGTCCAAAGGAATG	150	2.06	Communication, Membrane transport	Chapter 4
Jagged2	protein jagged-2	ATGCCAGCCCTACCAATGGG	TCCACCCGTACACAGCAGTC	130	1.98	Communication, Nucleic acid regulation, Metabolism	Chapter 4
BTBD2	BTB/POZ domain-containing protein 2	GGAACCGTCCACTCCACATCT	CCAGCACTTGGTCGCTCATC	99	2.14	Communication, Stress	Chapter 4
FilaminC	filamin-c	CCAATCCCGATGGTACTGTTGC	CGTGGGTCGGACTCTACATTTG	125	2.08	Cytoskeleton	Chapter 4
Titin	titin	AACGGTTATCCTTGCGGACG	GCACGTCACCACAGTGCTAG	81	1.90	Cytoskeleton	Chapter 4

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
Collagen	short-chain collagen c4	GGACGCCATAACGGAAGAAC	GGTCGTGAAGATGGGAATGG	97	2.03	Cytoskeleton, Metabolism	Chapter 4
Dynein	dynein heavy chain axonemal	GGACTGAGCGCCACATTGTC	TTGACCTGCCTGTGCTTTCC	115	1.97	Cytoskeleton, Cell cycle, Metabolism	Chapter 4
VWF	von Willebrand factor D and EGF domain-containing protein	CCCAGACAACAAGAGCGTGG	GGCAGATCCCGTCTCATGGA	123	1.98	Homeostasis, Cell cycle, Protein regulation	Chapter 4
HAAF	hemagglutinin/amebocyte aggregation factor	CGAGCTTCTTGGTCCACAGC	TTCCAGTGTCCAGGAGCAGG	105	2.16	Immune response	Chapter 4
Toll4	toll-like receptor 4	ACAGGAAACAGAGGCTGGCTAC	GGAACCACTCTCAGCAGGTGTC	93	1.98	Immune response, Communication	Chapter 4
Fibrocystin-L	fibrocystin-L	AGGCTGGTCACCTCCTTTCC	TTCTACACTCCGCCCTTGCC	74	1.99	Immune response, Translation	Chapter 4
MPI	mannose-6-phosphate isomerase	GCAAGGCCACTTCACTTTCAC	CAGAAGGCGATCTTGTGTTTCC	87	1.99	Metabolism	Chapter 4
ATP-binding cassette	ATP-binding cassette sub-family a member 3	GGGCTGTCCATCCAACAAGG	CGCTTCATGCCACCAGACAG	111	1.96	Metabolism	Chapter 4
COX1	cytochrome oxidase subunit I	TTTCCTACCACGGGATGTG	TGAGCTAATACCAGCCAAGTGA	65	2.04	Metabolism	Chapter 3
IAP2	inhibitor of apoptosis 2	GTATTCCTGCCCTGTGGTC	GGTCCCTTTGATGTTCTTCC	93	1.98	Cell cycle, Stress, Immune response	Chapter 4
IAP1	inhibitor of apoptosis 1	TGCGATACGTGTGCTGCTGA	CAGGCAGACACAAACAGGACAA	135	1.96	Cell cycle, Stress, Immune response	Chapter 4
Perlucin	perlucin	ATGACCAACGACAGCACCATTC	TCCGCGTCCAGCATTTATAGC	148	2.06	Immune response, Metabolism, Communication, Calcification	Chapter 4
M6P receptor	Cation-independent mannose-6-phosphate receptor	GGAATGGTGGCAAGGGTTGG	ACAGGGTGATCCAGACAGGC	97	2.01	Metabolism, Communication, Membrane transport, Cytoskeleton	Chapter 4
Fatty acid-binding protein	fatty acid-binding protein	TGTGAATGTTAGTGGCGGATCA	TCAGATAACGTGGCACCCAGTA	81	1.97	Metabolism, Membrane transport	Chapter 4
Kchannel	potassium channel subfamily t member 2	AGGATGGACACTCACAAGACCT	GCCAGTGTTAGGGACAGTAAGC	125	2.08	Metabolism, Membrane transport	Chapter 4
Sortilin	sortilin-related receptor	AGGGAGAGACCTGGAACACC	CCCTGGTTCTGTCAGTACACC	74	1.97	Metabolism, Membrane transport	Chapter 4
Two pore Ca channel	two pore calcium channel protein 1	TCCCAGCTCCTCCTTCTCCA	GAAGTTGTGGTCCCGCCCTA	151	2.03	Metabolism, Membrane transport	Chapter 4

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
Tricarboxylate mitochondrial	tricarboxylate transport protein mitochondrial-like	CATCCACAGGCCCG TTAACTG	GGTAATGTGCGTCTG ACAGGC	124	1.99	Metabolism, Membrane transport	Chapter 4
UDG	uracil-DNA glycosylase	ACAGATGCCGTCAT ATCGCA	GGCTGACAATGGAGA AGGATGG	147	2.08	Metabolism, Nucleic acid regulation	Chapter 4
Poly (ADP-ribose) polymerase 14	poly (ADP-ribose) polymerase 14	TCCACCCTGGTATC CGTTATGT	CCCAGCAGGTTGTAA ATGGAAG	120	1.98	Metabolism, Nucleic acid regulation	Chapter 4
Tho3	tho complex subunit 3	GTTTACGGTTGCCT GGCACC	CCTGCGTCCCGATTT CTGTC	87	1.96	Metabolism, Nucleic acid regulation, Protein regulation	Chapter 4
MEGF10	multiple epidermal growth factor-like domains protein 10	ATCCGTCATCGCAA CTCCCA	CGGATGGGATATGAC TGCAACG	130	2.08	Cell cycle, Metabolism, Protein regulation	Chapter 4
FGF	fibroblast growth factor receptor 2	TGGTCCTGCTTGGT TTGATG	CTGCTTGCTCTCAGC CTTTATG	98	2.00	Communication, Metabolism, Protein regulation	Chapter 4
Carboxypeptidase 2	cytosolic carboxypeptidase 2	GCTTGATGCTTGCA GAGGCT	CGTCTTCTCCTCTTGA TGGTCC	74	2.03	Metabolism, Protein regulation	Chapter 4
Transient receptor Ca channel	transient receptor potential cation channel subfamily m member 2	GAGCAACGGTCAAG AACAGG	AAGCACGTATGGTAT GGAGGAG	105	1.95	Metabolism, Protein regulation	Chapter 4
Phosphatase 1F	protein phosphatase 1F	GCTGCACTGGATTA ACGGTGT	ACCTGCCTCTCTGGTT TGTGG	133	2.06	Metabolism, Protein regulation	Chapter 4
Disintegrin Metalloproteinase	disintegrin and metalloproteinase domain-containing protein 10	TATCTCACAGACGC CGCCAG	CTACATGTTCGCGGA CCGTG	91	1.99	Metabolism, Protein regulation, Communication	Chapter 4
DCTN1	dynactin subunit 1	GCCACGTCTGAAAC CTTTGCT	CGGTCTCTTCCTGCCT GCTT	112	2.07	Cell cycle, Cytoskeleton, Protein regulation	Chapter 4
ECE1	endothelin-converting enzyme 1	GGCTTGGGACTGAC TAATGACC	GCACTGCGTTGTAAT AAGCGAC	97	2.03	Metabolism, Protein regulation	Chapter 4
P450	cytochrome P450 1A1	AAACGAAATCCAGG CACGGC	GATGCACATGGGAGG ATGCG	87	1.94	Stress, Metabolism, Protein regulation	Chapter 4
Dual oxidase	dual oxidase	TGTGTCTTTCCACTG GCTGTC	ACAGGAAACGCCGA ACCTTG	107	2.04	Stress, Metabolism	Chapter 4
Myosin	myosin-IIIb	CCCTGCAGTTTGTC GTTAAGCA	TGGACATACCTGGGT TTGAGGA	82	1.99	Cytoskeleton, Nucleic acid regulation, Metabolism	Chapter 4
Sentrin	sentrin-specific protease 8	AGCAGAGGGTCATT TGTCCTTG	TCCAAGAGCTGCTCA ACATGC	97	2.04	Protein regulation	Chapter 4
Leucine 15	leucine-rich repeat-containing protein 15	TGTACCGCGACTGC TTCATACA	TCTGTGAGCGGATTA CCCAACT	126	1.98	Protein regulation, Communication	Chapter 4

