LIVING COSTS OF OCEAN ACIDIFICATION AND WARMING IN HERBIVOROUS GASTROPODS AND THEIR ADAPTATIONS

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Cover image: *Nerita atramentosa* (black) and *Austrocochlea concamerata* (black with white stripes). Photo credit: Jonathan Yu Sing Leung

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ABSTRACT

Over the last century, atmospheric concentration of carbon dioxide (pCO_2) has been increasing at an unprecedented rate due to anthropogenic CO_2 emission. The elevated pCO_2 is predicted to cause substantial abiotic changes in future marine ecosystems, including ocean acidification and warming. In addition, extreme climatic events, such as heatwaves, will become more prevalent and persistent due to global warming. Thus, extensive studies have been conducted to determine how ocean acidification and warming affect marine organisms. It is generally considered that these climate change stressors will cause adverse effects on many marine organisms and hence disrupt ecosystem functioning in future. This prediction is, however, largely based on short-term experiments with simple experimental design that may have overestimated the impacts of climate change stressors. In fact, growing evidence shows that some marine organisms can acclimate to the predicted seawater conditions. Therefore, this thesis aims to examine the impacts of ocean acidification and warming on marine organisms and their potential adaptations. Herbivorous gastropods from intertidal to subtidal zones were chosen as the study animals in view of their substantial contribution to herbivory in their habitat.

Ocean acidification and warming can raise the energy demand of marine organisms, impacting their energy budget and ultimately survival. After a prolonged exposure period, I found that ocean acidification has limited effect on the energy budget and survival of subtidal gastropods (*Thalotia conica* and *Phasianella australis*), suggesting that they are able to cope with the elevated energy demand under ocean acidification. This response can be mediated indirectly by the positive effect of CO_2 enrichment on the nutritional quality (energy content and C:N ratio) of primary producers, which in turn boosts the energy gain of gastropods. In contrast, I found that ocean warming reduces the energy budget, growth and survival of these subtidal gastropods at temperature below their thermal tolerance, implying that prolonged exposure to sublethal thermal stress (e.g. persistent heatwaves) can already threaten their populations. While ocean acidification and warming enhanced the nutritional quality of primary producers, such indirect positive effect was shown to be outweighed by their combined direct negative effects on the energy budget of gastropods, culminating in energy depletion and mortality. Compared to subtidal gastropods, intertidal gastropods are considered to be more resistant to ocean acidification and warming in view of the large environmental fluctuations in their habitat, which make them have greater acclimation capacity through behavioural and physiological adaptations. Yet, their fitness and survival may still be challenged by the abrupt increase in temperature to an extreme level during heatwaves. I showed that intertidal gastropods (*Nerita*

atramentosa, *Austrocochlea concamerata* and *Austrocochlea constricta*) are able to exhibit adaptive behaviour (e.g. hiding) during heatwaves so that their body temperature can be maintained within their survivable range. Furthermore, their molecular defence mechanisms (e.g. production of heat shock proteins and antioxidative enzymes) were found to be activated to alleviate the impacts of heatwaves so that they can resist (*N. atramentosa*) or recover (*A. concamerata*) from thermal stress. Since heatwaves are mostly transient, these adaptive behavioural and physiological responses probably allow intertidal gastropods to maintain their populations and ecological functions in their habitat.

Calcification is predicted to be hindered by ocean acidification due to the reduced pH and carbonate saturation state of seawater, which can weaken shell mechanical strength. Contrary to this prediction, I found that ocean acidification has limited effect on the shell hardness of both intertidal (*N. atramentosa* and *A. constricta*) and subtidal gastropods (*Austrocochlea odontis*, *Turbo undulatus*, *Bulla quoyii*, *T. conica* and *P. australis*). Instead, ocean warming resulted in the production of softer shells in some subtidal gastropods (*T. undulatus*, *B. quoyii* and *P. australis*). Moreover, all the tested subtidal gastropods produced aragonite as the only carbonate mineral, which is more soluble than calcite. In contrast, a bimineralic intertidal gastropod (*A. constricta*) could reduce shell solubility by increasing precipitation of calcite under ocean acidification, which also minimizes the energy cost of calcification and allows faster shell growth. This finding suggests that calcification is primarily governed by energy budget rather than seawater carbonate chemistry. Overall, subtidal gastropods would be more vulnerable than intertidal gastropods to physical damage and shell dissolution in future marine ecosystems.

In conclusion, I demonstrated both positive and negative responses of herbivorous gastropods to ocean acidification and warming, where warming could trigger detrimental effects on the energy budget, calcification and survival of subtidal gastropods. In contrast, intertidal gastropods appeared to be robust to ocean acidification and warming, probably attributed to their adaptive responses (e.g. behavioural and physiological adaptations). Based on the findings in this thesis, the populations of subtidal gastropods and their ecological contributions would be undermined by climate change stressors, possibly leading to serious repercussions on the future subtidal environment, such as disrupted trophic dynamics and modifications in habitat structures.

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Chapter 2

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INFLUENCE OF ANTHROPOGENIC CO_2 EMISSION ON MARINE ECOSYSTEMS

Atmospheric carbon dioxide (pCO_2) concentration was relatively stable, ranging from about 172 ppm to 300 ppm, in the last 800,000 years (Lüthi et al., 2008). Since the onset of Industrial Revolution in the 18th century, however, pCO_2 concentration has increased drastically from 280 ppm (pre-industrial) to 400 ppm (current) primarily due to intensified anthropogenic activities, especially combustion of fossil fuels (Caldeira and Wickett, 2005). As such, it is conjectured that pCO_2 concentration will increase to approximately 1000 ppm by the end of this century if anthropogenic CO_2 emission is not regulated (i.e. business-as-usual emission scenario) (IPCC, 2013). The accelerated increase in pCO_2 concentration in the last 250 years has already caused detectable environmental changes not only in terrestrial ecosystems, but also marine ecosystems which have drawn substantial attention over the last decade (Feely et al., 2004; Doney et al., 2009).

Since CO_2 is a greenhouse gas, the heat from solar radiation is partially absorbed by CO_2 and trapped in the atmosphere, causing global warming. Meanwhile, part of the heat in the atmosphere is transferred to oceans and hence elevates seawater temperature (i.e. ocean warming). In fact, sea surface temperature has increased by approximately 0.76°C over the last century and is predicted to further increase by 1.8 to 3.5°C by the end of this century, depending on the CO_2 emission scenario (Caldeira and Wickett, 2005; IPCC, 2013). Apart from this climatic trend, global warming also increases the occurrence of heatwave events, which can be defined as three or more consecutive days above the 90th percentile for maximum temperature (Perkins and Alexander, 2013). Heatwaves are extreme climatic events that receive special concern because they can abruptly and substantially increase sea surface temperature for a long period of time. For example, the recent heatwave event in Western Australian coast in 2011 increased the seawater temperature along coastlines by ~3°C on average above normal level for more than eight weeks (Wernberg et al., 2013). In view of global warming, it is forecasted that heatwaves will be more intense, frequent and persistent in future (Meehl and Tebaldi, 2004; Perkins et al., 2012). Indeed, 71% of coastlines worldwide have already been warmed to some

extent, while 38% have experienced anomalously high seawater temperature due to extreme hot events (Lima and Wethey, 2012). Given the slow rate of change in seawater temperature by climatic trend (i.e. ocean warming), heatwaves would be more determining than ocean warming to influence marine ecosystems in future (Jentsch et al., 2007; Thompson et al., 2013).

In addition to ocean warming, elevated pCO_2 concentration can also modify seawater chemistry because CO_2 is a relatively soluble gas and thus approximately one third of atmospheric CO_2 is absorbed by oceans (Sabine et al., 2004). When atmospheric CO_2 is dissolved in seawater, carbonic acid (H_2CO_3) is formed, followed by dissociation into bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions by losing hydrogen ions (H^+), where bicarbonate and carbonate ions respectively account for about 90% and 9% of the inorganic carbon in seawater at pH 8.1 (Fabry et al., 2008; Doney et al., 2009). The elevated concentration of hydrogen ions leads to the reduction in seawater pH, known as ocean acidification. Since the pre-industrial time, seawater pH has dropped by 0.1 pH unit on average and is predicted to further decrease by approximately 0.3 pH units by the end of this century in the business-as-usual emission scenario (Caldeira and Wickett, 2005; IPCC, 2013). Given the pH reduction, ocean acidification leads to the ensuing reduction in the carbonate saturation state (Ω) of seawater (i.e. reduced concentration of carbonate ions) (Doney et al., 2009). Comparing the two most common carbonate minerals, aragonite has higher solubility than calcite and will become undersaturated ($\Omega < 1$) sooner than calcite under ocean acidification (Feely et al., 2004; Orr et al., 2005). In short, anthropogenic CO_2 emission will eventually result in ocean acidification and warming, which could disturb marine organisms and hence alter ecosystem functioning in the near future. Therefore, their potential impacts on marine ecosystems have received global attention in the last decade.

1.2 IMPACTS OF OCEAN ACIDIFICATION AND WARMING ON MARINE ORGANISMS

Ocean acidification and warming are regarded as climate change stressors to marine organisms because the altered seawater conditions can directly influence their physiology and behaviour (Fabry et al., 2008; Pörtner, 2008; Byrne, 2011). Based on meta-analyses, ocean acidification and warming could adversely affect the fitness and survival of marine organisms and hence modify the community structures and functioning of marine ecosystems in future

(Kroeker et al., 2013; Nagelkerken and Connell, 2015). In general, the direct negative effects are mainly associated with aerobic metabolism, energy homeostasis and calcification.

1.2.1 AEROBIC METABOLISM AND ENERGY HOMEOSTASIS

Aerobic metabolism is one of the most vital physiological processes (Brown et al., 2004), where metabolic energy is yielded to support cellular functions, maintenance, behaviour, growth and reproduction. However, this fundamental process could be directly impaired by ocean acidification and warming (Pörtner, 2008, 2012), thereby affecting energy homeostasis and eventually fitness and survival of marine organisms (Sokolova et al., 2012; Sokolova, 2013). Disturbance in the acid-base balance of extracellular fluid under ocean acidification is regarded as the principal mechanism influencing aerobic metabolism (Pörtner, 2008). Under acidified conditions, the pH of extracellular fluid is reduced after equilibrating with the external environment and thus marine organisms have to regulate their extracellular pH back to the normal level (e.g. via ion exchange, bicarbonate accumulation or buffering of intracellular and extracellular fluid) (Pörtner, 2008). The capacity for acid-base regulation is species-specific. In general, less active organisms (e.g. sipunculids, molluscs and echinoderms) with an open circulatory system have lower capacity for acid-base regulation because they have lower metabolic rate and larger volume of extracellular fluid, resulting in higher vulnerability to hypercapnia (Pörtner, 2008). When the change in extracellular pH is not compensated, adverse effects would be triggered, such as metabolic depression, reduced protein synthesis, impaired locomotory activity and hindered growth (Reipschläger et al., 1997; Michaelidis et al., 2005; Langenbuch et al., 2006; Fabry et al., 2008). Even if the change in extracellular pH is fully compensated, substantial metabolic energy is required for acid-base regulation (Pörtner, 2008). Given the elevated energy demand for acid-base regulation and reduced metabolic energy due to metabolic depression, energy budget would be reduced under ocean acidification, meaning that less energy can be allocated to growth and reproduction (Nisbet et al., 2012; Applebaum et al., 2014). In the long term, fitness and survival would be compromised due to the energy trade-offs (Wood et al., 2010; Sokolova et al., 2012; Calosi et al., 2013; Turner et al., 2015).

Ocean warming can also influence aerobic metabolism, which is a temperature-dependent process. How marine organisms are affected by the elevated temperature under ocean warming can be determined by their aerobic scope, defined as the excess metabolic energy available after basal metabolic cost is met (Sokolova, 2013). In general, marine

organisms can gain benefit from ocean warming when their aerobic scope is elevated (i.e. greater energy budget), and *vice versa*. Based on the oxygen- and capacity-limited thermal tolerance (OCLTT) concept (Pörtner, 2012), aerobic metabolism should increase with temperature to yield more metabolic energy so that the elevated energy demand for cellular functions and maintenance at higher temperature is met. When aerobic metabolism can no longer increase with temperature, aerobic scope decreases due to a mismatch between energy demand and energy supply. This can eventually diminish the fitness and survival of organisms, depending on the severity of thermal stress. If the reduction in aerobic scope is substantial, physiological and behavioural performance will be immediately weakened, such as loss of motor coordination (Lutterschmidt and Hutchison, 1997). In this situation, survival becomes time-limited as the metabolic energy produced by aerobic metabolism is inadequate to support fundamental cellular functions and activities (Pörtner, 2002, 2012). Moreover, energy gain by feeding is reduced and thus energy reserves need to be consumed as the temporary energy source (Ivanina et al., 2013; Sokolova, 2013). Provided energy deficit persists in the long term, energy reserves will be depleted, ultimately leading to mortality. Even though acute thermal stress is not triggered by the elevated temperature under ocean warming, marine organisms can still be impacted by sublethal thermal stress because enormous energy is required for somatic maintenance (Somero, 2002, 2010). For example, production of heat shock proteins, which is energy-costly, is induced by sublethal thermal stress in order to repair damaged proteins and stabilize denaturing proteins (Miller et al., 2009; Tomanek, 2010). While survival is sustained, less energy can be allocated to growth and reproduction, ultimately affecting population maintenance. In this regard, studying thermal tolerance of marine organisms can provide an initial insight into their aerobic scope and energy budget under ocean warming.

Importantly, both ocean acidification and warming are caused by elevated pCO_2 concentration, meaning that marine organisms in future will be subject to their combined effects, rather than isolated effects. Based on previous studies, it is generally recognized that ocean acidification and warming in combination can exacerbate their adverse effects (Kroeker et al., 2013). In particular, thermal tolerance can be reduced by hypercapnia as observed in some marine organisms (e.g. Metzger et al., 2007; Lannig et al., 2010; Lesser, 2016), suggesting their increased susceptibility to ocean warming. As such, aerobic metabolism will be impaired and energy budget will further decrease, possibly leading to reduced growth and survival (Sokolova et al., 2012; Sokolova, 2013). For example, the metabolic rate and activity level of jumbo squid *Dosidicus gigas* are reduced by the combined effects of ocean

acidification and warming (Rosa and Seibel, 2008); the reduced growth of sea star *Asterias rubens* under ocean acidification is further exaggerated by elevated temperature (Keppel et al., 2015); mortality of corals increases drastically under combined ocean acidification and warming probably due to their worsened cellular performance (Prada et al., 2017). Therefore, ocean acidification and warming should be studied together to provide a more realistic prediction for their impacts on marine organisms.

1.2.2 CALCIFICATION

Most marine organisms (e.g. corals, gastropods, bivalves and sea urchins) produce calcareous shells as the primary structure for defence against predators, harmful substances and adverse environmental conditions, suggesting that calcification is crucial for their survival. However, ocean acidification may compromise their ability to build calcareous shells in view of the reduced carbonate saturation state, where shell dissolution may occur if carbonate saturation state is lower than 1 (Fabry et al., 2008; Doney et al., 2009). Alarmingly, aragonite is predicted to become undersaturated in the entire Antarctic Ocean and subarctic Pacific Ocean by the year 2100 in the business-as-usual emission scenario (Orr et al., 2005), implying that the survival of aragonite-producing organisms would be seriously threatened in the near future (Feely et al., 2004). Previous studies generally demonstrate retarded shell growth under ocean acidification across marine taxa (Fabry et al., 2008; Pörtner, 2008; Doney et al., 2009). In addition, thinner and softer shells are produced (Shirayama and Thornton, 2005; Ivanina et al., 2013; Melatunan et al., 2013), indicating impaired calcification. One of the processes affecting shell growth and shell properties is crystallization of amorphous calcium carbonate (Fitzer et al., 2014, 2016), which occurs at the early stage of calcification. Amorphous calcium carbonate, the precursor of crystalline calcium carbonate, is unstable and highly soluble (Addadi et al., 2006); therefore, acidified seawater may render crystallization more difficult to occur (Fitzer et al., 2016). It is noteworthy that calcification is a biological process, meaning that shell growth and shell properties are also governed by physiology (Pörtner, 2008; Roleda et al., 2012). For example, crystallization of amorphous calcium carbonate may be hampered under ocean acidification due to the limited capacity of organisms to regulate intracellular and extracellular ion composition (Hüning et al., 2013); shell strength is weakened under acidified conditions possibly due to the reduced production of organic matrix occluded in the shell (Addadi et al., 2006; Marin et al., 2008); reduced shell growth may be mediated by the elevated

energy cost of calcification under ocean acidification due to reduced carbonate saturation state (Waldbusser et al., 2013; Thomsen et al., 2015). Apart from ocean acidification, shell growth and shell properties may be further modified by ocean warming as temperature plays an important role in physiology (Burton and Walter, 1987; Balthasar and Cusack, 2015). For instance, calcification of corals was retarded by both ocean acidification and warming (Horvath et al., 2016; Prada et al., 2017); shell hardness of bivalves was markedly reduced under combined elevated pCO_2 and temperature conditions (Ivanina et al., 2013). If calcification is impacted by ocean acidification and warming, the functionality of shells would be worsened (Fitzer et al., 2014, 2015), probably diminishing the survival of calcifying organisms. Indeed, the secular oscillations of seawater pH and temperature have caused mass mortality of calcifying organisms when production of their calcareous structures is not favoured by the seawater chemistry (Hautmann, 2006; Zhuravlev and Wood, 2009).

1.3 POTENTIAL MECHANISMS TO COUNTER THE IMPACTS OF OCEAN ACIDIFICATION AND WARMING

Based on the extensive studies in the last decade, it is generally recognized that ocean acidification and warming would threaten marine organisms and have serious repercussions on marine ecosystems in future (Kroeker et al., 2013; Nagelkerken and Connell, 2015). This pessimistic prediction is, however, predominantly based on short-term studies with simple experimental designs, which may have limited predictability in the real situation. Indeed, the temporal change in seawater chemistry is very slow so that marine organisms may be able to accommodate the impacts of climate change stressors and thus sustain their populations. Acclimation can be achieved by modifying physiology or behaviour (i.e. phenotypic plasticity) to maintain homeostasis under new environmental conditions. Apart from the direct effects, ocean acidification and warming can indirectly affect marine organisms by altering species interaction (e.g. plant-herbivore interaction) (O'Connor, 2009; Poore et al., 2013; Boyd and Brown, 2015), which may further complicate the ecological consequences. To date, how ocean acidification and warming affect species interaction remains largely unexplored, but it may be the overarching factor determining the fitness and survival of marine organisms in future.

1.3.1 PHENOTYPIC PLASTICITY OF MARINE ORGANISMS

Since maintaining energy surplus is the key for long-term survival (Sokolova et al., 2012; Sokolova, 2013), marine organisms may counter the elevated energy demand under ocean acidification and warming by altering their physiology or behaviour. For example, they can increase feeding rate to boost energy gain, which may outweigh the adverse effects of environmental stressors. Such compensatory response is exhibited in a variety of marine organisms, such as copepods (Li and Gao, 2012), sea stars (Gooding et al., 2009) and gastropods (Ghedini and Connell, 2016; McSkimming et al., 2016). In addition, aerobic metabolism can be upregulated to offset the additional energy demand under ocean acidification and warming (Wood et al., 2008; Li and Gao, 2012; Turner et al., 2015). This mechanism is particularly important for activating defence mechanism against short-term environmental stress. For example, production of heat shock proteins and antioxidative enzymes can be upregulated in response to short-term thermal stress (Lesser, 2006; Tomanek, 2010). Nevertheless, increasing aerobic metabolism indicates greater energy expenditure, which may not be adaptive in the long term. Thus, some marine organisms lower metabolic rate to minimize energy expenditure in order to persist under thermal stress (Marshall and McQuaid, 2011), or acidified conditions (Calosi et al., 2013). For instance, the energy gain of intertidal gastropod *Echinolittorina malaccana* becomes limited at high body temperature (35 – 46°C) and thus it downregulates aerobic metabolism to conserve energy (Marshall et al., 2011). Regardless of strategies, physiological plasticity (i.e. capacity of organisms to adaptively change their physiology in response to altered environmental conditions) may enable marine organisms to maintain their fitness and survival under ocean acidification and warming.

Given the reduced carbonate saturation state of acidified seawater, energy cost of calcification will increase (Waldbusser et al., 2013; Thomsen et al., 2015), possibly affecting shell growth and shell properties (Orr et al., 2005; Melzner et al., 2011; Melatunan et al., 2013). However, the response of calcifying organisms to ocean acidification has been shown to be more variable than previously predicted (Ries et al., 2009), implying that calcification may not be subject to carbonate saturation state. Growing evidence further reveals that many calcifying organisms can maintain normal shell growth under ocean acidification (e.g. Findlay et al., 2011; Rodolfo-Metalpa et al., 2011; Garilli et al., 2015; Ramajo et al., 2016). Indeed, most calcifying organisms do not directly utilize carbonate ions in seawater as the substrate for calcification (Ries, 2011; Roleda et al., 2012). Physiologically, calcification takes place in an isolated fluid compartment with alkaline conditions mediated by ion transport across epithelia (Pörtner, 2008). Thus, calcifying organisms have to use energy to regulate ion composition (especially

bicarbonate and calcium ions, rather than carbonate ions) in the compartment through proton pumps so that an optimal condition for calcification is obtained (Carre et al., 2006; Roleda et al., 2012). In other words, shell growth can be maintained under ocean acidification as long as adequate energy is allocated to calcification. Energy cost of calcification also depends on the quality of shells produced; therefore, changing mineralogical properties (e.g. carbonate polymorphs and magnesium content in calcite) may reduce the energy cost of calcification (Weiner and Addadi, 1997; Bentov and Erez, 2006). Such mineralogical plasticity, which also modifies the physicochemical properties of shells, may be fundamental to minimizing the impacts of ocean acidification on shell growth and accounting for the inconsistent responses of calcifying organisms to ocean acidification (Ries et al., 2009). A comprehensive investigation is required to test whether this potential compensatory mechanism can counter the impacts of ocean acidification on calcification.

1.3.2 INDIRECT POSITIVE EFFECTS THROUGH TROPHIC TRANSFER

Ocean acidification and warming not only influence marine organisms directly through physiological pathways, but also indirectly through modification in species interaction, especially plant-herbivore interaction (O'Connor, 2009; Connell et al., 2013; Poore et al., 2013). Indeed, CO_2 can act as a resource for primary producers which can gain benefit from CO_2 enrichment and hence indirectly favour herbivores (Connell et al., 2013). For example, the growth of primary producers can be facilitated by elevated pCO_2 and temperature (Connell and Russell, 2010; Connell et al., 2017), thereby increasing food availability to herbivores. More importantly, the nutritional quality of primary producers (e.g. energy and nitrogen contents) can also be modified by pCO_2 and temperature (Raven and Geider, 1988; Falkenberg et al., 2013; Ghedini and Connell, 2016), possibly leading to ensuing changes in feeding rate and eventually fitness of herbivores (Cruz-Rivera and Hay, 2001; Hemmi and Jormalainen, 2002). For example, herbivores preferentially feed on the food with lower C:N ratio because nitrogen is usually limited in their diet (Mattson, 1980; Falkenberg et al., 2013; Ghedini and Connell, 2016). Enhanced feeding rate on the more nutritious food also results in greater energy gain, which may compensate for the elevated energy demand under ocean acidification and warming. Such indirect effects of ocean acidification and warming on herbivores are difficult to predict and often lead to unexpected outcomes due to their complex interactive effects on primary producers (Connell et al., 2013), that could outweigh their direct negative effects on herbivores (Kamya et al., 2017) and even boost their populations (Connell et al., 2017; Goldenberg et al.,

2017). To date, research on how ocean acidification and warming indirectly affect marine organisms through trophic transfer is in its infancy and requires more attention.

1.4 THESIS SCOPE AND OUTLINE

In this thesis, the impacts of ocean acidification and warming on the fitness and survival of marine organisms are studied using grazing gastropods, which have important ecological roles in their habitats, such as maintenance of ecosystem health and nutrient dynamics (Poore et al., 2012; Ghedini et al., 2015). Additionally, grazing gastropods have a greater contribution to herbivory than other marine herbivores, especially in intertidal and subtidal rocky reefs (Poore et al., 2012). The impacts of ocean acidification and warming on gastropods are assessed using a bioenergetics-based approach, which allows to compare the effects of environmental stressors and evaluate their potential synergy (Sokolova et al., 2012; Sokolova, 2013). On the other hand, their impacts on calcification are assessed by analysing the shell properties of gastropods. Then, the potential mechanisms to counter the impacts of climate change stressors are examined, including phenotypic plasticity of gastropods and trophic transfer from primary producers to gastropods. Experiments are conducted by simulating the natural environment and manipulating the seawater chemistry to the predicted future conditions so that the following objectives can be addressed:

1. To examine whether prolonged exposure to elevated pCO_2 and temperature can diminish the energy budget of gastropods, which leads to reduced growth, energy depletion and eventually mortality (Chapters 2 and 3).

2. To investigate whether ocean acidification and warming can boost the nutritional quality of primary producers, which in turn increases the feeding rate and energy gain of gastropods so that their fitness and survival can be maintained under future seawater conditions (Chapter 3).

3. To examine whether gastropods can counter the impacts of acute thermal stress by exhibiting adaptive physiological, molecular and behavioural responses, which may allow them to resist or recover from short-term heatwaves (Chapter 4).

4. To assess the impacts of ocean acidification and warming on the mechanical and geochemical properties of shells so that their functionality in future marine ecosystems is evaluated (Chapter 5).

5. To assess whether changing mineralogical properties can help compensate for the adverse effect of ocean acidification on shell growth (Chapter 6).

1.5 THESIS SUMMARY

The background information and objectives of each chapter are summarized below. Chapters 2 – 6 have been written in the form of an individual scientific manuscript and follow journal style and format. Chapters 2, 5 and 6 have been published in journals. A list of co-authors and their contributions to these chapters have been shown in the statement of authorship.

Chapter 2

Heatwaves are predicted to be more frequent and persistent in future (Meehl and Tebaldi, 2004). The sudden increase in seawater temperature due to heatwaves has been shown to jeopardize a variety of marine organisms (Wernberg et al., 2013). Ocean acidification may exaggerate the devastating impacts of heatwaves on subtidal organisms by reducing their energy budget and thermal tolerance. Therefore, the impacts of prolonged exposure to heatwaves and ocean acidification on energy budget are examined by measuring the scope for growth of a subtidal gastropod, where respiration rate, absorption rate and excretion rate are quantified. The energy budget is then linked to the body condition, growth and survival of gastropods to elucidate the impacts of elevated pCO_2 and temperature.

Chapter 3

Ocean acidification and warming may modify plant-herbivore interaction by changing the properties of primary producers (Falkenberg et al., 2013; Poore et al., 2013; Boyd and Brown, 2015). As such, herbivores may indirectly gain benefit via consumption of more nutritious food, which possibly offsets the direct negative effects of ocean acidification and warming on energy budget so that fitness and survival can be sustained. In this chapter, therefore, the energy content and C:N ratio of turf algae growing under ocean acidification and warming are analysed. The feeding rate of a subtidal gastropod is then examined to see if the nutritional quality of turf algae can alter its feeding behaviour. To integrate direct and indirect effects, energy budget is estimated to correlate with the body condition, growth and survival of gastropods.

Chapter 4

Intertidal organisms are considered to be particularly susceptible to heatwaves because many of them are living close to their thermal limit (Somero, 2010). However, the temperature profile of intertidal environment is highly heterogeneous so that intertidal organisms can avoid thermal stress by hiding. In addition, they may counter the transient impacts of thermal stress by activating molecular defence mechanisms (Lesser, 2006; Tomanek, 2010), which enable them to resist or recover from heatwaves. In this chapter, the impacts of heatwaves on three intertidal gastropods are examined by integrating physiological (feeding rate and respiration rate), molecular (heat shock proteins and antioxidative capacity) and behavioural (habitat preference) responses. The recoverability of gastropods from heatwaves is then evaluated.

Chapter 5

Shell properties, such as solubility and mechanical strength, are highly associated with the survival of calcifying organisms (Hautmann, 2006; Zhuravlev and Wood, 2009). These properties are subject to the calcifying process, which can be indicated by the mechanical and geochemical properties of shells. Calcifying organisms may be able to modify these properties in response to ocean acidification and warming so that the functionality of their shells can be maintained. As such, the mechanical and geochemical properties of shells are analysed. A total of seven species collected from intertidal to subtidal zones are examined. Given the greater environmental fluctuations in the intertidal zone, it is hypothesized that the shell properties of intertidal gastropods are more plastic and less susceptible than those of subtidal gastropods to the impacts of ocean acidification and warming.

Chapter 6

Energy cost of calcification is elevated by ocean acidification due to the kinetic constraints, such as reduced carbonate saturation state (Waldbusser et al., 2013), possibly retarding shell growth and even somatic growth. Yet, calcifying organisms may alter mineralogical properties of shells to reduce the energy cost of calcification and make their shells functionally suitable under ocean acidification so that their fitness can be maintained. To test this hypothesis, the aerobic metabolism, feeding performance, body condition, shell growth and shell properties of an intertidal gastropod are examined under ocean acidification.

Chapter 7

The key findings in the previous chapters are reviewed and generally discussed, whereas the potential research gaps are suggested for future investigation.

1.6 REFERENCES

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Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Heatwaves diminish the survival of a subtidal gastropod through reduction in energy budget and depletion of energy reserves

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Extreme climatic events, such as heatwaves, are predicted to be more prevalent in future due to global climate change. The devastating impacts of heatwaves on the survival of marine organisms may be further intensified by ocean acidification. Here, we tested the hypothesis that prolonged exposure to heatwave temperatures (24 °C, +3 °C summer seawater temperature) would diminish energy budget, body condition and ultimately survival of a subtidal gastropod (*Thalotia conica*) by pushing close to its critical thermal maximum (CT_{max}). We also tested whether ocean acidification (pCO_2: 1000 ppm) affects energy budget, CT_{max} and hence survival of this gastropod. Following the 8-week experimental period, mortality was markedly higher at 24 °C irrespective of pCO_2 level, probably attributed to energy deficit (negative scope for growth) and concomitant depletion of energy reserves (reduced organ weight to flesh weight ratio). CT_{max} of *T. conica* appeared at 27 °C and was unaffected by ocean acidification. Our findings imply that prolonged exposure to heatwaves can compromise the survival of marine organisms below CT_{max} via disruption in energy homeostasis, which possibly explains their mass mortality in the past heatwave events. Therefore, heatwaves would have more profound effects than ocean acidification on future marine ecosystems.

Since the onset of Industrial Revolution, atmospheric carbon dioxide (pCO_2) concentration has been increasing rapidly and caused observable changes in marine ecosystems. For example, sea surface temperature increased by ~0.76 °C in the last century and is predicted to increase by 1.8–3.5 °C by the end of this century, depending on the CO_2 emission scenario[1]. The increase in pCO_2 is also leading to ocean acidification (OA), which is expected to have profound repercussions on a variety of marine organisms[2,3]. Indeed, meta-analyses suggest that OA and warming will likely modify community structures and functions of future marine ecosystems[4,5].

Given the slow rate of change in seawater chemistry, however, growing evidence shows that marine organisms may be able to acclimate to the predicted climatic conditions[6–8]. As such, it has been suggested that extreme climatic events, rather than climatic trends, will lead to more significant changes in future marine ecosystems due to their instant and catastrophic effects[9]. Heatwaves, one of the most devastating extreme climatic events, are of special concern because they can abruptly increase the sea surface temperature along coastlines by ~3 °C on average and persist for more than two months, causing substantial changes in marine ecosystems[10–12]. In view of global warming, it is conjectured that heatwaves will be more frequent and persistent in future[9,13]. Indeed, 71% of coastlines worldwide have already been warmed to some extent, while 38% have experienced anomalously high seawater temperature due to extreme hot events[14].