Gene symbol	Gene name	Fw (5'→ 3')	Rv (5'→ 3')	Product size (bp)	Effici-ency	Functional group	Sequence source
Kelch3	kelch-like protein 3	TTGTGCCGCAGAGC TGAATG	CATCTGTCAGTGCTT GGCTGG	117	1.95	Immune response, Protein regulation, Communication	Chapter 4
TubulinC	tubulin-specific chaperone C	GTAGCTCCCACGTA TGCCAC	GCTTCAGCGTCCTCTT CTCC	95	1.97	Protein regulation, Cytoskeleton	Chapter 4
H3	histone H3	CTCTTACGGGCTGC CTTGGT	ACACTCTGCCCTCGT CATTCA	128	2.03	Nucleic acid regulation, Cell cycle	Chapter 4
H4	histone H4	AAACCCGTGGTGTC CTGAAAG	TTGGCGTGCTCTGTG TAGGTG	73	1.99	Nucleic acid regulation, Cell cycle	Chapter 4
Universal stress	universal stress protein A	TAGATCCGCCTCCA CCTCCT	GACGCCAAGATAAAC GGCACT	93	2.02	Stress	Chapter 4
ecSOD	extracellular superoxide dismutase	TAGCCAATGAACGT CCCAGA	GATCTCCATGACGAC GACCA	100	1.99	Stress	Chapter 4
Multidrug resistance	multidrug resistance protein 1	GTATTGGCCGGGTG CTGAAG	AGATGATGGCGAAGG CTGGT	108	2.01	Stress	Chapter 4
Glutaredoxin-2 mitochondrial-like	glutaredoxin-2 mitochondrial-like	TGGAACCTGTATTG GTGGAGCA	TCCACTTGAGGGTTA TGGCACT	96	2.06	Stress, Metabolism	Chapter 4
Glutathione S-transferase	glutathione S-transferase omega	CCTGTGACGTAGCA AAGCCAGT	CCGAGGTAGGGAAAC AGCTTGA	126	1.97	Stress, Metabolism	Chapter 4
Hsp70b	heat shock 70 kDa protein 12b	CGTGACTGAGAAAG GGTGTTTG	CATACAAGTGGCTTT GGCTTGG	136	2.00	Stress, Protein regulation	Chapter 4
Hsp70a	heat shock 70 kDa protein 12a	CGTGACTGAGAAAG GGTGTTTG	CAAGTGGCTTTGGCT TGGA	132	2.04	Stress, Protein regulation	Chapter 4
Serine arginine	serine arginine repetitive matrix protein 1	CGCTGAACCGATTG TCCTGG	AAATGGTGAGAGTGG GTGTTGG	86	2.01	Nucleic acid regulation	Chapter 4
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	ACCGCGCCAGTCTT TGTTG	GGCATTGTTGAGGGT CTGATG	90	1.94	Metabolism, Potential reference gene	Chapter 3
β-actin	β-actin	GCACCTGAATCGCT CGTTG	CAGCAGCATCGTCAT CATCC	86	1.95	Cytoskeleton, Reference gene	Chapter 3
EF1α	elongation factor 1 alpha	CCATAGCGGCATCT CCACTC	CCTTGATTGCCACAC TGCTC	125	1.97	Nucleic acid regulation, Reference gene	Chapter 3

CHAPTER 6

SYNTHESIS AND CONCLUSIONS

Oceans represent the main repository for the CO_2 emitted by human activity. Ocean uptake of anthropogenic CO_2 is crucial for life on Earth since it moderates the atmospheric impacts of climate change (Sabine *et al.* 2004). However, it comes at the cost of ocean acidification (OA) and warming (Hoegh-Guldberg & Bruno 2010; Watson *et al.* 2011). OA produces predominantly negative effects on marine organisms across different species, populations, life-history stages and levels of biological complexity (Byrne *et al.* 2011; Fabry 2008; Hofmann *et al.* 2010). Reduction of calcification, as well as metabolic shifts, abnormal development and growth, are among the most evident impacts of OA in marine calcifiers (Gazeau *et al.* 2013; Harvey *et al.* 2013; Kroeker *et al.* 2013; Kroeker *et al.* 2010). In addition to these overt effects, exposure to elevated CO_2 has also been shown to elicit substantial changes in intracellular and molecular responses in numerous calcifying species (Pörtner 2012; Somero 2010). The physiological effects of CO_2 stress can be attributed to complex rearrangements in cellular functions and molecular processes (Evans & Hofmann 2012; Franks & Hoffmann 2012; Somero 2010). These effects may be complicated by the impacts of elevated temperature, which is the other main climate change variable that affects marine ecosystems (Brierley & Kingsford 2009; Hoegh-Guldberg & Bruno 2010). The existing evidence suggests that the concurrent effects of OA and ocean warming may be even more detrimental than the impacts of each stressor in isolation (Byrne 2011; Harvey *et al.* 2013; Kroeker *et al.* 2013; Rodolfo-Metalpa *et al.* 2011).

The effects of climate change on keystone species and ecosystem engineers are of particular concern because they are likely to compromise whole communities. Corals are already known to be affected by current changes in seawater temperature and pH, as are some species of bivalves (Harvey *et al.* 2013; Kroeker *et al.* 2013; Kroeker *et al.* 2010). The risk of substantial impact is especially high for corals and bivalves, even if temperature and CO_2 increments are kept within the most stringent emission-mitigation scenario proposed by the

Intergovernmental Panel on Climate Change (IPCC) (Representative Concentration Pathway 2.6; RCP2.6) (IPCC 2013). If temperature and CO_2 concentrations follow the current high-carbon-emission business-as-usual scenario (RCP8.5), very high risks of impact are expected for most marine organisms (Gattuso *et al.* 2015).

The detrimental effects of climate change will extend beyond impacts on individual species and their environments to large-scale changes in ecosystem function and the provision of ecosystem services. Coastal communities, in particular, depend heavily on fisheries resources for food and economic security. They will be at particular risk from shifts in species distribution and abundance resulting from climate change (Barange *et al.* 2014; Branch *et al.* 2013; Cheung *et al.* 2010).

Decreasing productivity is also expected to affect aquaculture industries worldwide. In addition to the impacts of OA and warming on the performance of farmed species, coastal aquaculture is threatened by sea level rise. Rising sea levels will affect salinity in deltas and estuaries where aquaculture commonly occurs. Such environmental perturbations may also facilitate the spread of invasive species, as well as increase the incidence of infectious diseases and harmful algal blooms (Burge *et al.* 2014; De Silva 2012; Hoegh-Guldberg & Bruno 2010; Rodrigues *et al.* 2015). Molluscs currently represent the second largest group of aquaculture animals (behind finfish). They comprised 23% of world aquaculture production for food in 2012 (15.2 million tonnes) (FAO 2014). In this context, the global economic cost of losses in the capture and aquaculture of molluscs resulting from OA is estimated to be over US$ 100 billion by the year 2100 based on the current high-carbon-emission business-as-usual scenario (RCP8.5) (Narita *et al.* 2012). A reduction in mussel (*Mytilus edulis*) production of 50% to 70% is anticipated in the United Kingdom under the RCP2.6 or RCP8.5 scenarios, respectively (Ferreira *et al.* 2008).

Oyster production will also decline in response to OA and warming. A recent assessment of climate change impacts on aquaculture indicated that the edible oyster industry is at highest potential risk in south-eastern Australia (Doubleday *et al.* 2013). This is primarily due to increases in temperature and heat wave-related mortalities that are already emerging as an issue in the states of New South Wales (NSW) and South Australia (SA). The study also revealed that the Australian oyster species, Sydney rock oyster (*Saccostrea glomerata*), is likely to suffer stronger overall negative impacts than the exotic Pacific oyster (*Crassostrea gigas*), particularly farmed *S. glomerata* derived from wild spat (compared to hatchery-produced spat) (Doubleday *et al.* 2013). Given this susceptibility of farmed species to global climate change, aquaculture practices will require interventions comparable to those for wild populations to ensure a reliable supply of seafood in the future (Evans & Hofmann 2012).

Despite the potential severity of climate change impacts on marine calcifiers, such as molluscs, some organisms exhibit a notable phenotypic plasticity or a capacity for rapid heritable changes in response to environmental stress (Donelson *et al.* 2012; Dupont *et al.* 2013; Parker *et al.* 2015; Sanford & Kelly 2011; Schoville *et al.* 2012). Signs of tolerance to stressful conditions may reflect distinctive ecological and evolutionary trajectories in many of these organisms. Coastal species may be particularly amenable to beneficial change because their habitats are naturally subjected to constant fluctuations in pH and temperature independent of climate change (Zhang *et al.* 2012).

In addition to differences in susceptibility between broad taxonomic groups, variation in responses to stress has also been observed between spatially and/or genetically distinct populations within species, as well as between offspring of parents exposed to different CO_2 environments (Fabry 2008; Harvey *et al.* 2013; Miller *et al.* 2009; Parker *et al.* 2010, 2011; Thompson *et al.* 2016; Thomsen & Melzner 2010; Wright *et al.* 2014). Populations of Sydney rock oysters (*S. glomerata*) produced through selective breeding for fast growth and disease

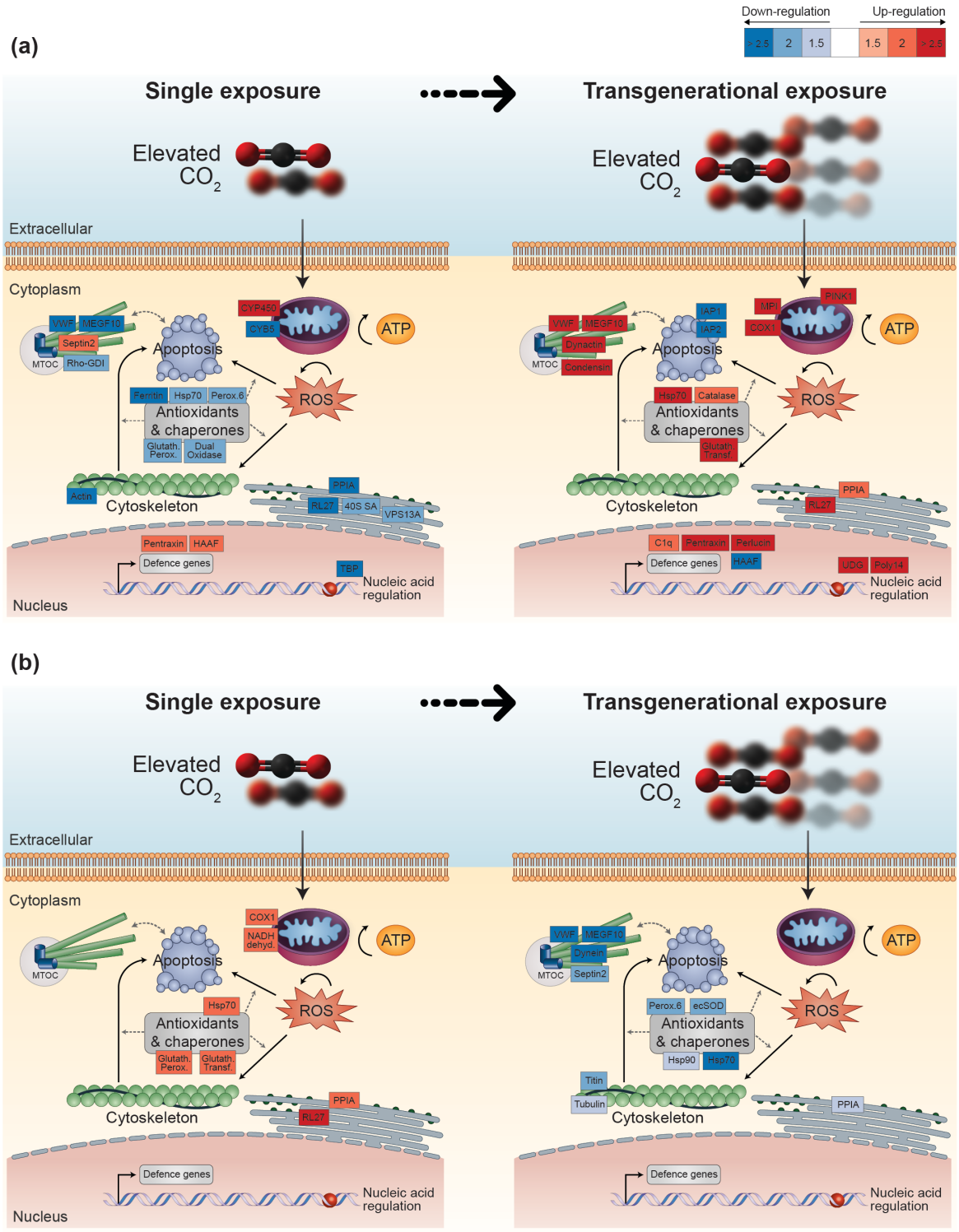
resistance have shown greater resilience to CO_2 stress than their non-selected (wild type) counterparts. Oysters resistant to winter mortality and those resistant to both winter mortality and QX disease (the B2 line) exhibited less pronounced changes in shell growth under OA when compared to oysters that had not been subjected to selective breeding (Parker *et al.* 2011). Further analyses of larval development, shell growth and the metabolic rates (SMR) of adults revealed the unique capacity of B2 oysters, in particular, to better cope with the effects of CO_2 stress (Parker *et al.* 2012). This thesis has exploited this unique and distinctive performance of selectively bred Sydney rock oysters by investigating the intracellular processes driving their resilience to CO_2 stress.