Heatwaves can greatly influence the survival and distribution of marine organisms, and hence functions of marine ecosystems[11,12]. Cool-affinity species with low mobility are particularly vulnerable due to their limited ability to escape from thermal stress, while heatwaves favour the proliferation of warm-affinity species[12]. This implies that thermal stress is the key factor causing the mass mortality in the past heatwave events. Therefore, studying thermal tolerance of marine organisms can provide an initial insight into how heatwaves affect their fitness and survival[15]. Nevertheless, thermal tolerance could be modulated by OA through disruption in aerobic

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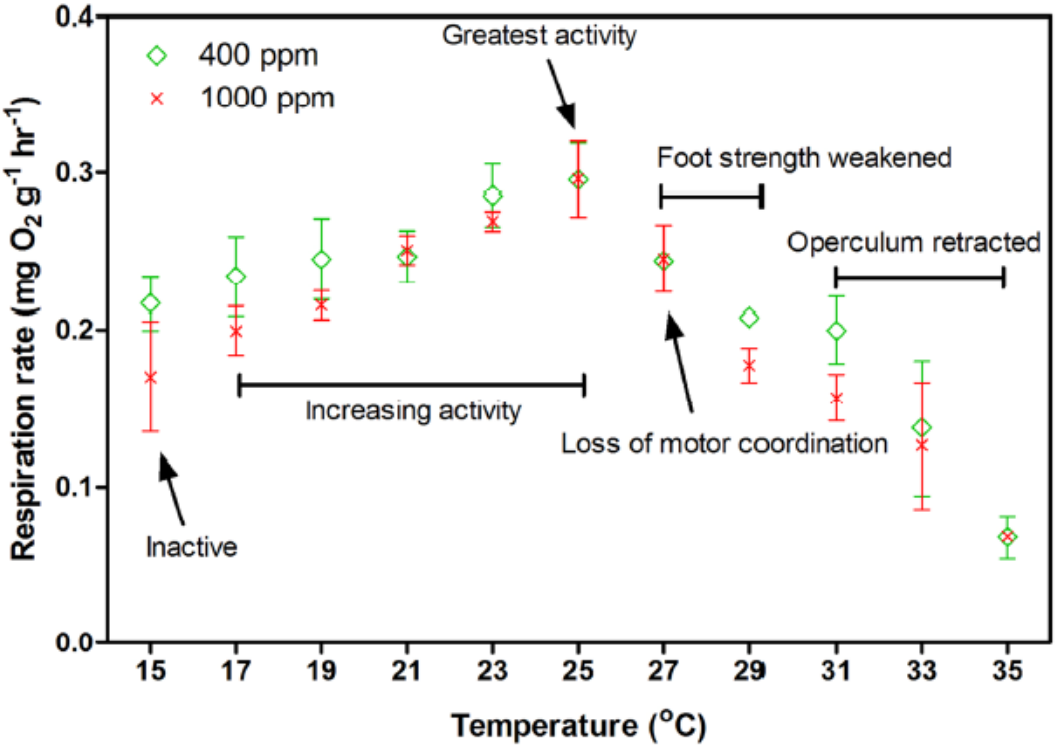

Figure 1. The respiration rate of *Thalotia conica* along an increasing temperature ramp (+2 °C hr^{-1}) from 15 °C to 35 °C at different pCO_2 levels (mean ± S.E.; $n = 3$).

performance or energy homeostasis[16,17], possibly increasing the susceptibility of marine organisms to heatwaves. Indeed, marine organisms are impacted by environmental stressors commonly via reduction in aerobic scope, defined as the excess metabolic energy available after basal metabolic cost is met[18]. As such, studying bioenergetics can shed light on the combined effects of multiple stressors on the performance, fitness and survival of marine organisms[18]. If heatwaves or OA causes energy deficit (i.e. negative energy budget) in the long term, energy reserves would be consumed or even depleted, eventually leading to mortality.

Here, we examined the effects of prolonged exposure to elevated pCO_2 and temperature on the energy budget, body condition and survival of a subtidal grazing gastropod *Thalotia conica*, which is widely distributed in temperate Australia. Given its high abundance, this seagrass-associated gastropod plays a crucial role in energy flow in the seagrass community by regulating the abundance of epiphytes on seagrass leaves[19], which can enhance the survival of seagrasses[20]. We first assessed the performance of aerobic metabolism of *T. conica* along a temperature gradient and determined its critical thermal maximum (CT_{max}), at which motor coordination is lost[21], as a proxy for thermal tolerance. Then, we hypothesized that (1) prolonged exposure (8 weeks) to heatwave temperatures would lead to increased mortality of *T. conica* by diminishing its energy budget and body condition, when the temperature is close to CT_{max}; and (2) OA would reduce energy budget and thermal tolerance, resulting in greater mortality. Provided prolonged exposure to heatwaves diminishes the survival of this subtidal gastropod through energy depletion even below its thermal tolerance, this mechanism not only helps explain the mass mortality of marine organisms in the past heatwave events, but also suggests that persistent heatwaves are the main driver of the functions of both contemporary and future marine ecosystems.

Results

Performance of aerobic metabolism and CT_{max}. The respiration rate of *T. conica* gradually increased from 15 °C to 25 °C and then dropped from 25 °C to 35 °C (Fig. 1). The effect of pCO_2 on respiration rate was not discernible, with few exceptions at some temperatures (29 °C and 31 °C). Regarding locomotory behaviour, individuals were inactive at 15 °C, but became gradually more active as temperature increased with the greatest activity at 25 °C. At 27 °C, foot strength was weakened and motor coordination was lost. Beyond 29 °C, the operculum was retracted, indicating acute thermal stress. Based on the locomotory behaviour, the CT_{max} of *T. conica* appeared at 27 °C.

Energy budget. The ingestion rate, absorption rate and scope for growth of *T. conica* at 24 °C were lower than those at 21 °C irrespective of pCO_2 level (Fig. 2a,c and f; Table S1). Assimilation efficiency and excretion rate were not affected by both pCO_2 and temperature (Fig. 2b and e; Table S1). Respiration rate at 24 °C was lower than that at 21 °C when pCO_2 was at 1000 ppm (Fig. 2d; Table S1). Based on the patterns of energy budget parameters, absorption rate was the key factor determining scope for growth. Energy deficit was found at 24 °C, indicated by the negative scope for growth (Fig. 2f).

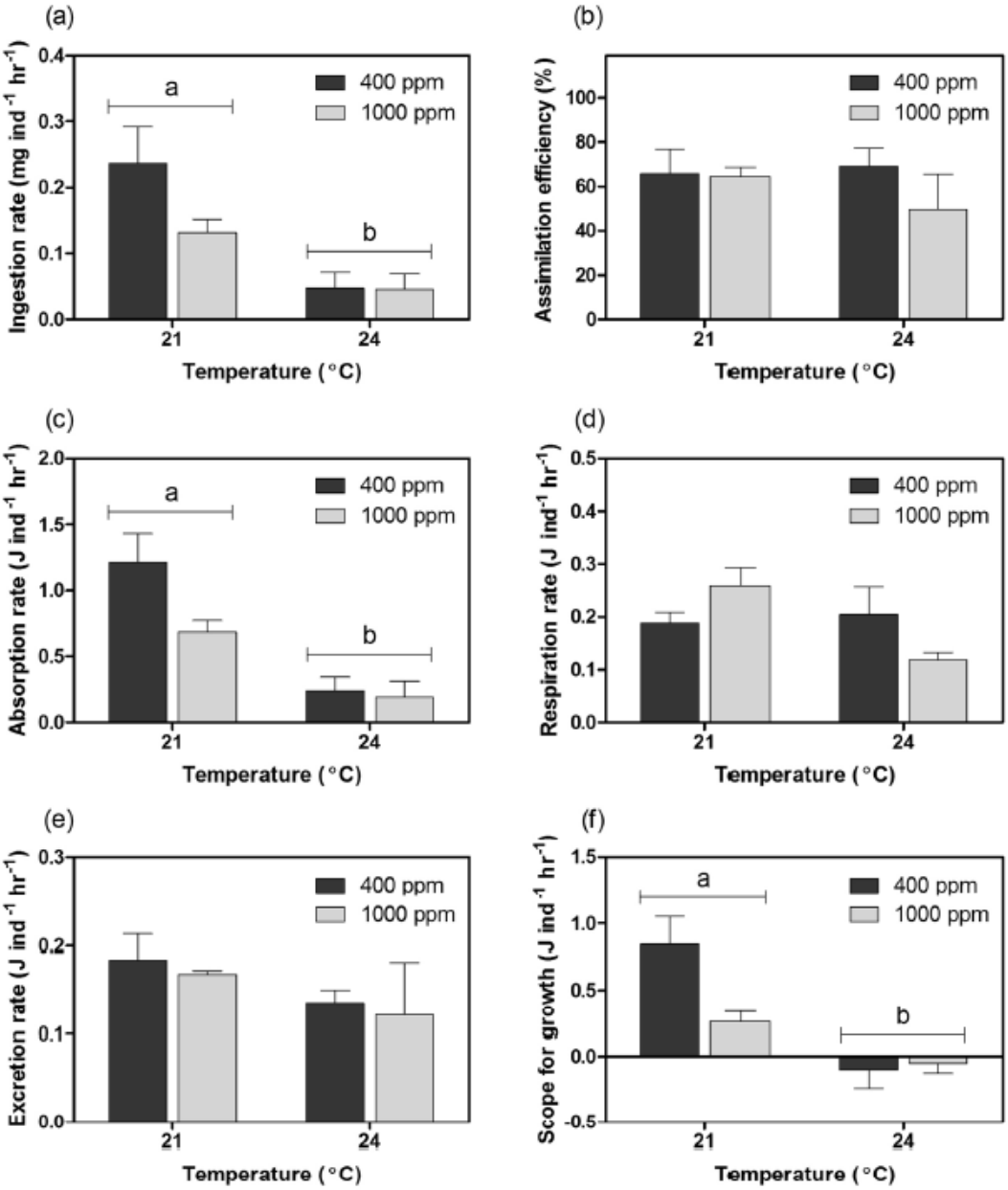

Figure 2. (a) Ingestion rate, (b) assimilation efficiency, (c) absorption rate, (d) respiration rate, (e) excretion rate and (f) scope for growth of *Thalotia conica* under different treatment conditions at the end of the experimental period (mean ± S.E.; $n = 3$). Different letters indicate significant difference between temperature groups ($p < 0.05$).

Body condition and survival. Following the 8-week experimental period, the total weight of *T. conica* increased at 21 °C, but decreased at 24 °C regardless of pCO_2 level (Fig. 3a; Table S1). Organ weight to flesh weight ratio did not change significantly at 21 °C, but decreased at 24 °C at both pCO_2 levels (Fig. 3b; Table S1), indicating the use of energy reserves. Mortality at 21 °C (~20%) was much lower than that at 24 °C (~70% at 400 ppm; ~80% at 1000 ppm) after the experimental period (Fig. 4; Table S1).

Discussion

Studying bioenergetics can help decipher the fitness and survival of marine organisms in response to multiple environmental stressors[18]. Contrary to theory[22], we revealed that OA did not significantly affect the thermal tolerance, energy budget, body condition and survival of a subtidal gastropod, even though the individuals had limited time to acclimate. The slightly reduced energy budget may be elicited by the extra energy required for the maintenance of acid-base balance and digestion efficiency under OA conditions and thus less energy can be allocated for foraging activity[22], resulting in reduced energy gain by feeding. In contrast, we found that prolonged exposure to heatwave temperature markedly reduces energy budget, body condition and eventually survival.

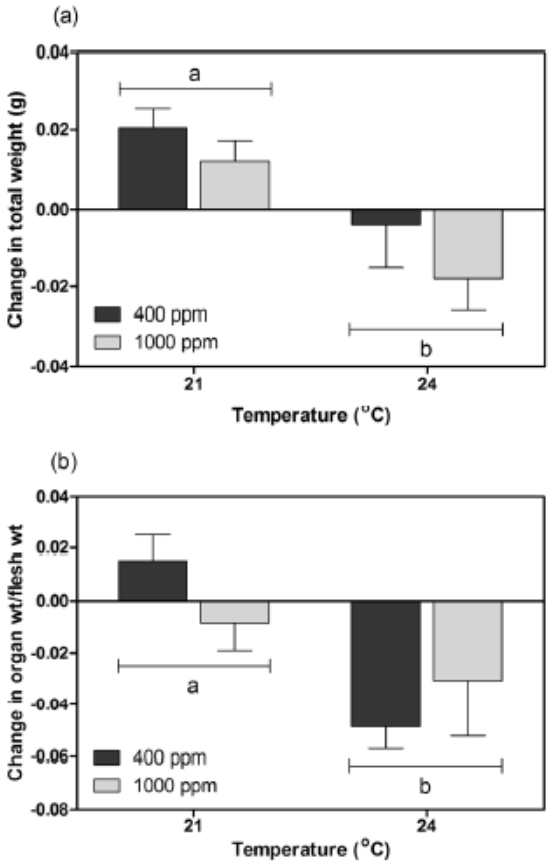

Figure 3. The change in (**a**) total weight and (**b**) organ weight to flesh weight ratio of *Thalotia conica* after the experimental period under different treatment conditions (mean + S.E.; $n = 22$ individuals for 21 °C, 400 ppm; $n = 21$ individuals for 21 °C, 1000 ppm; $n = 8$ individuals for 24 °C, 400 ppm; $n = 5$ individuals for 24 °C, 1000 ppm). Different letters indicate significant difference between temperature groups ($p < 0.05$).

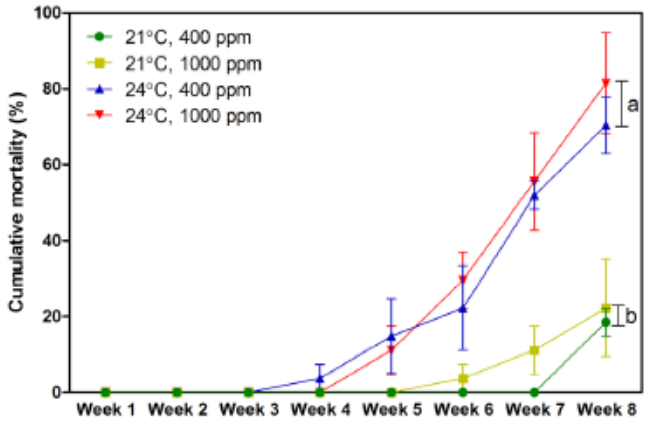

Figure 4. The cumulative mortality of *Thalotia conica* across the experimental period under different treatment conditions (mean ± S.E.; $n = 3$). Different letters indicate significant difference between temperature groups in Week 8 ($p < 0.05$).

Therefore, heatwaves can probably pose greater adverse consequences than OA on the fitness and survival of marine organisms.

The detrimental effect of heatwaves on organisms can be mediated by reducing their aerobic scope (or excess metabolic energy)[22]. In general, energy demand increases with temperature and thus aerobic respiration needs to be upregulated to produce more metabolic energy and meet the elevated energy demand so that energy homeostasis can be maintained. When metabolic energy produced by aerobic respiration is insufficient to meet the elevated energy demand, aerobic scope will decrease[18,23], which can be indicated when aerobic respiration starts

to decline with increasing temperature[24,25]. As such, the foot strength of *T. conica* starts to weaken and its equilibrium is lost at 27 °C (i.e. CT_{max}), where the respiration rate can no longer increase with temperature probably due to reduced capacity of ventilatory and circulatory systems to deliver oxygen to foot tissues[23,24]. Consequently, the excess metabolic energy would not be adequate to support normal physiological functions and locomotory activity, and hence survival becomes time-limited, depending on the tolerance of individuals to starvation and thermal stress[24]. To avoid the impact of acute thermal stress, therefore, *T. conica* maximized locomotory activity at 25 °C, potentially as an escape behaviour. Since the respiration rate at 24 °C (i.e. heatwave temperature) was very close to the maximum, *T. conica* would be under sublethal thermal stress, which can compromise fitness and survival in the long term via disruption in energy homeostasis[23,26].