In the current study, intracellular effects of elevated CO_2 and elevated temperature were examined at different levels of biological complexity to determine the molecular processes and cellular pathways that are potentially involved in acclimation or adaptation to such stressful conditions. These analyses revealed that a number of cellular functions involving a variety of genes and their encoded proteins are substantially altered following exposure to elevated CO_2 and/or elevated temperature. Such changes in subcellular processes were found to differ between genetically distinct populations of Sydney rock oysters. By comparing the molecular responses of B2 oysters with those of wild type oysters in Chapters 3 and 4, I identified contrasting expression patterns for molecules (genes and proteins) associated primarily with control of the cell cycle, maintenance of cellular homeostasis, metabolism and intracellular stress responses (Fig. 6.1) (Supplementary Table 6.1). The differentially regulated genes/proteins included heat shock proteins (Hsp70 and 90), cytochromes (b5 and P450) and glutathione regulating enzymes (glutathione peroxidase and transferase). These results are consistent with previous studies suggesting that adaptive responses to climate change variables are likely to involve the differential regulation of specific molecular systems responsible for

preserving cellular homeostasis, as well as preventing and/or repairing cellular and macromolecular damage (Pörtner 2008; Somero 2010).

By identifying these processes, the thesis also builds upon the generalised framework of intracellular stress responses in bivalves proposed by Anderson *et al.* (2015) and Tomanek (2014). These consensus models highlight the roles of metabolic and antioxidant processes as primary targets for stress-induced responses. The current study extends those models by revealing additional alterations in other fundamental cellular functions induced by OA and warming. The additional biological functions affected by CO_2 and/or thermal stresses included control of the cell cycle, protein synthesis and trafficking, nucleic acid regulation, intracellular signalling and immune responses. All of these functions are consistent with the complex and dynamic regulation of metabolism, oxidative stress and resultant apoptosis. Their relationship to the consensus model of environmental stress responses in oysters is shown in Figure 6.1. A presumptive list of molecular markers potentially associated with adaptive responses to OA and warming (warranting further investigation) is shown in Supplementary Table 6.1.

The magnitude and direction of changes in this suite of intracellular functions were found to differ between selectively bred (B2) and wild type (non-selected) oysters (Fig. 6.1). Variability in responses between populations was evident following single (Chapter 3) or multiple (transgenerational; Chapters 3 and 4) exposures to elevated CO_2. Oysters from the B2 breeding line exposed to CO_2 stress over a single generation (1 week to 1 month exposure) exhibited lower expression for the majority of differentially regulated genes/proteins, with the exception of some genes/proteins involved in immune responses and cytochrome P450 (relative to B2 oysters held at ambient CO_2 conditions) (Fig. 6.1a). A single exposure to elevated CO_2 induced less evident changes in the molecular processes of wild type oysters. Most of the changes observed in this population reflected an up-regulation of genes in response to elevated CO_2 (relative to wild type oysters held at ambient CO_2 conditions) (Fig. 6.1b).


(a)
Down-regulation
Up-regulation
> 2.5
2
1.5
1.5
2
> 2.5
Single exposure
Transgenerational exposure
Elevated CO2
Elevated CO2
Extracellular
Cytoplasm
VWF
MEGF10
Septin2
MTOC
Rho-GDI
Apoptosis
CYP450
CYB5
ATP
ROS
Ferritin
Hsp70
Perox.6
Antioxidants & chaperones
Glutath. Perox.
Dual Oxidase
Actin
Cytoskeleton
PPIA
RL27
40S SA
VPS13A
Pentraxin
HAAF
Defence genes
TBP
Nucleic acid regulation
Nucleus
VWF
MEGF10
Dynactin
Condensin
MTOC
IAP1
IAP2
MPI
PINK1
COX1
Apoptosis
ATP
Hsp70
Catalase
Antioxidants & chaperones
Glutath. Transf.
ROS
Cytoskeleton
PPIA
RL27
C1q
Pentraxin
Perlucin
Defence genes
HAAF
UDG
Poly14
Nucleic acid regulation
(b)
Single exposure
Transgenerational exposure
Elevated CO2
Elevated CO2
Extracellular
Cytoplasm
MTOC
COX1
NADH dehyd.
Apoptosis
ATP
Hsp70
Antioxidants & chaperones
Glutath. Perox.
Glutath. Transf.
ROS
Cytoskeleton
PPIA
RL27
Defence genes
Nucleic acid regulation
Nucleus
VWF
MEGF10
Dynein
MTOC
Septin2
Apoptosis
ATP
Perox.6
ecSOD
Antioxidants & chaperones
Hsp90
Hsp70
ROS
Titin
Tubulin
Cytoskeleton
PPIA
Defence genes
Nucleic acid regulation

Transgenerational conditioning to CO_2 stress also resulted in contrasting intracellular profiles between breeding lines (Chapters 3 and 4). B2 oysters exhibited higher expression of genes related to a variety of cellular processes, while gene expression in wild type oysters was generally down-regulated. Genes/proteins that participate in the control of the cell cycle and the maintenance of cellular homeostasis were particularly affected (*e.g.* multiple epidermal growth factor-like domains protein 10 - MEGF10; and von Willebrand factor D and EGF domain-containing protein - VWF). These molecules were overexpressed in CO_2-conditioned B2 oysters and suppressed in CO_2-conditioned wild types (relative to their respective ambient controls) (Fig. 6.1). Genes related to immune function (*e.g.* perlucin and pentraxin fusion protein) were also found to be induced in CO_2-conditioned B2 oysters, while no substantial change in their expression was observed among wild type oysters. The allocation of these genes to the "immune response" category traditionally implies defence against invading pathogens. However, in the context of abiotic stress, it is likely that many putative immune-response genes are associated with damage repair involving the clearance of dysfunctional somatic cells.

Figure 6.1. (opposite page) Intracellular responses induced by ocean acidification in Sydney rock oysters. Major alterations in gene expression or protein concentration in B2 line **(a)** and wild type **(b)** oysters following single or transgenerational exposures to elevated CO_2 (-0.3 to -0.4 pH unit relative to ambient conditions). Changes were particularly evident in molecules involved in metabolism, intracellular stress responses, antioxidant defence, the cytoskeleton, the cell cycle (cell replication and death through apoptosis), protein and nucleic acid regulation, as well as immune responses. Examples of differentially regulated genes/proteins associated with each function are shown in coloured boxes. The colour represents the fold change in expression of genes/proteins relative to control, ambient conditions. Red boxes reflect up-regulation in the CO_2 stress treatment relative to ambient controls, while blue boxes indicate down-regulation. Gene and protein names, as well as their biological functions are listed in Supplementary Table 6.1.

Overall, the differential intracellular responses of B2 and wild type oysters following single or transgenerational exposures to CO_2 stress reflect their different strategies for coping with OA. B2 oysters appear to be able to direct much energy on gene regulation under short-term to moderate OA (Fig. 6.1a; single exposure), and have full capacity to acclimate to more severe CO_2 stress (Fig. 6.1a; transgenerational exposure). In contrast, our findings indicate that wild type oysters need to divert much energy into acclimation under short-term to moderate stress (Fig. 6.1b; single exposure) and may be unable to acclimate to long-term, more severe stress conditions (Fig. 6.1b; transgenerational exposure). Similar transcriptional responses and differential adaptive potential have been observed among conspecific clades of dinoflagellates of the genus *Symbiodinium* with different levels of tolerance to heat stress (Barshis *et al.* 2014).

Interestingly, the effects of OA appeared to vary not only between selectively bred and wild type oysters, but also between different selectively bred populations. The modifications observed in B2 oysters at the level of individual genes, as well as whole transcriptomes and proteomes, are consistent with inducible responses that may be beneficial to the oyster. In contrast, cytological analyses of a different oyster population selected for fast growth and resistance to winter mortality (WM) indicated that CO_2 exposure resulted in deleterious oxidative cellular stress (Chapter 2). The winter mortality-resistant population exhibited increased levels of reactive oxygen species (ROS) and reduced mitochondrial integrity under elevated CO_2 conditions (Supplementary Table 6.1). These cytological changes are likely caused by an enhanced demand for cellular energy in response to OA. The most evident and immediate outcome of such a condition would be cellular dysfunction due to excess ROS production and resulting mitochondrial damage, particularly within the first hours post-CO_2 exposure. These detrimental cytological modifications in oysters bred for resistance to winter mortality are at least superficially distinct from the differential regulation of genes and proteins seen in B2 oyster responding to elevated CO_2. However, it needs to be acknowledged that the

apparent differences between WM and B2 oysters may simply reflect the different levels of biological organisation at which analyses were undertaken (cytology versus molecular analysis), and that WM oysters have shown a degree of tolerance to CO_2 stress at the physiological level (Parker *et al.* 2011).

The analysis of oysters subjected to a single CO_2 exposure suggests that they are capable of substantial inducible responses to elevated CO_2. Transgenerational experiments performed in this thesis indicated that oysters also have the capacity for rapid heritable change, and that this capacity differs between oyster populations. Transgenerational exposure to elevated CO_2 revealed that *S. glomerata* may be able to undergo rapid acclimation or adaptation to CO_2 stress at the transcriptional level (Chapters 3 and 4). A combination of parental and larval conditioning affected the expression profiles of key target genes, and this capacity for heritable change appeared to be more evident in oysters from the B2 breeding line (Chapter 3). These findings indicate that short-term evolutionary responses (over few generations) have the potential to mitigate the adverse effects of OA in oysters. Traditionally, transgenerational acclimation to environmental stress is considered to occur over shorter periods of time than genetic adaptation (natural selection). Acclimation may also persist for multiple generations, making it difficult to distinguish from genetic adaptation (Evans & Hofmann 2012; Sunday *et al.* 2014; van Oppen *et al.* 2015). For example, exposure to acute heat stress causes substantial declines in aerobic scope (the difference between resting and maximal rates of oxygen consumption) of the reef fish *Acanthochromis polyacanthus*. However, conditioning of parents and F1 offspring to elevated temperature resulted in complete restoration of metabolic performance in the F2 offspring (Donelson *et al.* 2012). Similarly, heritable change to CO_2 stress was reflected by positive or neutral effects of elevated CO_2 on metabolic rate, length, weight and survival in F1 juveniles of the anemonefish *Amphiprion melanopus* following parental conditioning (Miller *et al.* 2012). The capacity of Sydney rock oysters for acclimation or adaptation to CO_2 stress

within a few generations and the intrinsic genetic differences between their populations represent key features that may contribute to the viability of the species in acidifying environments. Such genetic variation and plasticity to generate phenotypes with different pH tolerances have the potential to replenish populations of conspecifics that face suboptimal performance or local extinction in the near future (Somero 2010). Whether or not these changes arise from acclimation or adaptation is important because it implies different strategies for achieving the desired outcome of OA-resilient oysters. However, the current thesis does not make such a distinction. The experimental protocols used here to investigate transgenerational change were not designed to differentiate between non-genetic processes (acclimation), such as epigenetic modifications, and genetic adaptation (differential reproductive success). Such distinctions will be best addressed through detailed genomic analysis in the future.