As maintaining energy surplus is the key for long-term survival, bioenergetics-based models can be used to examine how environmental stressors affect the fitness and survival of marine organisms by changing their energy budget[18]. In this study, scope for growth was estimated to indicate energy budget, which was subject largely to absorption rate. We found that the heatwave temperature led to reduced absorption rate, which was in turn caused by reduced ingestion rate. The worsened feeding performance is likely attributed to the impact of sublethal thermal stress on aerobic metabolism. Based on the respiration rate along the temperature ramp, *T. conica* should have higher respiration rate at 24 °C than at 21 °C. After the experimental period, however, *T. conica* could not maintain higher respiration rate at 24 °C, which is likely caused by the reduced oxygen delivery capacity or impaired mitochondrial functions under long-term sublethal thermal stress[23,27,28]. As a result, energy production is suppressed, impairing various physiological functions and activities, including feeding[29,30]. Whether the reduced energy gain by feeding compromises growth and body condition depends on the net energy balance (i.e. scope for growth). We found that scope for growth is negative after the prolonged exposure to heatwave temperatures, indicating energy deficit which probably accounts for the loss of total weight. As shell weight is generally constant, the reduction in total weight plus negative energy budget suggest consumption of energy reserves, which is further substantiated by the reduced body condition (i.e. reduced organ weight to flesh weight ratio), indicating that energy supply was inadequate to support energy demand. Indeed, using energy reserves for basal maintenance becomes inevitable when energy deficit persists for a longer term. For instance, Ivanina *et al.*[31] demonstrated that prolonged exposure to elevated temperature can cause depletion of energy reserves in bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Yet, using energy reserves can only provide a temporary relief against environmental stressors and is considered as the last resort for survival. When energy reserves are depleted, physiological processes will be hindered, or even arrested, explaining the rapid increase in the mortality of *T. conica* after six weeks of exposure to the heatwave temperature.

To date, heatwaves have received less attention due to their historical rarity in nature, but they can pose destructive impacts on marine ecosystems, especially survival of marine organisms. For example, recent heatwave events have abruptly increased the seawater temperature along coastlines by ~3 °C on average above normal level for more than eight weeks and caused mass mortality of marine organisms with little sign of recovery, possibly culminating in irreversible damage to ecosystem functions (Northwest Mediterranean coast in 1999 and 2003[10,11,32]; Western Australian coast in 2011[12,33]). Although underlying mechanisms (e.g. development of pathogens and modified species interactions) have been postulated to account for the mass mortality[9,12,34], we suggest that persistent thermal stress is the overarching factor because it can directly impair aerobic metabolism, worsen feeding performance and eventually cause energy depletion[18,23]. Although subtidal organisms, especially in temperate regions, were shown to be resilient to elevated temperature because most of them are living below their thermal tolerance and have high acclimation capacity[35,36], we showed that the adverse effects of thermal stress can be manifested after prolonged exposure to temperature below thermal tolerance. As many subtidal organisms are not highly mobile or even sessile (e.g. corals, polychaetes, gastropods, bivalves, sea urchins, etc.), they are subject to the instant thermal stress caused by heatwaves and have limited time to respond, ultimately leading to mortality.

In the past decade, a plethora of studies have investigated the potential impacts of OA on future marine ecosystems. Despite the merits of these studies, it is still difficult and challenging to accurately predict the impacts of OA in view of the inconsistent results and complexity of marine ecosystems[4,8,37,38]. Given the slow rate of change in seawater chemistry, the acclimation capacity of marine organisms will further make the consequences of future marine ecosystems unpredictable. In contrast, extreme climatic events, such as heatwaves, can remarkably modify the functions of marine ecosystems in a relatively short period of time[11,12]. In view of the dominance and role of *T. conica*, our findings imply that the ecological functions of seagrass community would be substantially altered by heatwaves, whereas OA poses negligible effects in the near future[39,40].

Owing to global climate change, the frequency and persistence of heatwaves are predicted to increase in future, possibly jeopardizing the survival of marine organisms and modifying the functions of marine ecosystems. We revealed that prolonged exposure to heatwaves significantly diminishes the survival of a subtidal gastropod through reduction in energy budget and depletion of energy reserves, which are manifested at temperature below its thermal tolerance. This bioenergetics-based mechanism could help explain the mass mortality of marine organisms in past heatwave events and suggest that many subtidal organisms in temperate regions are vulnerable to persistent thermal stress, which would be the major driver affecting the integrity of both contemporary and future marine ecosystems.

Methods

Collection of gastropods and rearing conditions. Adult *Thalotia conica* were collected in summer from the subtidal region (0.5 to 4 m depth) at Wirrina Cove, South Australia (35°29′S, 138°15′E), where the seawater pH and temperature were similar to our laboratory control conditions at ~8.10 and 21 °C, respectively. Individuals were temporarily kept in a plastic holding aquarium (1 m × 40 cm × 30 cm) filled with natural seawater and allowed to acclimate under ambient conditions (pH: 8.10 ± 0.10, temperature: 21 ± 1 °C, salinity: 35 ± 1

psu and dissolved oxygen concentration: 6.9 ± 0.2 mg O_2 L^{-1}) with natural day/night cycles for two weeks prior to experimentation. Food was provided as epiphytes on rocks from the collection site. Seawater (ca. 50%) was replaced weekly.

Performance of aerobic metabolism and CT_{max} of gastropods. The performance of aerobic metabolism of *T. conica* was assessed by measuring respiration rate from 15 °C to 35 °C with an increasing temperature ramp of 2 °C h^{-1} [25], whereas the locomotory behaviour was observed to estimate CT_{max}, indicated by the loss of motor coordination as the end point[21,36]. Prior to experimentation, individuals were transferred into plastic holding aquaria for a 2-day acclimation period at lower temperature. To avoid the shock effect due to a sudden decrease in temperature, the aquaria were submerged in a water bath and the seawater temperature in the aquaria was reduced from 21 °C to 15 °C at a decreasing rate of 3 °C day^{-1} using heater/chiller units (TC60, TECO, Italy). After the 2-day acclimation period, two individuals (shell height: ~11 mm) were transferred into a 73 mL airtight chamber filled with experimental seawater (i.e. pCO_2 and temperature manipulated) at 15 °C and allowed to rest for one hour to minimize the effect of handling stress ($n = 3$ replicate chambers per pCO_2 level, see section below for manipulation of pCO_2 level). After the 1-hour resting period, the chamber seawater was fully replaced with oxygen-saturated experimental seawater with initial dissolved oxygen concentration measured using an automatic temperature compensation oxygen optode (LDO101, HACH, USA), which was calibrated at room temperature (~23 °C) following the manufacturer's instructions. The chamber was then closed and put into a water bath at the target temperature to maintain the temperature of chamber seawater. After one hour, the chamber was opened to measure the final dissolved oxygen concentration, which was above 5.2 mg O_2 L^{-1} for all samples. Then, the chamber seawater was fully replaced with oxygen-saturated experimental seawater at the next temperature level (i.e. +2 °C) immediately. The procedure for measuring respiration rate was repeated and locomotory behaviour was observed using the same individuals until measurement for the last temperature level (i.e. 35 °C) was completed. Blank samples without individuals were used to correct the background change in dissolved oxygen concentration. Respiration rate was expressed as mg O_2 g^{-1} (flesh weight) h^{-1}, where flesh weight was obtained after dissection.

Experimental setup for exposure to elevated pCO_2 and temperature. Individuals of *T. conica* (shell height: 11.0 ± 1.30 mm, mean ± S.D.) were transferred into plastic aquaria (30 cm × 20 cm × 18 cm; $n = 9$ individuals per aquarium), which contained 5 L natural seawater, *Ulva* sp. and rocks from the collection site. They were then exposed to one of the crossed combinations of pCO_2 (400 ppm vs. 1000 ppm) and temperature (21 °C vs. 24 °C) for eight weeks ($n = 3$ replicate aquaria per treatment). The "ambient" temperature (21 °C) is the average seawater temperature at the collection site in summer (average annual range: ~14 °C–22 °C), while the elevated temperature (+3 °C) simulates the average increase in seawater temperature based on the past heatwave events[11,12]. The desired seawater temperatures were achieved by submerging the aquaria in water baths maintained by heater/chiller units (TC60, TECO, Italy). The elevated pCO_2 level, which simulates the predicted RCP8.5 scenario for the year 2100[1], was maintained by aerating the seawater with CO_2-enriched atmospheric air using a gas mixer (Pegas 4000 MF, Columbus Instruments, USA). Food was provided as epiphytes on rocks from the collection site and replenished before totally consumed. Seawater (ca. 50%) was renewed weekly to prevent accumulation of metabolic waste. The seawater carbonate chemistry parameters throughout the experimental period are shown in Table S2.

Energy budget of gastropods. To estimate the effect of prolonged exposure to elevated pCO_2 and temperature on energy budget, scope for growth was measured at the end of the experimental period (i.e. Week 8), where respiration rate, absorption rate and excretion rate were determined[41]. To measure respiration rate, two individuals from each aquarium were transferred into a 73 mL airtight chamber filled with seawater adjusted to their respective treatment conditions, and allowed to rest for one hour ($n = 3$ replicate chambers per treatment). The experimental procedures for measuring respiration rate are described in the section above. To quantify absorption rate (i.e. energy gain from the ingested food in a given period of time), two individuals which had been starved for one day were allowed to consume the epiphytes on rocks in their aquarium for one hour under their respective treatment conditions ($n = 3$ replicate aquaria per treatment). The initial and final total weights of each individual were measured by an electronic balance to the nearest 0.0001 g after blotting and removing the seawater inside the shell by gently tapping the operculum to obtain the fresh weight of epiphytes consumed[8]. The water content of epiphytes was determined by oven-drying so that ingestion rate was expressed as dry weight of epiphytes consumed per individual per hour. Then, the two individuals from the same feeding trial were put into a clean container filled with 200 mL natural seawater maintained under their respective treatment conditions. After four hours, the faeces in the container were collected, rinsed with deionized water and dried on a pre-weighed aluminium foil to obtain the dry weight of faeces. The ash-free dry weights of epiphytes and faeces were determined by weight loss on ignition at 550 °C in a muffle furnace for six hours so that assimilation efficiency was calculated according to equation (1) [ref.[42]]:

$$AE(\%) = \frac{F' - E'}{(1 - E')(F')} \times 100 \quad (1)$$

where AE is the assimilation efficiency; F' is the ash-free dry weight to dry weight ratio of food; E' is the ash-free dry weight to dry weight ratio of faeces.

Absorption rate is calculated by multiplying ingestion rate by assimilation efficiency, where the ingestion rate is converted into energy equivalent by the calorific value of epiphytes (8227 J g^{-1}), determined by bomb calorimeter (C2000 Basic, IKA, Germany). To quantify excretion rate, the ammonium concentration of seawater in

the container was analysed using a flow injection analyser (QuikChem 8500, Lachat Instruments, USA). Blank samples (i.e. natural seawater) were measured for correction of excretion rate. Scope for growth was calculated according to equation (2) [ref.[41]]:

$$SfG = AR - RR - ER \quad (2)$$

where SfG is scope for growth (J ind^{-1} hr^{-1}); AR is absorption rate; RR is respiration rate; ER is excretion rate. Conversion factors of 14.14 J mg O_2^{-1} and 0.025 J µg NH_4^{-1} were used to convert respiration rate and excretion rate, respectively, into energy equivalent[43].

Body condition and survival of gastropods. Total weight of each individual was measured before and after the exposure experiment using an electronic balance to indicate the change in biomass. Mortality was checked daily and dead individuals were removed from the aquarium. Following the aforementioned measurements, all individuals were dissected to obtain the flesh weight which was further separated into organ weight and foot weight. Before the exposure experiment, 20 additional individuals which were collected at the same time as the experimental animals were dissected and weighed to obtain the initial organ weight to flesh weight ratio (i.e. body condition) so that the change in this ratio could be estimated[8]. All the weights were measured on a fresh weight basis.

Statistical analysis. Two-way permutational analysis of variance (PEMANOVA) was applied to examine the effects of pCO_2 and temperature on ingestion rate, assimilation efficiency, absorption rate, respiration rate, excretion rate, scope for growth, total weight, organ weight to flesh weight ratio and mortality using software PRIMER 6 with PERMANOVA + add-on.

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Author Contributions

J.Y.S.L. designed and conducted the experiment, analysed the data and wrote the manuscript. S.D.C. helped revise the manuscript. B.D.R. designed the experiment and helped revise the manuscript.

Additional Information

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CHAPTER 3

BOOSTING EFFECT OF CO_2 ENRICHMENT ON THE NUTRITIONAL QUALITY OF ALGAE FAILS TO OFFSET THE ELEVATED ENERGY DEMAND OF HERBIVORES UNDER OCEAN WARMING

3.1 ABSTRACT

The CO_2-boosted trophic transfer from primary producers to herbivores has been increasingly discovered at natural CO_2 vents and in laboratory experiments. Despite the emerging knowledge of this boosting effect, we do not know the extent to which it may be enhanced or dampened by ocean warming. We investigated whether ocean acidification and warming enhance the nutritional quality (C:N ratio) and energy content of turf algae, which should in turn translate into higher feeding rate, energy budget and growth of a common grazing gastropod. Following a 6-month exposure period in mesocosms, we found that the isolated effects of ocean acidification or warming enhanced the energy budget of gastropods by either increasing feeding rate on the more nutritious algae or increasing energy gain per feeding effort so that their survival was unaffected (c.f. control conditions). However, growth was retarded by ocean warming which posed sublethal thermal stress. When both climate change stressors were combined, mortality was elevated due to depletion of energy reserves, suggesting that the boosting effect through trophic transfer is inadequate to fully compensate for the increased energy demand. In circumstances where the elevated energy demand under ocean acidification and warming outweighs the enhanced energy gain via their enriching effects on primary producers, the capacity of herbivores to control their blooming resources fails and runaway primary production will likely ensue.

3.2 INTRODUCTION

Given the global acceleration in anthropogenic carbon dioxide emission, marine organisms are predicted to be threatened by ocean acidification and warming, possibly altering the

functioning of marine ecosystems in the near future (Fabry et al., 2008; Doney et al., 2009; Nagelkerken and Connell, 2015). Nevertheless, there is concern that the impacts of these climate change stressors may have been overestimated because the measured responses are predominantly based on short-term studies with oversimplified experimental designs (Wernberg et al., 2012; Gazeau et al., 2013), where the capacity for stability via acclimation and species interactions have been largely overlooked. In fact, growing evidence shows that some marine organisms can maintain their physiological performance and reproductive output (Russell et al., 2013; Suckling et al., 2015), or even boost their populations (Heldt et al., 2015), after prolonged exposure to ocean acidification or warming so that some ecosystem processes are stabilized (Ghedini et al., 2015).

Recent studies reveal that ocean acidification can facilitate the trophic transfer of carbon from primary producers to herbivores (Goldenberg et al., 2017; Vizzini et al., 2017), suggesting that herbivores can indirectly gain benefit from the enhanced primary production via CO_2 enrichment (Connell et al., 2017). Indeed, CO_2 can act as a resource for primary producers and hence propagate throughout food webs (Ghedini and Connell, 2017). Therefore, the nutritional quality of primary producers (e.g. turf algae) may be modified by CO_2 enrichment (Falkenberg et al., 2013; Vizzini et al., 2017), possibly leading to ensuing changes in feeding rate, energy gain and eventually fitness of herbivores (Cruz-Rivera and Hay, 2001; Hemmi and Jormalainen, 2002; Poore et al., 2013; Ghedini and Connell, 2016; Kamya et al., 2017). As such, the elevated energy demand of herbivores under ocean acidification, caused by acid-base regulation and kinetic constraints on calcification (Fabry et al., 2008; Pörtner, 2008; Thomsen et al., 2015), may be offset through trophic transfer. Such boosting effect on energy gain may be an overarching mechanism allowing some herbivores to maintain their fitness and survival under ocean acidification (Rosenblatt and Schmitz, 2016).

Similarly, the trophic transfer from primary producers to herbivores may also be facilitated by ocean warming, which can modify the properties of primary producers (e.g. nutrient content) and hence possibly favour the fitness and survival of herbivores (Raven and Geider, 1988; Renaud et al., 2002; O'Connor, 2009; Harley et al., 2012). Nevertheless, ocean warming can directly affect marine organisms via physiological pathways, where the influence depends on their thermal tolerance. When temperature exceeds the thermal tolerance of marine organisms, aerobic scope, defined as the excess metabolic energy available after basal metabolic demand is met (Sokolova,

2013), will decrease due to the elevated energy demand for basal maintenance and reduced energy supply from aerobic metabolism (Pörtner, 2002, 2012). Consequently, the physiological and behavioural performance of marine organisms deteriorate, leading to reduced fitness and survival in the long term (Sokolova, 2013; Leung et al., 2017a). Thus, studying thermal tolerance and aerobic metabolism can shed light on the direct effect of ocean warming on the performance of marine organisms. More importantly, whether the boosting effect through trophic transfer possibly compensates for the elevated energy demand under ocean warming, and thus allows population maintenance, remains poorly known, especially when ocean acidification may lower thermal tolerance and further raise energy demand (Metzger et al., 2007; Zittier et al., 2015).