The thesis also shows that the regulation of a distinct suite of genes and proteins is altered when B2 oysters are exposed to a combination of elevated CO_2 and elevated temperature (Chapter 5) (Supplementary Table 6.1). Concurrent exposure to CO_2 and thermal stresses indicated that these environmental factors do not interact to produce synergistic or additive effects on oyster molecular processes. Previous studies have already demonstrated the role of thermal stress in ameliorating some impacts of elevated CO_2. Increased temperature minimised the negative effects of elevated CO_2 on growth, calcification rate and photosynthesis in a number of calcifying marine organisms (Harvey *et al.* 2013), including the oyster *C. gigas* (Harney *et al.* 2016), corals (McNeil *et al.* 2004), and the sea star *Pisaster ochraceus* (Gooding *et al.* 2009). In line with these studies, findings in this thesis could suggest that the addition of warming may alleviate or at least not aggravate the adverse effects of OA on the molecular responses of oysters. Alternatively, my data may indicate that the addition of thermal stress to OA overwhelms the stress response system of B2 oysters, such that the molecular profiles of B2 oysters exposed to both stressors simultaneously more closely resemble those of oysters

under fully ambient conditions. This would imply a hormetic stress response, where inducible and beneficial changes are possible until the combined level of stress reaches a threshold beyond which cellular dysfunction overwhelms the favourable response.

Findings from the concurrent exposure to elevated CO_2 and elevated temperature may also indicate that the resilience of B2 oysters to OA does not extend to ocean warming (Chapter 5). Selection for a specific trait can result in positive side effects, such as B2 oysters artificially selected for faster growth and disease resistance also being resilient to CO_2 stress. However, trait evolution may also lead to trade-offs that can compromise a variety of energy-demanding processes (Kelly & Hofmann 2013; van Oppen *et al.* 2015). For instance, marine organisms may need to divert energy from other fundamental processes, such as reproduction and immune responses, to be able to maintain and/or regulate pH balance under CO_2 stress (primary effect of OA) (Pörtner *et al.* 2004). Such energy reallocation could also affect the response of organisms to additional and/or novel environmental disturbances, such as changes in temperature, salinity or infectious diseases. Therefore, resilience to a selective stressor and the costs associated with it may compromise the capacity of the organism to cope with other forms of stress (Sunday *et al.* 2014). Negative genetic correlations between traits that provide resilience against OA and warming, for example, could decrease the rate of adaptive evolution and result in long-term consequences for population viability (Kelly & Hofmann 2013; Sunday *et al.* 2014). This possibility seems unlikely given that the molecular responses to OA and warming were found to be generally similar when the stressors were applied in isolation. However, future studies should be conducted to test for competitive effects of OA and warming by evaluating the performance of B2 oysters under a combination of OA and warming at the physiological level (*i.e.* growth, development, mortality, metabolic rate).

The degree to which transgenerational exposure to stress ameliorates adverse impacts and the nature of the evolutionary trade-offs associated with those changes is likely to depend

on the genetic background of the founder populations (Evans & Hofmann 2012; Harley *et al.* 2006). Throughout this thesis, I have shown that oysters from the B2 breeding line have distinctive responses to elevated CO_2 at the subcellular level when compared to other populations within the same species. This complements and extends the findings of Parker *et al.* (2011) and Parker *et al.* (2012) who have characterised the differential performance of these oysters at the physiological level. Additional analysis investigating the genetic differences (*e.g.* SNP allele frequency estimation) between the two breeding lines is currently being conducted. This will provide a better understanding of the distinct responses that were observed at both physiological and molecular levels.

Depending on the physiological trade-offs involved in achieving resilience, selectively bred populations could have the potential to preserve species from extinction and to maintain ecosystem function in a rapidly changing ocean. Geological records show that historic carbon perturbation events occurred at rates slower than those projected for the coming decades (Friedrich *et al.* 2012; Luthi *et al.* 2008). However, even these more gradual changes did not prevent mass extinctions, leading to major widespread alterations in marine ecosystems (Gattuso *et al.* 2015). For instance, the Permian-Triassic boundary (~60,000 years) harboured the most catastrophic loss of biodiversity in geological history and is characterised by extreme environmental changes, including OA (decrease by up to 0.7 pH unit) (Clarkson *et al.* 2015). These changes in ocean chemistry and physics resulted in the elimination of over 90% of marine species, with preferential loss of sessile shell-forming organisms (Song *et al.* 2013). Even though that event exceeded the current anthropogenic perturbation in magnitude, it is widely recognised that marine calcifiers, including oysters, are again among the organisms at the greatest risk from current climate change (Doubleday *et al.* 2013; Gattuso *et al.* 2015; Kroeker *et al.* 2013). Therefore, improved understanding that leads to the development of appropriate

management strategies is urgently required to mitigate the impacts of projected near-future ocean temperature and pH on these organisms.

This thesis has analysed the response of oysters from three genetically different population following exposure to ambient or elevated CO_2 conditions. This work was conducted sequentially as oyster populations and CO_2 facility became available. As a result, it was impossible to analyse all oyster populations using the same set of techniques. The sequential nature of this thesis also reflected in constant optimisation of the experimental setup. This resulted in slightly different physico-chemical conditions among the exposures, particularly in regard to the elevated CO_2 treatments (625-933 μatm pCO_2, pH 7.70-7.87). In addition to the small differences in the experimental setup, the studies presented in this thesis have analysed the response of oysters at different levels of biological complexity (cellular and molecular impacts at either protein or transcriptional levels). Given that each of these levels of biological organisation would be affected by environmental stress at different times, we have analysed oyster response following short-term (4 to 15 days), moderate (1 week to 1 month) and long-term (transgenerational) exposures to elevated CO_2.

The studies reported in this thesis are among the first to investigate evolutionary and phenotypically plastic responses through long-term, transgenerational experiments. The work provides evidence for rapid acclimation and/or adaptation of oysters to the adverse effects of climate change at the most fundamental level (transcriptional regulation). My data indicate that the biological systems involved in response to OA (and warming) are far more complex and dynamic than currently understood. Despite that complexity, the thesis reports novel insights into the intracellular processes driving the response of marine organisms to OA and ocean warming. This understanding could be used to predict which species or populations are more or less vulnerable to climate change based on the expression profiles of genes and proteins revealed herein.

In addition, the thesis reports the discovery of functional molecular markers that could be applied in marker-assisted selection programs to climate-proof oysters and other economically important bivalves. Assisted evolution towards resilience to climate change has been proposed for highly threatened species, such as corals (van Oppen *et al.* 2015). These initiatives involve a deep screening of traits and mechanisms potentially associated with tolerance to climate change variables and the development of biological toolkits for enhancing resilience to such stressors. The knowledge gained from this thesis represents the most comprehensive and integrative resource available for the development of such programs in oysters. Marker-assisted breeding programs could be developed to enhance climate change resilience in oysters based on selection of parents with inherent up-regulation of key target genes identified in this study (*e.g.* genes involved in mitochondrial energy production and anti-oxidant processes). Following breeding, resilience against climate change variables could then be assessed in terms of larval development and adult performance over successive generations. The evaluation of these parameters over multiple generations would reveal whether the improved, selected traits have a genetic basis and are heritable. Identification and enhancement of genotypes that are fittest in these environments will be essential for the sustainability of marine organisms and their associated ecological and economical value in the face of increasing human impacts and intensifying climate change.

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SUPPLEMENTARY INFORMATION

Supplementary Table 6.1. Summary of the major intracellular responses to ocean acidification and/or warming revealed in this thesis. CO_2 refers to exposure to elevated CO_2 conditions (-0.2 to -0.4 pH unit relative to ambient conditions), Heat refers to exposure to elevated temperature (+3.6 °C relative to ambient conditions), and CO_2 + Heat refers to exposure to both elevated CO_2 (-0.36 pH unit) and elevated temperature (+2.7 °C). Exposure to stress was performed over single or multiple generations (Transgen^2 indicates 2 consecutive generations of exposure and Transgen^3 indicates 3). Populations of Sydney rock oysters analysed in this study were wild type (non-selected), B2 line (fast-growing, resistant to QX disease and Winter Mortality) and Winter Mortality resistant (WM; fast-growing and resistant to Winter Mortality). Annotation of primary functional group/cellular process was based on Blast2GO results and UniProtKB database. Fold changes (FC) were calculated from relative gene expression or normalised intensities of protein spots compared to the control, ambient conditions. FC values > 1 reflect up-regulation (↑) in the stress treatment relative to ambient controls, while values < 1 indicate down-regulation (↓).

Stress	No. exposures	Popula-tion	Functional group/ Cellular process[1]	Gene or Protein	Direction	FC
CO_2	Single	Wild	Metabolism	NADH dehydrogenase	↑	1.6
CO_2	Single	Wild	Metabolism	Cytochrome c oxidase I (COX1)	↑	2.3
CO_2	Single	Wild	Metabolism	Mitochondrial membrane potential	↑	2.9
CO_2	Single	Wild	Stress	Extracellular superoxide dismutase (ecSOD)	↓	0.5
CO_2	Single	Wild	Stress	Glutathione peroxidase	↑	1.6
CO_2	Single	Wild	Stress	Heat shock 70 kDa protein (Hsp70)	↑	1.9
CO_2	Single	Wild	Stress	Glutathione S-transferase omega	↑	1.6
CO_2	Single	Wild	Protein regulation	Peptidylprolyl isomerase A (PPIA)	↑	2.0
CO_2	Single	Wild	Protein regulation	Ribosomal protein RL27	↑	3.2
CO_2	Single	B2	Metabolism	NADH dehydrogenase	↓	0.4
CO_2	Single	B2	Metabolism	ATP synthase	↑	1.5

Stress	No. exposures	Popula-tion	Functional group/ Cellular process[1]	Gene or Protein	Direction	FC
CO_2	Single	B2	Stress	Cytochrome b5	↓	0.4
CO_2	Single	B2	Stress	Ferritin	↓	0.3
CO_2	Single	B2	Stress	Glutathione peroxidase	↓	0.5
CO_2	Single	B2	Stress	Peroxiredoxin 6	↓	0.5
CO_2	Single	B2	Stress	Dual oxidase	↓	0.5
CO_2	Single	B2	Stress	Heat shock 70 kDa protein 12a (Hsp70a)	↓	0.6
CO_2	Single	B2	Stress	Stress-70 protein, mitochondrial	↑	2.3
CO_2	Single	B2	Stress	Extracellular superoxide dismutase (ecSOD)	↑	2.5
CO_2	Single	B2	Stress	Cytochrome P450	↑	6.9
CO_2	Single	B2	Protein regulation	Vacuolar protein sorting-associated protein 13A (VPS13A)	↓	0.5
CO_2	Single	B2	Protein regulation	Peptidylprolyl isomerase A (PPIA)	↓	0.4
CO_2	Single	B2	Protein regulation	Ribosomal protein RL27	↓	0.3
CO_2	Single	B2	Protein regulation	Ribosomal protein 40S SA	↓	0.5
CO_2	Single	B2	Nucleic acid regulation	TATA-binding protein (TBP)	↓	0.3
CO_2	Single	B2	Cell cycle	Rho GDP dissociation inhibitor 1 (Rho-GDI)	↓	0.5
CO_2	Single	B2	Cell cycle	Multiple epidermal growth factor-like domains protein 10 (MEGF10)	↓	0.4
CO_2	Single	B2	Cell cycle	von Willebrand factor D and EGF domain-containing protein (VWF)	↓	0.2
CO_2	Single	B2	Cell cycle	Structural maintenance of chromosomes protein 3	↓	0.3
CO_2	Single	B2	Cell cycle	Septin 2	↑	2.2
CO_2	Single	B2	Cytoskeleton	Actin	↓	0.2
CO_2	Single	B2	Communication	14-3-3 protein	↑	2.3
CO_2	Single	B2	Immune response	Pentraxin fusion protein	↑	2.4
CO_2	Single	B2	Immune response	Hemagglutinin/amebocyte aggregation factor (HAAF)	↑	1.8

Stress	No. exposures	Popula-tion	Functional group/ Cellular process[1]	Gene or Protein	Direction	FC
CO_2	Single	WM	Metabolism	Mitochondrial membrane potential	↓	2.8
CO_2	Single	WM	Stress	Production of reactive oxygen species (ROS)	↑	3.0
CO_2	Transgen[2]	Wild	Stress	Heat shock 70 kDa protein 12a (Hsp70a)	↓	0.4
CO_2	Transgen[2]	Wild	Cell cycle	Multiple epidermal growth factor-like domains protein 10 (MEGF10)	↓	0.3
CO_2	Transgen[2]	Wild	Cell cycle	von Willebrand factor D and EGF domain-containing protein (VWF)	↓	0.1
CO_2	Transgen[2]	Wild	Cell cycle	Septin-2	↓	0.5
CO_2	Transgen[2]	Wild	Cytoskeleton	Dynein heavy chain axonemal	↓	0.2
CO_2	Transgen[2]	Wild	Cytoskeleton	Titin	↓	0.6
CO_2	Transgen[2]	Wild	Communication	Two pore calcium channel protein 1	↓	0.5
CO_2	Transgen[2]	Wild	Immune response	Complement C1q/tumor necrosis factor-related protein 7	↓	0.2
CO_2	Transgen[2]	Wild	Immune response	Hemagglutinin/amebocyte aggregation factor (HAAF)	↑	2.2
CO_2	Transgen[3]	Wild	Stress	Heat shock 90 kDa protein (Hsp90)	↓	0.7
CO_2	Transgen[3]	Wild	Stress	Extracellular superoxide dismutase (ecSOD)	↓	0.5
CO_2	Transgen[3]	Wild	Stress	Peroxiredoxin 6	↓	0.6
CO_2	Transgen[3]	Wild	Protein regulation	Peptidylprolyl isomerase A (PPIA)	↓	0.7
CO_2	Transgen[3]	Wild	Cytoskeleton	β-tubulin	↓	0.7
CO_2	Transgen[2]	B2	Metabolism	Mannose-6-phosphate isomerase (MPI)	↑	48
CO_2	Transgen[2]	B2	Stress	Extracellular superoxide dismutase (ecSOD)	↓	0.5
CO_2	Transgen[2]	B2	Stress	Heat shock 70 kDa protein 12a (Hsp70a)	↑	87
CO_2	Transgen[2]	B2	Stress	Glutathione S-transferase omega	↑	3.5
CO_2	Transgen[2]	B2	Nucleic acid regulation	Uracil-DNA glycosylase (UDG)	↑	138
CO_2	Transgen[2]	B2	Nucleic acid regulation	Poly (ADP-ribose) polymerase 14	↑	3.3
CO_2	Transgen[2]	B2	Cell cycle	Inhibitor of apoptosis 1 (IAP1)	↓	0.05