In this study, we examined the changes in body condition, biomass and survival of a common subtidal grazing gastropod following a long-term exposure period to ocean acidification and warming. We used a bioenergetics-based approach to integrate the direct and indirect effects of these climate change stressors on the energy budget of gastropods, which is the key for long-term survival (Sokolova, 2013). The direct effect was determined by the respiration rate, feeding rate and absorption rate (i.e. energy gain by feeding) of gastropods, whereas the indirect effect was indicated by the energy content and C:N ratio (i.e. nutritional quality) of turf algae. In addition, respiration rate and locomotory activity were assessed along a temperature gradient to determine the performance of aerobic metabolism against temperature and critical thermal maximum (CT_{max}) as a proxy for thermal tolerance, which can indicate whether thermal stress is posed on gastropods by ocean warming. If ocean acidification and warming could increase the nutritional quality of turf algae, we hypothesized that (1) the feeding rate and energy budget of gastropods would be elevated under ocean acidification so that their body condition, biomass and survival are unaffected or even promoted (c.f. control); (2) the possible increase in energy gain under ocean warming would be insufficient to offset the increased energy demand when the elevated temperature is close to the thermal tolerance of gastropods, causing reduced body condition and biomass; and (3) mass mortality would be incurred when energy deficit persists, as indicated by reduced body condition. If the boosting effect of ocean acidification and warming through trophic transfer is sufficient to compensate for their direct negative effects on the energy budget of herbivores, habitat integrity and top-down control on primary producers can likely be maintained in future marine ecosystems.

3.3 MATERIALS AND METHODS

3.3.1 EXPERIMENTAL SETUP

A common subtidal grazing gastropod, *Phasianella australis*, was chosen as the study species due to its substantial contribution to herbivory in shallow seagrass and rocky reef habitats (Kinloch et al., 2007; Poore et al., 2012). Individuals were collected in summer from the subtidal zone (~5 – 10 m depth) at Wirrina Cove, South Australia (35°29′S, 138°15′E). Mesocosm systems were constructed in a constant temperature room (air temperature: 23°C) to offer a more realistic simulation of the subtidal habitat (Goldenberg et al., 2017). Each of the 12 mesocosm systems was composed of a large circular mesocosm (1.8 m diameter; 1 m depth; ~1,800 L) and a small enrichment tank (60 L) where seawater pH and temperature were regulated. Two header tanks (800 L) were made to maintain the inflow of seawater with pre-treatment conditions to the mesocosms. To simulate the soft-bottom seabed, a layer of sand (~10 cm thick) collected *in situ* was added into each mesocosm. There were eight circular habitat patches (42.5 cm in diameter) in each mesocosm: four consisted of live rocks harbouring macroalgae (e.g. *Cystophora* sp., *Exallosorus* sp., *Sargassum* sp. and *Zonaria* sp.) and four harboured artificial seagrasses made of green polypropylene ribbon strips. The rocks were collected *in situ*, whereas the artificial seagrasses were soaked *in situ* for two weeks to allow the growth of natural epibiont communities. A 250 W high pressure metal halide lamp (Powerstar HQI-T 250 W/D PRO, Osram, Germany) was hung ~1 m above the centre of each mesocosm for illumination with day/night cycles of 14:10 hours. The illuminance was ~3800 lx at the substratum surface, which is similar to that in local waters at ~10 m depth (Phillips et al., 1981). To maintain the nutrient and mineral contents of seawater in each mesocosm, a continuous inflow of natural, unfiltered seawater (~1,800 L day^{-1}) was provided through an offshore pipeline from the coast. A central filter column (mesh size: 20 μm) was built to allow excess seawater to flow out of the mesocosm and into the enrichment tank by gravity. Two pumps (~1.8 m^3 hr^{-1}) were installed in the enrichment tank to pump the seawater back to the mesocosm. A hole was made on the enrichment tank to allow an outflow of excess seawater so that the volume of seawater in the mesocosm remained stable.

The mesocosm system had a 2 × 2 crossed design of pCO_2 (400 ppm vs. 1000 ppm) and temperature (21°C vs. 24°C) with three replicate mesocosms per treatment combination. 21°C is the average seawater temperature at the collection site in summer, and the elevated pCO_2 and

temperature levels simulate the RCP8.5 scenario for the year 2100 (IPCC, 2013). The elevated pCO_2 level was achieved by bubbling the seawater in the header tanks with pure CO_2 and then maintained by aerating the seawater in the enrichment tank with CO_2-enriched air using a gas mixer (Pegas 4000 MF, Columbus Instruments, USA). The desired seawater temperature was maintained using two titanium heaters (300 W × 1 and 500 W × 1), which were put into the enrichment tank. The mesocosm systems were allowed to run for three weeks to ensure that the seawater and habitat conditions were stabilized before experimentation. The seawater carbonate system parameters for the experimental period are shown in Table S1.

Twenty individuals of juvenile *P. australis* (shell length: 10 ± 3.5 mm; total biomass: 4.0 ± 0.25 g) were transferred into each mesocosm and allowed to acclimate for one week prior to commencement of the experiment. They were then maintained in the mesocosms for six months. After this exposure period, mortality was determined by collecting and enumerating the individuals in each mesocosm (n = 3 replicate mesocosms per treatment). An electronic balance was used to measure the total weight of each individual so that the change in total biomass in each mesocosm was determined (n = 3 replicate mesocosms per treatment). Following the energetics experiment (see section 2b below), all individuals were dissected to obtain the flesh weight which was further separated into organ weight and foot weight. The organ weight to flesh weight ratio of each individual was calculated to indicate body condition (Leung et al., 2017b). The initial organ weight to flesh weight ratio was obtained by dissecting additional 20 individuals before the exposure period so that the change in this ratio could be calculated. Body condition is improved when this ratio has positive change and *vice versa*. All the weights were measured on a fresh weight basis.

3.3.2 ENERGETICS OF GASTROPODS FOLLOWING EXPOSURE

Following the 6-month exposure period, energy loss by respiration and energy gain by feeding were estimated because they are the key processes determining energy budget in marine molluscs (Widdows and Johnson, 1988; Navarro et al., 2013; Zhang et al., 2015). To measure respiration rate, one individual was transferred into a 73 mL airtight chamber filled with seawater adjusted to the respective pCO_2 and temperature levels of the mesocosm, and allowed to rest for one hour to eliminate potential handling stress. Then, the seawater in the chamber was fully replaced with oxygen-saturated experimental seawater (i.e. pCO_2 and temperature manipulated)

with initial dissolved oxygen concentration measured by an optical dissolved oxygen probe (Fibox 4, PreSens, Germany). The chamber was then closed and put into a water bath at the target temperature so that the temperature of seawater in the chamber was maintained. After one hour, the chamber was opened to measure the final dissolved oxygen concentrations of seawater. Two individuals from each mesocosm were tested and the average respiration rate was taken as a replicate for each mesocosm (n = 3 replicate mesocosms per treatment). The background change in dissolved oxygen concentration was corrected by blank samples (i.e. no individual in the chamber). Respiration rate was expressed as oxygen consumed per flesh weight per hour, where the flesh weight was obtained following dissection. The same procedure was also applied before the exposure period (i.e. following the 1-week acclimation period) to examine the temporal change in respiration rate, which can indicate the health status of gastropods.

Before feeding trials, tiles were put into each mesocosm for one month which allows adequate quantity of turf algae to grow on the tile surface for experimentation. To standardize hunger level, gastropods in each mesocosm were temporarily transferred into a nylon mesh cage without food, which was then put back to the respective mesocosm. After starving for one day, one individual was placed into a clean, tailor-made plastic cage (12 cm × 12 cm × 5 cm), which contained a tile (5 cm × 10 cm) with turf algae growing under the respective treatment conditions. Both ends of the cage were covered by nylon mesh to allow continuous water flow. The individual was then put back to the respective mesocosm and allowed to feed in the cage for three hours. Two to four individuals from each mesocosm were tested in separate cages and their feeding rate was averaged as a replicate for each mesocosm (n = 3 replicate mesocosms per treatment). Each tile was photographed at a fixed distance of 37 cm using a high-definition camera (Canon EOS 1100D) before and after the feeding trial to estimate the total grazed space on the tile (i.e. no turf algae). The photographs were then analysed using software eCognition 9 (Trimble, Germany) which provides high accuracy for measuring spatial coverage. Feeding rate was expressed as the dry weight of turf algae consumed per flesh weight of gastropod per hour, where the dry weight of turf algae per area was measured by scraping the tile with a scalpel to collect the turf algae in a known area, which were then oven-dried and weighed. To assess the nutritional quality of turf algae, energy content and C:N ratio were measured using a bomb calorimeter (AC-350, LECO, USA) and CHNS elemental analyser (2400 Series II, Perkin Elmer, USA) (n = 3 replicate mesocosms per treatment; 2 trials per replicate), respectively.

To estimate absorption rate (i.e. energy gain by feeding), assimilation efficiency was determined by measuring the organic matter content in turf algae and faeces. The faeces of gastropods in the cages were collected, rinsed with deionized water and dried on a pre-weighed aluminium foil to obtain the dry weight of faeces. The ash-free dry weights of turf algae and faeces were determined by weight loss on ignition at 550°C in a muffle furnace for six hours so that assimilation efficiency was calculated using Conover ratio (Conover, 1966):

$$AE(\%) = \frac{F' - E'}{(1 - E')(F')} \times 100$$

Where *AE* is the assimilation efficiency; *F'* is the ash-free dry weight to dry weight ratio of food; *E'* is the ash-free dry weight to dry weight ratio of faeces.

Absorption rate can then be calculated by multiplying the feeding rate by assimilation efficiency, whereas energy budget is given by energy gain by feeding minus energy loss by respiration. The energy content of turf algae and a conversion factor of 14.14 J mg O_2^{-1} were used to convert feeding rate and respiration rate into energy equivalent (Elliott and Davison, 1975), respectively.

3.3.3 THERMAL TOLERANCE AND AEROBIC METABOLISM ALONG A TEMPERATURE GRADIENT

Aerobic metabolism of gastropods was assessed by measuring respiration rate from 15°C to 35°C with an increasing temperature ramp of 2°C hr^{-1} (Giomi et al., 2016), whereas thermal tolerance was indicated by CT_{max} at which motor coordination is lost (Lutterschmidt and Hutchison, 1997). The procedure for this experiment has been previously described (Leung et al., 2017a). Briefly, individuals that were not used for the 6-month exposure were transferred into aquaria for a 3-day acclimation period with seawater temperature at 15°C and pCO_2 at either 400 or 1000 ppm. Then, one individual was put into a 73 mL airtight chamber containing experimental seawater at 15°C and allowed to rest for one hour (n = 3 replicate chambers per pCO_2 level). Respiration rate was then measured using the same procedure described above. After each measurement, the seawater in the chamber was fully replaced with oxygen-saturated experimental seawater at the next temperature level (i.e. +2°C) immediately so that oxygen consumption was measured after another one hour. The procedure for respiration rate measurement was repeated with the same

individual until measurement for the last temperature level (i.e. 35°C) was completed. The behaviour of gastropods in the chambers was observed continuously to estimate CT_{max}.

3.3.4 STATISTICAL ANALYSIS

Two-way permutational analysis of variance with pCO_2 and temperature as fixed factors was applied to test their effects on the aforementioned response variables (i.e. calorific value, C:N ratio, total biomass, mortality and respiration rate) with each mesocosm as a replicate, except for organ weight to flesh weight ratio with each individual as a replicate in view of the large number of individuals used. Given the substantial mortality under combined elevated pCO_2 and temperature conditions, feeding trials were not performed for this treatment. Therefore, *t*-test was applied to examine the effects of pCO_2 and temperature on feeding rate and energy budget.

3.4 RESULTS

The energy content of turf algae increased at elevated temperature, while pCO_2 had no significant effect (Table 1, Table S2). C:N ratio was reduced by elevated pCO_2 at ambient temperature, meaning that CO_2 enrichment can increase the relative nitrogen content in turf algae (Table 1, Table S2). Elevated temperature also reduced the C:N ratio of turf algae compared to the control, but this positive effect of nitrogen enrichment was slightly reduced when combined with elevated pCO_2.

The organ weight to flesh weight ratio of *P. australis* increased by ~7% under control and by ~5% under elevated CO_2 conditions, but remained close to the value recorded before the exposure for elevated temperature in isolation (Figure 1a). This ratio significantly dropped by ~12% under combined elevated pCO_2 and temperature conditions (Fig. 1a, Table S2), indicating reduced energy reserves and body condition. Elevated temperature, but not elevated pCO_2, negatively impacted the increase in total biomass of *P. australis* in the mesocosm (Fig. 1b, Table S2). Mortality was not significantly affected by elevated pCO_2 or temperature in isolation compared to the control, but increased drastically under combined elevated pCO_2 and temperature conditions (Fig. 1c, Table S2).

Compared to the control, the feeding rate of *P. australis* was raised by elevated pCO_2 (t = 3.81, p = 0.019), but remained unchanged at elevated temperature (t = 1.58, p = 0.189) following the 6-month exposure period (Fig. 2a). Assimilation efficiency was neither affected by elevated pCO_2 (t = 0.96, p = 0.391) nor temperature (t = 2.75, p = 0.051) (Fig. 2b). Energy budget was higher at both elevated pCO_2 (t = 4.59, p = 0.010) and temperature (t = 3.37, p = 0.028) than the control (Fig. 2c). A significant negative correlation was found between C:N ratio and feeding rate (Fig. 2d), meaning that the feeding rate of gastropods had a positive relationship with the relative N content of their food.

After the 6-month exposure to the treatment conditions, the respiration rate of *P. australis* was substantially reduced by nearly 40% under combined elevated pCO_2 and temperature conditions (Fig. 3, Table S2). Yet, elevated pCO_2 or temperature in isolation had limited effect on the change in respiration rate (Fig. S1 for the respiration rate before and after exposure).

Along an increasing temperature ramp, *P. australis* was inactive below 17°C but became more active with increasing temperature from 17°C to 25°C (Fig. 4). Motor coordination was lost at 27°C which represents the CT_{max} (i.e. upper thermal tolerance) of *P. australis*. Respiration rate increased with temperature from 15°C to 25°C (maximum), above which the respiration rate declined gradually with increasing temperature irrespective of pCO_2 level (Fig. 4). The effect of pCO_2 on respiration rate was not discernible.

Table 1 Energy content and C:N ratio of turf algae growing under different treatment conditions (mean ± S.E., n = 3).

	Control	OA	Temp	OA × Temp
Energy content (kJ g^{-1})	9.77 ± 0.23	10.7 ± 0.35	15.0 ± 0.50	13.9 ± 1.12
C:N ratio	12.0 ± 0.49	8.12 ± 0.33	6.74 ± 0.80	9.35 ± 0.42

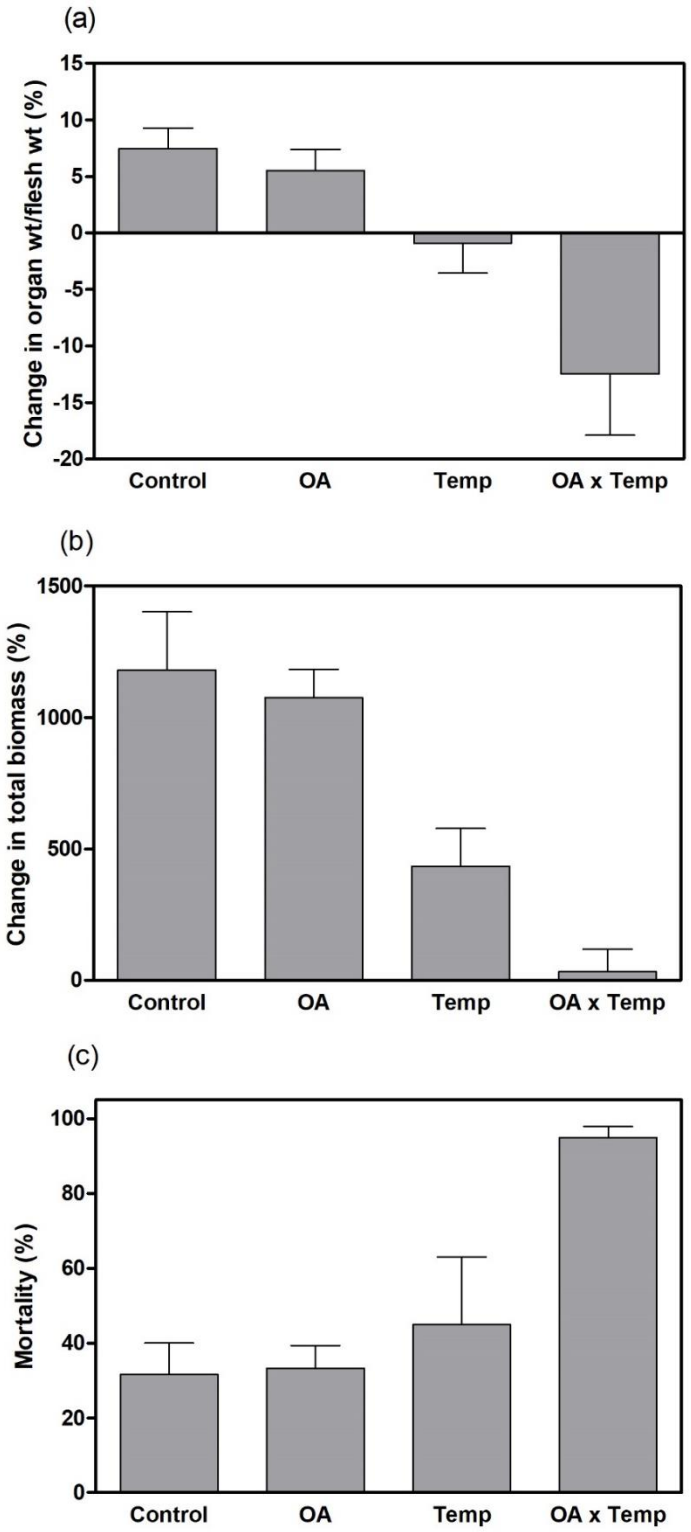

Fig. 1 (a) Change in organ weight to flesh weight ratio (mean + S.E.; *n* = 41 individuals for Control, *n* = 40 individuals for OA, *n* = 32 individuals for Temp and *n* = 3 individuals for OA × Temp), (b) change in total biomass and (c) mortality of *P. australis* following the 6-month exposure period (mean + S.E.; *n* = 3 replicate mesocosms). Control: 21°C, 400 ppm; OA: 21°C, 1000 ppm; Temp: 24°C, 400 ppm; OA × Temp: 24°C, 1000 ppm.

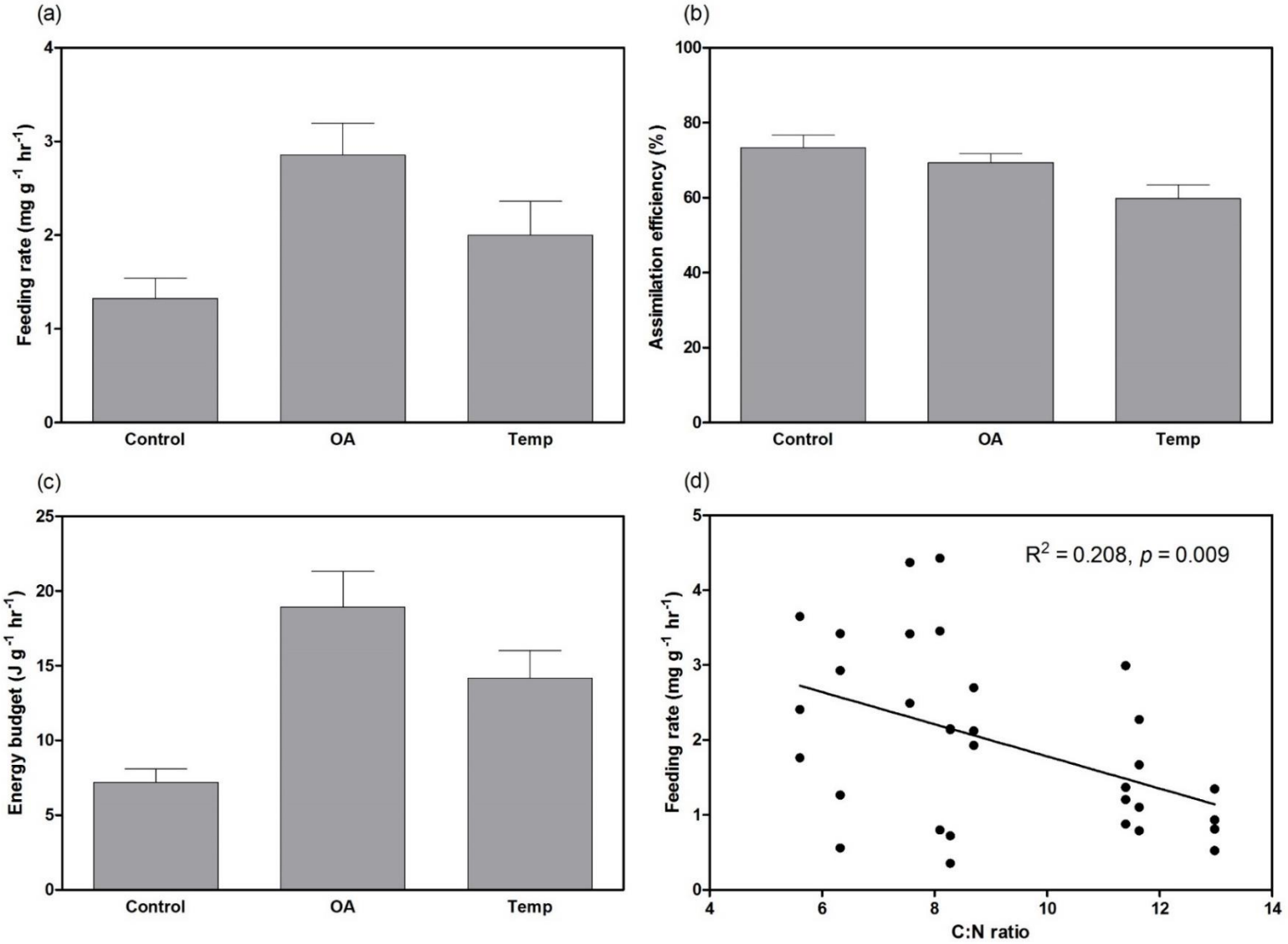

Fig. 2 (a) Feeding rate, (b) assimilation efficiency and (c) energy budget of *P. australis* under different treatment conditions (mean + S.E.; $n = 3$); (d) Relationship between feeding rate of *P. australis* and C:N ratio of turf algae.

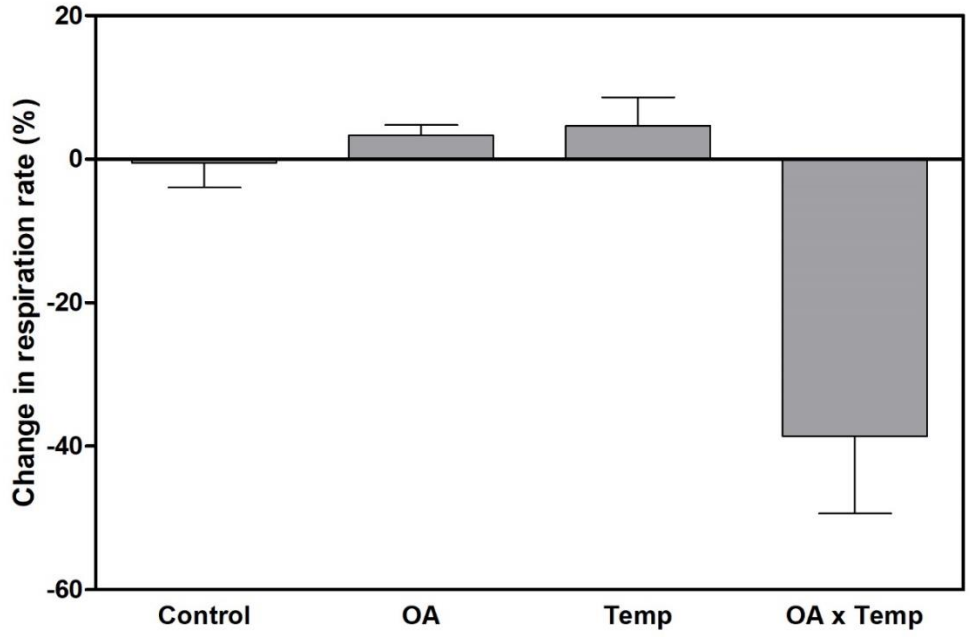

Fig. 3 Change in respiration rate of *P. australis* following the 6-month exposure period under different treatment conditions (mean + S.E.; n = 3).

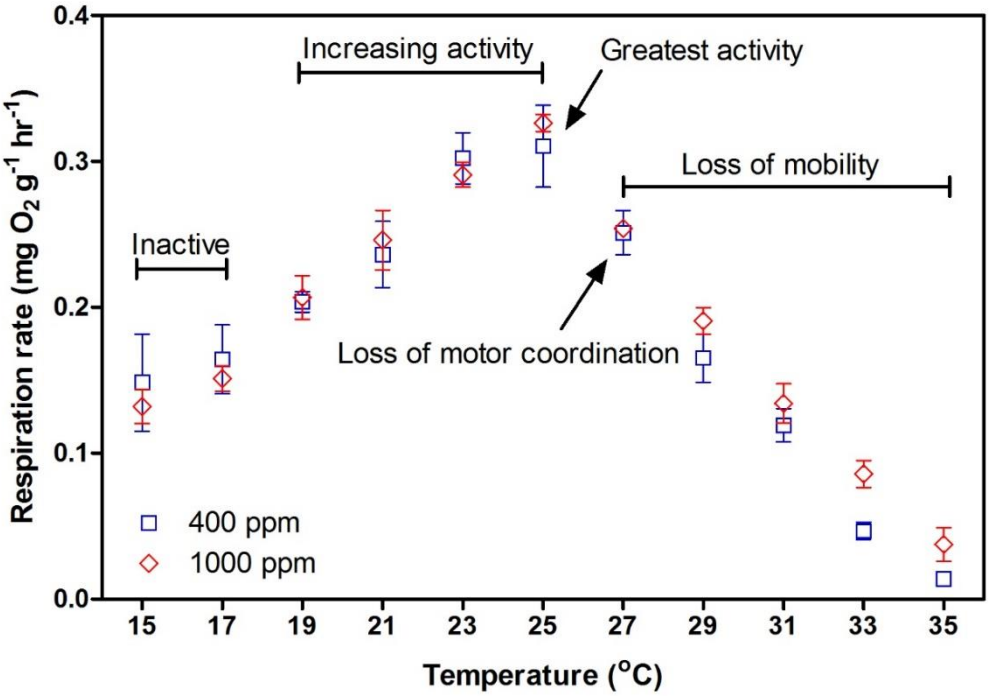

Fig. 4 Respiration rate of *P. australis* along an increasing temperature ramp from 15°C to 35°C at different pCO_2 concentrations (mean ± S.E.; n = 3).

3.5 DISCUSSION

Enhanced trophic transfer from primary producers to herbivores may be the key mechanism allowing herbivores to maintain their populations under ocean acidification and warming (Boyd and Brown, 2015; Rosenblatt and Schmitz, 2016). Here, we demonstrated that the boosting effect through trophic transfer elevates the energy budget of a common marine herbivore under ocean acidification or warming in isolation, which is favourable for long-term survival. However, such indirect positive effect was overwhelmed by the direct negative effect of these climate changes stressors in combination, leading to a substantial decline in fitness and survival.

It has been well-recognized that ocean acidification disrupts the energy homeostasis of marine organisms, especially calcifying molluscs, because they require extra metabolic energy for acid-base regulation and calcification (Pörtner, 2008; Thomsen et al., 2015). In addition, reduced pH can undermine their feeding performance, energy gain and eventually growth (Fabry et al., 2008; Gazeau et al., 2013; Leung et al., 2015). Nevertheless, we found that ocean acidification had little influence on the body condition, biomass and survival of *P. australis*, suggesting that energy homeostasis was maintained. In other words, the elevated energy demand under ocean acidification was probably offset by the increased feeding rate and hence energy gain (Ghedini and Connell, 2017). Based on the metabolic theory of ecology (Brown et al., 2004), increased feeding rate can be driven by elevated metabolic rate, as shown in some grazing gastropods (Mertens et al., 2015; McSkimming et al., 2016), but the respiration rate of our study gastropod was not upregulated by ocean acidification, meaning that the feeding rate is not driven by metabolic rate. We suggest that the increased feeding rate under ocean acidification is mediated by the reduced C:N ratio of turf algae, which could be attributed to the either higher assimilation of nitrogen by the algae or more efficient photosynthesis that allows reallocation of nitrogen in metabolic processes (Zou, 2005; Hurd et al., 2009; Koch et al., 2013; Xu et al., 2017). As nitrogen is often limiting for herbivores (Mattson, 1980), higher relative nitrogen content in turf algae can enhance the feeding rate of gastropods, as also shown in other herbivores (Falkenberg et al., 2013; Ghedini and Connell, 2016; Kamya et al., 2017). Regardless of the underlying mechanism, we showed that the increased feeding rate on the more nutritious turf algae promotes energy budget, ultimately enabling the gastropods to maintain fitness under ocean acidification. Indeed, such boosting effect through trophic transfer could outweigh the direct negative effects of ocean

acidification (Falkenberg et al., 2013; Kamya et al., 2017), and even boost the population of herbivores (Connell et al., 2017; Goldenberg et al., 2017; Vizzini et al., 2017).

Ocean warming in isolation was also shown to promote the energy budget of gastropods, but their body condition and biomass were lower than those in the control, suggesting that less energy was allocated to growth. Thermal tolerance is often the overriding factor determining the effect of elevated temperature on aerobic scope (or energy budget) and hence growth. We found that the CT_{max} of *P. australis* appears at 27°C where motor coordination is lost and respiration rate is declining. As such, aerobic scope and overall performance are diminished according to the oxygen- and capacity-limited thermal tolerance concept (Pörtner, 2002, 2012). To prevent this acute thermal stress, locomotory activity was maximized at 25°C as the avoidance behaviour. Since the respiration rate at elevated temperature (i.e. 24°C) was close to the maximum at 25°C, the gastropods likely suffered from sublethal thermal stress in the mesocosms, which can reduce energy budget and growth in the long term (Pörtner, 2002, 2012; Sokolova, 2013; Gianguzza et al., 2014; Leung et al., 2017a). To compensate for the greater energy demand at elevated temperature, energy gain by feeding should be promoted. Here, the gastropods failed to increase their feeding rate possibly due to the sublethal thermal stress (Leung et al., 2017a). Nevertheless, the higher energy content of turf algae provides a mechanism of partial compensation by boosting energy gain, and hence energy budget, per feeding effort. Despite the advantage of this boosting effect, growth was still retarded, implying that the energy budget of gastropods is allocated mostly to somatic maintenance and less to growth and reproduction based on the dynamic energy budget model (Nisbet et al., 2012). Indeed, survival under sublethal thermal stress requires a disproportionate energy allocation for somatic maintenance because cellular and structural damage can already be inflicted (Pörtner, 2002; Somero, 2002, 2010). For example, production of heat shock proteins, which incurs substantial energy cost, is upregulated under sublethal thermal stress to repair damaged proteins and stabilize denaturing proteins (Miller et al., 2009; Tomanek, 2010). Consequently, the boosting effect via trophic transfer was inadequate to fully compensate for the direct negative effects of sublethal thermal stress on energy budget, resulting in reduced body condition.