Stress	No. exposures	Popula-tion	Functional group/ Cellular process[1]	Gene or Protein	Direction	FC
CO_2	Transgen[2]	B2	Cell cycle	Inhibitor of apoptosis 2 (IAP2)	↓	0.03
CO_2	Transgen[2]	B2	Cell cycle	Multiple epidermal growth factor-like domains protein 10 (MEGF10)	↑	81
CO_2	Transgen[2]	B2	Cell cycle	Serine/threonine-protein kinase mitochondrial (PINK1)	↑	72
CO_2	Transgen[2]	B2	Cell cycle	von Willebrand factor D and EGF domain-containing protein (VWF)	↑	2.7
CO_2	Transgen[2]	B2	Cell cycle	Condensin-2	↑	3.1
CO_2	Transgen[2]	B2	Cytoskeleton	Dynactin subunit 1 (DCTN1)	↑	34
CO_2	Transgen[2]	B2	Immune response	Hemagglutinin/amebocyte aggregation factor (HAAF)	↓	0.03
CO_2	Transgen[2]	B2	Immune response	Pentraxin fusion protein	↑	52
CO_2	Transgen[2]	B2	Immune response	Perlucin	↑	36
CO_2	Transgen[2]	B2	Immune response	Complement C1q/tumor necrosis factor-related protein 7	↑	1.8
CO_2	Transgen[3]	B2	Metabolism	Cytochrome c oxidase I (COX1)	↑	3.9
CO_2	Transgen[3]	B2	Stress	Catalase	↑	1.9
CO_2	Transgen[3]	B2	Stress	Extracellular superoxide dismutase (ecSOD)	↑	1.9
CO_2	Transgen[3]	B2	Stress	Peroxiredoxin 6	↑	1.4
CO_2	Transgen[3]	B2	Protein regulation	Peptidylprolyl isomerase A (PPIA)	↑	2.0
CO_2	Transgen[3]	B2	Protein regulation	Ribosomal protein RL27	↑	2.8
Heat	Single	B2	Metabolism	Enolase (ENO)	↓	0.4
Heat	Single	B2	Stress	Dual oxidase	↓	0.4
Heat	Single	B2	Stress	Heat shock 70 kDa protein 12a (Hsp70a)	↓	0.4
Heat	Single	B2	Stress	Heat shock 70 kDa protein 12b (Hsp70b)	↓	0.5
Heat	Single	B2	Stress	Cytochrome P450	↑	3.9
Heat	Single	B2	Stress	Protein disulfide isomerase (PDI)	↑	3.9
Heat	Single	B2	Stress	Stress-induced protein 1	↑	2.3

Stress	No. exposures	Popula-tion	Functional group/ Cellular process[1]	Gene or Protein	Direction	FC
Heat	Single	B2	Nucleic acid regulation	Uracil-DNA glycosylase (UDG)	↓	0.4
Heat	Single	B2	Nucleic acid regulation	Histone H4	↑	2.2
Heat	Single	B2	Cell cycle	Rho GDP dissociation inhibitor 1 (Rho-GDI)	↓	0.5
Heat	Single	B2	Cytoskeleton	Actin	↓	0.5
Heat	Single	B2	Immune response	Hemagglutinin/amebocyte aggregation factor (HAAF)	↑	1.6
CO_2 + Heat	Single	B2	Stress	Multidrug resistance protein 1	↓	0.6
CO_2 + Heat	Single	B2	Stress	Stress-induced protein 1	↑	1.9
CO_2 + Heat	Single	B2	Stress	Extracellular superoxide dismutase (ecSOD)	↑	1.9
CO_2 + Heat	Single	B2	Stress	Cytochrome P450	↑	3.3
CO_2 + Heat	Single	B2	Protein regulation	Kelch 3	↑	1.6
CO_2 + Heat	Single	B2	Cell cycle	von Willebrand factor D and EGF domain-containing protein (VWF)	↓	0.3
CO_2 + Heat	Single	B2	Cell cycle	Rho GDP dissociation inhibitor 1 (Rho-GDI)	↓	0.7
CO_2 + Heat	Single	B2	Cell cycle	Charged multivesicular body protein 4 (CHMP4)	↑	2.0
CO_2 + Heat	Single	B2	Cytoskeleton	Actin	↓	0.4
CO_2 + Heat	Single	B2	Communication	IgGFc-binding protein	↓	0.6
CO2 + Heat	Single	B2	Immune response	Perlucin	↓	0.6

[1] Metabolism includes metabolic and catabolic processes, oxidative and reductive reactions, glycolysis and lipid metabolism. Stress includes response to biotic and abiotic stimuli, and antioxidant response. Protein regulation includes protein synthesis, modification, folding, phosphorylation and dephosphorylation, polymerisation, targeting and trafficking. Nucleic acid regulation includes transcription, DNA replication and other DNA- and RNA-specific processes. Cell cycle includes regulation of cell division, cell growth and differentiation, cell migration, cell death (apoptosis and autophagy) and maintenance of cellular homeostasis. Communication includes cell signalling, cell adhesion and cell-cell interactions..

APPENDIX

OTHER RELEVANT PUBLICATIONS DURING CANDIDATURE

Outreach:

Goncalves P (2016) Understanding climate change impacts on oysters. *The Malacological Society of Australasia Newsletter* **156**, 1-3.

Goncalves P (2014) Integrating cell biology and climate change: Can oysters adapt to ocean acidification? *The Malacological Society of Australasia Newsletter* **150**, 1-3.

Peer-reviewed:

Goncalves P, Guertler C, Bachère E, de Souza CR, Rosa RD, *et al*. (2014) Molecular signatures at imminent death: Hemocyte gene expression profiling of shrimp succumbing to viral and fungal infections. *Developmental & Comparative Immunology* **42**, 294-301.

Rosa RD, Vergnes A, de Lorgeril J, **Goncalves P**, Perazzolo LM, *et al*. (2013) Functional divergence in shrimp anti-lipopolysaccharide factors (ALFs): From recognition of cell wall components to antimicrobial activity. *PLoS One* **8**, e67937.