The adverse effects of elevated temperature were exacerbated when combined with elevated pCO_2, as indicated by the mass mortality of gastropods. This suggests that the boosting

effect through trophic transfer was outweighed by the synergistic effect of ocean acidification and warming. Under stressful conditions (e.g. sublethal thermal stress), aerobic metabolism should be promoted to provide sufficient metabolic energy for molecular defence mechanisms, such as enhanced production of heat shock proteins and antioxidative enzymes (Pörtner, 2002; Somero, 2010; Tomanek, 2010). However, the aerobic metabolism of gastropods dropped substantially under these combined stressors (c.f. elevated temperature in isolation), implying not only insufficient metabolic energy for basal maintenance, but also impairment in cellular functions (e.g. energy production by mitochondria) (Pörtner, 2002; Sokolova et al., 2012). The considerable reduction in metabolic energy would worsen overall biological performance (Pörtner, 2012; Sokolova, 2013), including feeding (Mertens et al., 2015). Although we did not examine feeding rate due to the mass mortality under combined elevated pCO_2 and temperature, energy reserves (i.e. organ weight to flesh weight ratio) were markedly reduced, indicating a critical mismatch between energy gain and energy demand. Using energy reserves only provides temporary relief under adverse environmental conditions and is maladaptive in the long term (Sokolova, 2013). When energy reserves are depleted, vital cellular functions and physiological processes will be arrested, culminating in mortality and thus affecting population persistence (Leung et al., 2017a).

Herbivory is fundamental to trophic dynamics and often shapes the habitat structures of marine ecosystems, for which grazing gastropods can play a crucial role (Poore et al., 2012). Indeed, these herbivores have the capacity to counter the CO_2-driven boosted growth of primary producers through trophic compensation and hence sustain ecosystem stability (Ghedini et al., 2015). For example, Falkenberg et al. (2014) demonstrated that the top-down control by grazing gastropods can prevent the expansion of fast-blooming turf algae caused by nutrient and CO_2 enrichment; Mertens et al. (2015) showed that the feeding rate of grazing gastropods increases in response to enhanced primary production. As such, if the fitness and survival of herbivores are diminished by the combination of ocean acidification and warming, it is possible that habitat structures would be more susceptible to shifts from large canopy-forming algae to low-lying opportunistic algae (e.g. turf), thereby modifying ecosystem functioning (Connell and Russell, 2010; Kroeker et al., 2013).

In conclusion, ocean acidification and warming can increase the nutritional quality of primary producers to boost the energy budget of herbivores. However, this boosting effect through

trophic transfer can be overwhelmed by the combined direct negative effects of climate change stressors, culminating in reduced fitness and survival of herbivores. Certainly, compensatory mechanisms that buffer against changes can be strong enough to maintain homeostasis of individuals and population growth (Ghedini and Connell, 2016), but there inevitably will be a tipping point beyond which these mechanisms fail. In these cases, the innate capacity of herbivores to control their blooming resources (e.g. turf algae) fails and runaway primary production is likely to ensue.

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3.7 SUPPLEMENTARY INFORMATION

Table S1 Seawater carbonate system parameters in different treatments throughout the 6-month exposure period. Temperature and pH were daily measured using a pH/temperature meter (HI 98128, HANNA Instruments, Germany), calibrated using NBS buffers. Salinity and total alkalinity were weekly measured using a hand-held refractometer and potentiometric titrator (888 Titrando, Metrohm, Switzerland), respectively. The pCO_2, dissolved inorganic carbon (DIC) and saturation states of calcite (Ω_{cal}) and aragonite (Ω_{ara}) were calculated using the CO2SYS program (Pierrot et al., 2006), with dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

	Measured parameters				Calculated parameters			
Treatment	Salinity (ppt)	Temperature (°C)	pH (NBS scale)	Total alkalinity (µmol kg^{-1})	pCO_2 (ppm)	DIC (µmol kg^{-1})	Ω_{cal}	Ω_{ara}
Control	36.3 ± 0	21.0 ± 0.14	8.14 ± 0.004	2482 ± 4.1	465 ± 5.0	2195 ± 8.3	4.74 ± 0.05	3.09 ± 0.04
OA	36.3 ± 0	20.9 ± 0.04	7.89 ± 0.009	2485 ± 5.2	905 ± 6.9	2309 ± 4.3	2.91 ± 0.02	1.90 ± 0.01
Temp	36.3 ± 0	23.7 ± 0.19	8.12 ± 0.002	2486 ± 6.0	500 ± 8.5	2191 ± 4.4	4.90 ± 0.05	3.22 ± 0.03
OA × Temp	36.3 ± 0	23.7 ± 0.08	7.89 ± 0.009	2493 ± 3.0	915 ± 25.1	2301 ± 12.0	3.20 ± 0.07	2.10 ± 0.05

Pierrot, D., Lewis, E., Wallace, D.W.R. 2006. MS Excel Program Developed for CO_2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

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Table S2 Results of permutational analysis of variance showing the effects of pCO_2 and temperature on the calorific value and C:N ratio of turf algae, as well as the organ weight to flesh weight ratio, total biomass, mortality and respiration rate of *P. australis*. N.S.: Not significant.

	df	Mean square	F	*p*	Pairwise comparisons
Calorific value					
pCO_2	1	7.15×10^{-3}	5.67×10^{-3}	0.942	
Temperature	1	53.0	42.0	**< 0.001**	24°C > 21°C
pCO_2 × Temperature	1	3.09	2.45	0.156	
Residual	8	1.26			
C:N ratio					
pCO_2	1	1.21	1.39	0.283	Within 21°C: 400 ppm > 1000 ppm
					Within 24°C: 1000 ppm > 400 ppm
Temperature	1	12.2	14.0	**0.017**	Within 400 ppm: 21°C > 24°C
					Within 1000 ppm: N.S.
pCO_2 × Temperature	1	31.7	36.4	**0.005**	
Residual	8	6.98			
Organ/Flesh					
pCO_2	1	563.0	3.44	0.066	
Temperature	1	1.45×10^3	8.88	**0.004**	21°C > 24°C
pCO_2 × Temperature	1	316.2	1.93	0.167	
Residual	112	163.7			
Total biomass					
pCO_2	1	1.94×10^5	2.91	0.126	
Temperature	1	2.40×10^6	36.1	**< 0.001**	21°C > 24°C
pCO_2 × Temperature	1	6.52×10^4	0.98	0.351	
Residual	8	6.65×10^4			
Mortality					
pCO_2	1	2.00×10^3	6.08	**0.039**	Within 21°C: N.S.
					Within 24°C: 400 ppm > 1000 ppm
Temperature	1	4.22×10^3	12.8	**0.004**	Within 400 ppm: N.S.
					Within 1000 ppm: 21°C > 24°C
pCO_2 × Temperature	1	1.75×10^3	5.32	**0.049**	
Residual	8	329.2			
Change in respiration rate					
pCO_2	1	720	4.59	**0.005**	Within 21°C: N.S.
					Within 24°C: 1000 ppm > 400 ppm

Temperature	1	1022	7.84	**0.002**	Within 400 ppm: N.S. Within 1000 ppm: 24°C > 21°C
pCO_2 × Temperature	1	1502	35.4	< **0.001**	
Residual	7	42.4			

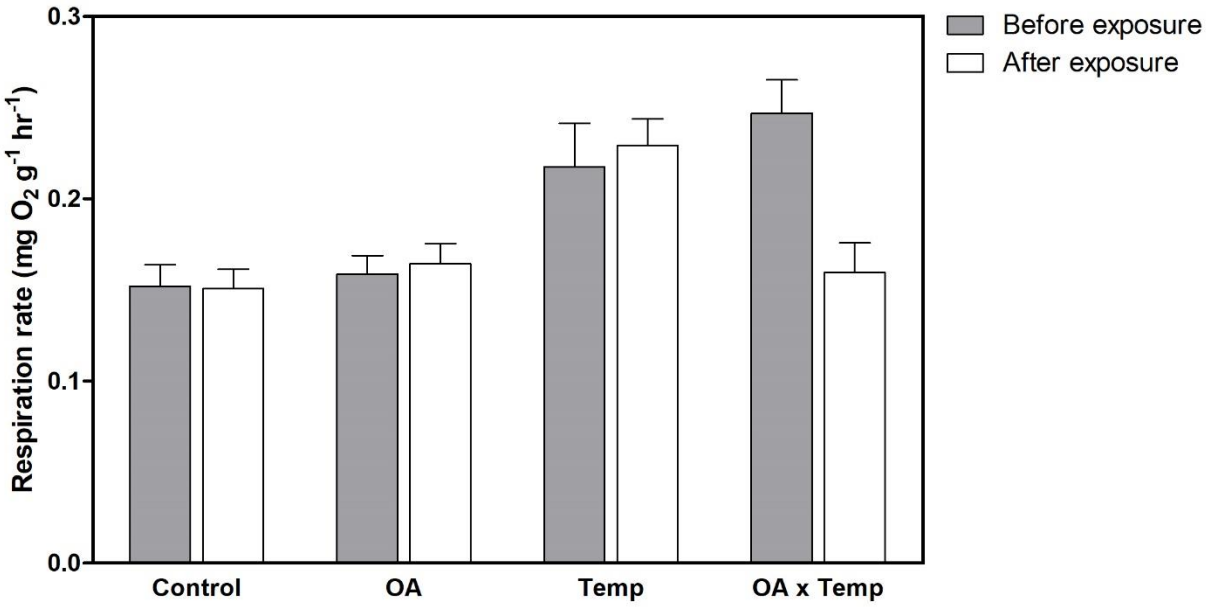

Fig. S1 Respiration rate of *P. australis* before and after the 6-month exposure period under different treatment conditions (mean + S.E.; n = 3).

CHAPTER 4

PHYSIOLOGICAL AND BEHAVIOURAL ADAPTATIONS ALLOW INTERTIDAL ORGANISMS TO COUNTER AND RECOVER FROM THE THERMAL STRESS POSED BY HEATWAVES

4.1 ABSTRACT

Concomitant with global warming, heatwaves are forecasted to be more frequent in future, possibly causing devastating impacts on coastal and marine organisms. While many of them are predicted to be threatened by heatwaves based on their thermal limit, growing evidence reveals their potential acclimation capacity to acute thermal stress. The underlying mechanism remains largely unexplored, but may be associated with adaptability and recoverability. Therefore, we examined the physiological, molecular and behavioural responses of three intertidal gastropods to acute thermal stress, and evaluated whether these responses confer them resistance and resilience to heatwaves (air temperature: 40°C; seawater temperature: 32°C). Three types of species were classified based on their responses and recoverability: (1) resistant species (*Nerita atramentosa*) increased aerobic metabolism to activate molecular defence mechanisms (production of heat shock proteins and antioxidative enzymes) so that feeding performance was unaffected by heatwaves; (2) resilient species (*Austrocochlea concamerata*) was impaired by heatwaves, but could recover when temperature returned to the ambient level; (3) sensitive species (*Austrocochlea constricta*) failed to recover after heatwaves because antioxidative defence was inadequate to counter the oxidative stress, leading to cellular damage. To avoid excess heat absorption during low tide, all gastropods hid under rocks so that body temperature was maintained within their survivable range. Our findings suggest that physiological and behavioural adaptations could be the cornerstones for intertidal organisms to accommodate the impacts of heatwaves. Since heatwaves are transient, their populations and ecological functions would be more robust to acute thermal stress than previously thought.

4.2 INTRODUCTION

In view of the accelerated increase in global temperature over the past few decades, an increasing number of studies have been conducted to predict the future impacts of ocean warming on marine organisms (Byrne, 2011). Despite the scientific merits, there is concern that these trend-based studies may have overestimated the impacts of ocean warming since marine organisms can possibly acclimate to the slow increasing rate of seawater temperature (Suckling et al., 2015; Veilleux et al., 2015; Drost et al., 2016). In contrast, heatwaves can rapidly elevate seawater temperature to a level that causes devastating impacts on marine organisms and hence ecosystem functioning (Garrabou et al., 2009; Wernberg et al., 2013; Leung et al., 2017a). Heatwaves, which can be defined as three or more consecutive days above the 90^{th} percentile for maximum temperature (Perkins and Alexander, 2013), are forecasted to be more intense, frequent and persistent in future due to global warming (Meehl and Tebaldi, 2004; Perkins et al., 2012; Hobday et al., 2016). Therefore, studying heatwaves (extreme climatic events) becomes increasingly important than ocean warming (climatic trends) to decipher the impacts of elevated temperature on marine ecosystems (Jentsch et al., 2007; Thompson et al., 2013).

While heatwaves are expected to threaten marine organisms by pushing close to their thermal limit, growing evidence reveals that they may not be affected by acute thermal stress possibly due to their potential acclimation capacity (e.g. Sorte et al., 2010; Smale et al., 2015; Giomi et al., 2016; Vinagre et al., 2016), which could be mediated by physiological adaptation (Williams et al., 2008; Magozzi and Calosi, 2015). For instance, aerobic metabolism can be downregulated as an energy-conserving mechanism when energy gain by feeding is impaired by thermal stress (Marshall and McQuaid, 2011). However, some physiological functions can be activated by thermal stress, such as production of heat shock proteins and antioxidative enzymes (Pörtner, 2002, 2012; Somero et al., 2017). These molecular defence responses are vital not only for protecting biomolecules (e.g. lipids and proteins) from thermal and oxidative stress, but also for recovering cellular and physiological functions when the stressor is removed (Lesser, 2006; Tomanek, 2010; Lushchak, 2011). Since the capacity for physiological adaptation is species-specific, it could be fundamental to determining whether marine organisms can accommodate the impacts of acute thermal stress posed by heatwaves.

Compared to subtidal organisms, intertidal organisms would be particularly vulnerable to heatwaves because solar radiation not only directly raises their body temperature during low tide, but also heats up the substrate and shallow seawater in the intertidal zone to extreme levels (Marshall et al., 2010). As heat dissipation is reduced by the extreme air temperature of heatwaves, intertidal organisms may be jeopardized by this abnormally hot environment because many of them are already living close to their thermal limit (Stillman, 2003; Somero, 2010). Nevertheless, the temperature profile in the intertidal zone can be highly heterogeneous at small spatial scales (Gilman et al., 2006; Helmuth et al., 2011), possibly allowing intertidal organisms to avoid thermal stress. For example, the less thermotolerant species can move to thermally favourable locations (e.g. crevices, shaded areas, etc.) during low tide as a short-term response to minimize heat absorption (Muñoz et al., 2005; Kearney et al., 2009; Chapperon and Seuront, 2011). Such avoidance behaviour can also lower body temperature and hence conserve metabolic energy for activating molecular defence mechanisms. Thus, behavioural adaptation (e.g. habitat preference) should be taken into consideration to accurately elucidate the impacts of heatwaves on intertidal organisms.

In this study, we examined how heatwaves impact intertidal gastropods, which are often dominant in the intertidal community, by integrating physiological, molecular and behavioural responses. Their recoverability from acute thermal stress was then evaluated. As such, we tested whether they can exhibit adaptive behaviour in response to heatwaves by determining their habitat preference and body temperature in the field during low tide. As for physiological response, feeding rate and respiration rate were measured to represent biological performance and aerobic metabolism, respectively. Molecular defence response was determined by heat shock protein concentration and total antioxidant capacity, while cellular damage by malondialdehyde concentration. We classified the species into three types based on their possible responses to heatwaves and recoverability, and hypothesized that (1) the performance of resistant species can be maintained by increasing aerobic metabolism to strengthen molecular defence; (2) resilient species are compromised by heatwaves due to weaker molecular defence than that of resistant species, but can recover upon removal of thermal stress due to recovery of aerobic metabolism; (3) sensitive species are vulnerable to heatwaves and unable to recover in view of serious cellular damage by acute thermal stress. The hypothetical relationship among biological performance, aerobic metabolism, defence response and cellular damage for different species types is illustrated

in Fig. 1. Provided physiological and behavioural adaptations allow intertidal organisms to accommodate the impacts of acute thermal stress, their populations and ecological functions would be more persistent to heatwaves than previously thought.

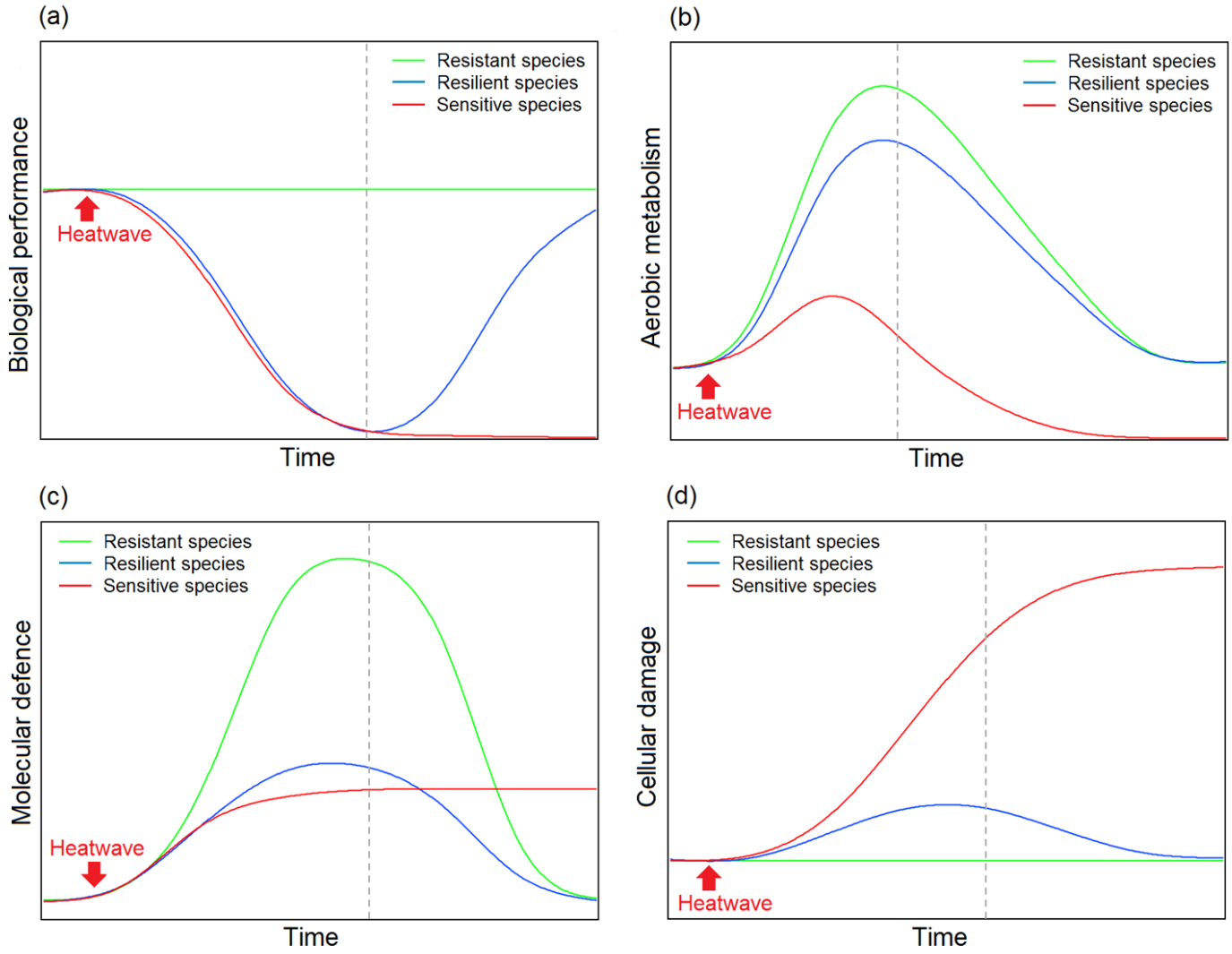

Fig. 1 Hypothetical diagrams showing the changes in (a) biological performance, (b) aerobic metabolism, (c) molecular defence and (d) cellular damage for different species types during and after heatwaves. The dotted vertical line indicates the time when temperature returns to the ambient level.

4.3 MATERIALS AND METHODS

4.3.1 HABITAT PREFERENCE AND BODY TEMPERATURE OF GASTROPODS

The rocky shore at Marino Rocks (35°20′39″S, 138°30′30″E), South Australia, was selected as the study site, where *Nerita atramentosa*, *Austrocochlea concamerata* and *Austrocochlea constricta* are the dominant grazing gastropods in the intertidal zone. These three gastropods were chosen as the study species because of their substantial contribution to the top-down control of primary production and hence trophic dynamics in their habitat (Poore et al., 2012). To examine the effect of temperature on their habitat preference, the ratio between the number of individuals on rock surface and under rock surface was estimated in a 50 cm × 50 cm quadrat at one of the following air temperatures during low tide: 21°C, 26°C, 31°C, 36°C and 41°C (n = 15 replicate quadrats per temperature). The sampling points are shown in Fig. S1. Field investigation was conducted on sunny calm days from January to February 2017 to minimize the effects of climatic and temporal variation on the behaviour of gastropods. To determine the amount of heat absorbed by gastropods on rock surface and under rock surface, their body temperature was measured using a thermal imaging camera (CompactXR, Seek Thermal, USA) (n = 10 – 20 individuals per species). The temperatures of rock surface, substrate under rocks and seawater in the intertidal zone were also recorded (Table 1).

4.3.2 EXPERIMENTAL DESIGN FOR HEATWAVE EXPOSURE

Adult *N. atramentosa*, *A. concamerata* and *A. constricta* (shell length: 13 – 16 mm) were collected from the rocky shore in January 2017 and allowed to acclimate in a plastic aquarium (1 m × 40 cm × 30 cm) filled with natural seawater under laboratory conditions (pH: 8.03 ± 0.04, temperature: 26.0 ± 0.5°C, salinity: 35.0 ± 0.5 psu and dissolved oxygen concentration: 6.30 ± 0.10 mg O_2 L^{-1}). Air temperature was maintained at 32°C in a controlled temperature room with day/night cycles of 14:10. Seawater temperature was maintained by submerging the aquarium in a water bath, where the water temperature was adjusted using heater/chiller units (TC60, TECO, Italy). Food was provided as epiphytes on rocks from the rocky shore. Following a 1-week acclimation period, six individuals of each species were put into a plastic aquarium (30 cm × 20 cm × 18 cm), which contained 5 L natural seawater and rocks with epiphytes (n = 6 replicate

aquaria). The exposure lasted for one week. On Day 1, air and seawater temperatures were maintained at 32°C and 26°C (i.e. ambient levels in summer), respectively. From Day 2 to Day 4, air and seawater temperatures were adjusted to 40°C and 32°C, respectively. The selected duration and air temperature were based on the definition of heatwaves, provided by the Bureau of Meteorology of Australia (http://www.bom.gov.au). From Day 5 to Day 7, air and seawater temperatures returned to their respective ambient levels. A control experiment was conducted by maintaining air and seawater temperatures at their ambient levels from Day 1 to Day 7. The environmental conditions during the exposure period are shown in Fig. S2.

4.3.3 BIOLOGICAL PERFORMANCE OF GASTROPODS

Respiration rate was daily measured from Day 1 to Day 7 to indicate aerobic metabolism. Two individuals from each aquarium were transferred into a 73 mL airtight chamber containing seawater adjusted to the daily temperature (i.e. 26°C or 32°C), and allowed to rest for one hour (n = 6 replicate chambers per species). The chamber seawater was then fully replaced with oxygen-saturated seawater, followed by closing the chamber for one hour. The temperature of chamber seawater was maintained by putting the chamber in a water bath at the desired temperature. The initial and final dissolved oxygen concentrations of chamber seawater were measured using an optical dissolved oxygen probe (LDO101, HACH, USA). Blank samples without individuals were prepared to correct the background change in dissolved oxygen concentration. Feeding rate was determined on Day 1 (before heatwaves), Day 3 (during heatwaves) and Day 6 (after heatwaves) of the exposure period. Two individuals from each aquarium, which had been starved for six hours before the feeding trial, were allowed to consume the epiphytes on rocks in their aquarium for one hour (n = 6 replicate aquaria per species). The amount of food consumed was indicated by the change in total weight before and after the feeding trial (Leung et al., 2017b). The initial and final total weights of individuals were measured to the nearest 0.0001 g after blotting the shell and removing the seawater inside the shell by gently tapping the operculum. Feeding rate is calculated according to the following formula:

$$Feeding\ rate = \frac{Final\ total\ weight - Initial\ total\ weight}{Feeding\ time}$$

4.3.4 MOLECULAR DEFENCE RESPONSES OF GASTROPODS

Molecular defence responses were determined on Day 1 (before heatwaves), Day 4 (during heatwaves) and Day 7 (after heatwaves) of the exposure period. Two individuals of each species were removed from each aquarium on these days, frozen immediately by liquid nitrogen and preserved at –80°C before analysis. Heat shock protein 70 (Hsp70) concentration and total antioxidant capacity (TAC) were determined to indicate molecular defence response, while malondialdehyde (MDA) concentration was measured to indicate cellular damage. To determine these biomarkers, 10% tissue homogenate (0.1 g foot tissue in 0.9 mL phosphate-buffered saline) was prepared in an ice bath and centrifuged at 2500 rpm for 10 min (n = 6 individuals per species). The supernatant was used for analysis and its protein concentration was measured by Coomassie Brilliant Blue staining method. Hsp70 concentration was analysed using the ELISA kit purchased from Fine Biotech, China (Catalogue No. EM1137), whereas TAC and MDA concentration using the assay kits purchased from Nanjing Jiancheng Bioengineering Institute, China. Multi-well plates precoated with anti-Hsp70 antibody is used for the enzyme-linked immunosorbent assay and biotin conjugated anti-Hsp70 antibody is used as detection antibodies. The antioxidative compounds in the tissue homogenate can reduce Fe^{3+} to Fe^{2+} which can then react with phenanthroline to form a stable complex with an absorption peak at 520 nm, whereas MDA reacts with thiobarbituric acid to form a pink compound with an absorption peak at 532 nm. The experimental procedures of each analysis followed the instruction manuals of manufacturers.

4.3.5 THERMOTOLERANCE OF GASTROPODS

The thermotolerance of gastropods was assessed by measuring their respiration rate and observing their motor coordination from 24°C to 40°C (seawater) with an increasing temperature ramp of 2°C hr^{-1} (Giomi et al., 2016). The experimental procedures were the same as described in Leung et al. (2017a), except the temperature range used. Briefly, individuals which were not used for the exposure experiment were allowed to acclimate at 24°C for two days. Then, two individuals were transferred into a 73 mL airtight chamber filled with seawater at 24°C and allowed to rest for one hour (n = 5 replicate chambers per species). Same procedures described in the above section were applied to measure respiration rate. Following each measurement, the chamber seawater was fully replaced with oxygen-saturated seawater at the next temperature level (i.e. +2°C)

immediately so that oxygen consumption was measured after another one hour. This procedure was repeated using the same individuals until measurement for the last temperature level (i.e. 40°C) was completed, or when the individuals lost their motor coordination as the end point (i.e. CT_{max}) (Lutterschmidt and Hutchison, 1997).

4.3.6 DATA ANALYSIS

Effect size for the aforementioned parameters was calculated to compare the responses of gastropods to heatwaves and evaluate their recoverability. Permutational analysis of variance with sampling time as the fixed factor was applied to test the temporal change in effect size. Effect size was given by:

$$Effect\ size = \frac{x_{heatwave} - \bar{x}_{control}}{\bar{x}_{control}}$$

Where $x_{heatwave}$ is the measured value of a parameter in the heatwave treatment on a particular day and $\bar{x}_{control}$ is the average value of that parameter in the control on the same day.

4.4 RESULTS

The percentage of gastropods appearing on the rock surface gradually decreased when the air temperature increased from 21°C to 31°C (*N. atramentosa*: 23% to 2%; *A. concamerata*: 7% to 0%; *A. constricta*: 77% to 38%) (Fig. 2). No gastropods appeared on the rock surface when the air temperature was ≥ 36°C. *A. constricta* was not even found under the rock surface when the air temperature was 41°C. The body temperature of gastropods increased with air temperature, regardless of their position (Table 1). *A. constricta* had slightly lower body temperature than *N. atramentosa* and *A. concamerata* by ~3°C when they were on the rock surface.

The feeding rate of *N. atramentosa* was insignificantly changed throughout the exposure period (Fig. 3a). Heatwaves reduced the feeding rate of *A. concamerata* and *A. constricta*, but the feeding rate of the former returned to baseline level after heatwaves (Fig. 3a). The respiration rate of *N. atramentosa* and *A. concamerata* increased during heatwaves and returned to the baseline level after heatwaves (Fig. 3b). The respiration rate of *A. constricta* increased during heatwaves

on Day 2, but gradually decreased with time (Fig. 3b). The decline continued after heatwaves and the respiration rate was lower than the baseline level from Day 5 to Day 7. Only *N. atramentosa* significantly increased Hsp70 concentration during heatwaves, while no significant change was found in *A. concamerata* and *A. constricta* throughout the exposure period (Fig. 4a). All gastropods increased TAC during heatwaves, but only *A. constricta* maintained elevated TAC after heatwaves (Fig. 4b). MDA concentration was insignificantly changed in *N. atramentosa* and *A. concamerata* throughout the exposure period, but increased markedly in *A. constricta* during and after heatwaves (Fig. 4c). *N. atramentosa*, *A. concamerata* and *A. constricta* lost equilibrium at 38°C, 34°C and 34°C, respectively, which indicate their respective thermotolerance (Fig. S3). Based on the overall results, *N. atramentosa*, *A. concamerata* and *A. constricta* are classified as resistant, resilient and sensitive species, respectively.

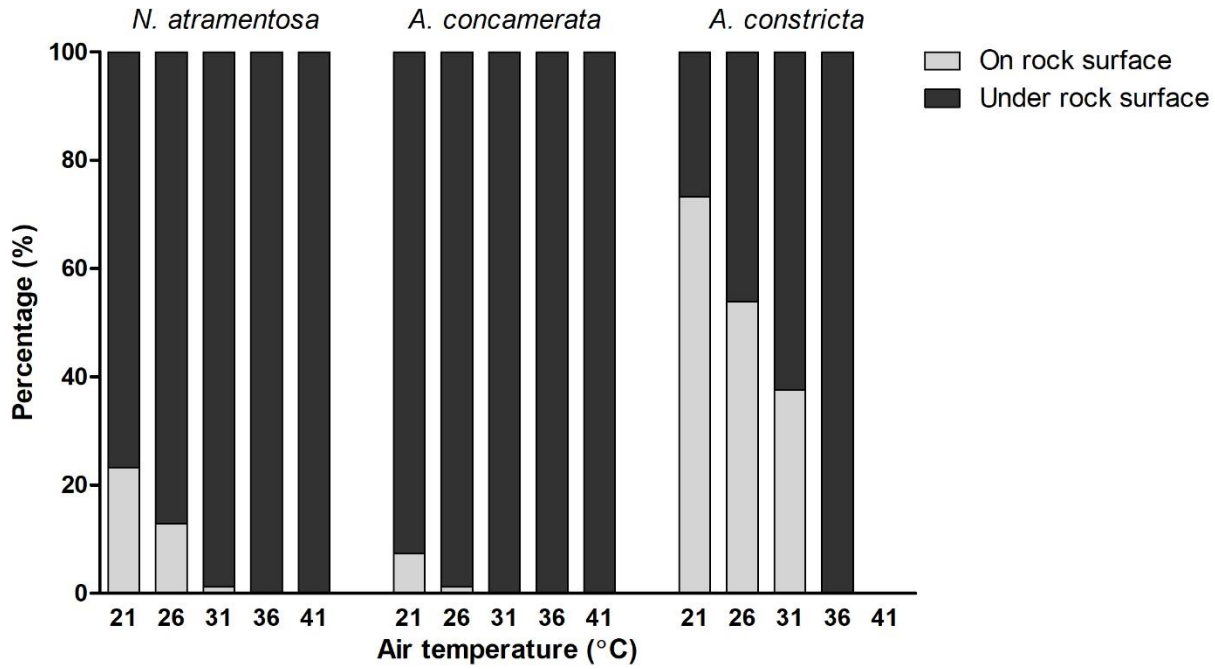

Fig. 2 Habitat preference of *N. atramentosa*, *A. concamerata* and *A. constricta* at different air temperatures. No individual of *A. constricta* was found at 41°C.

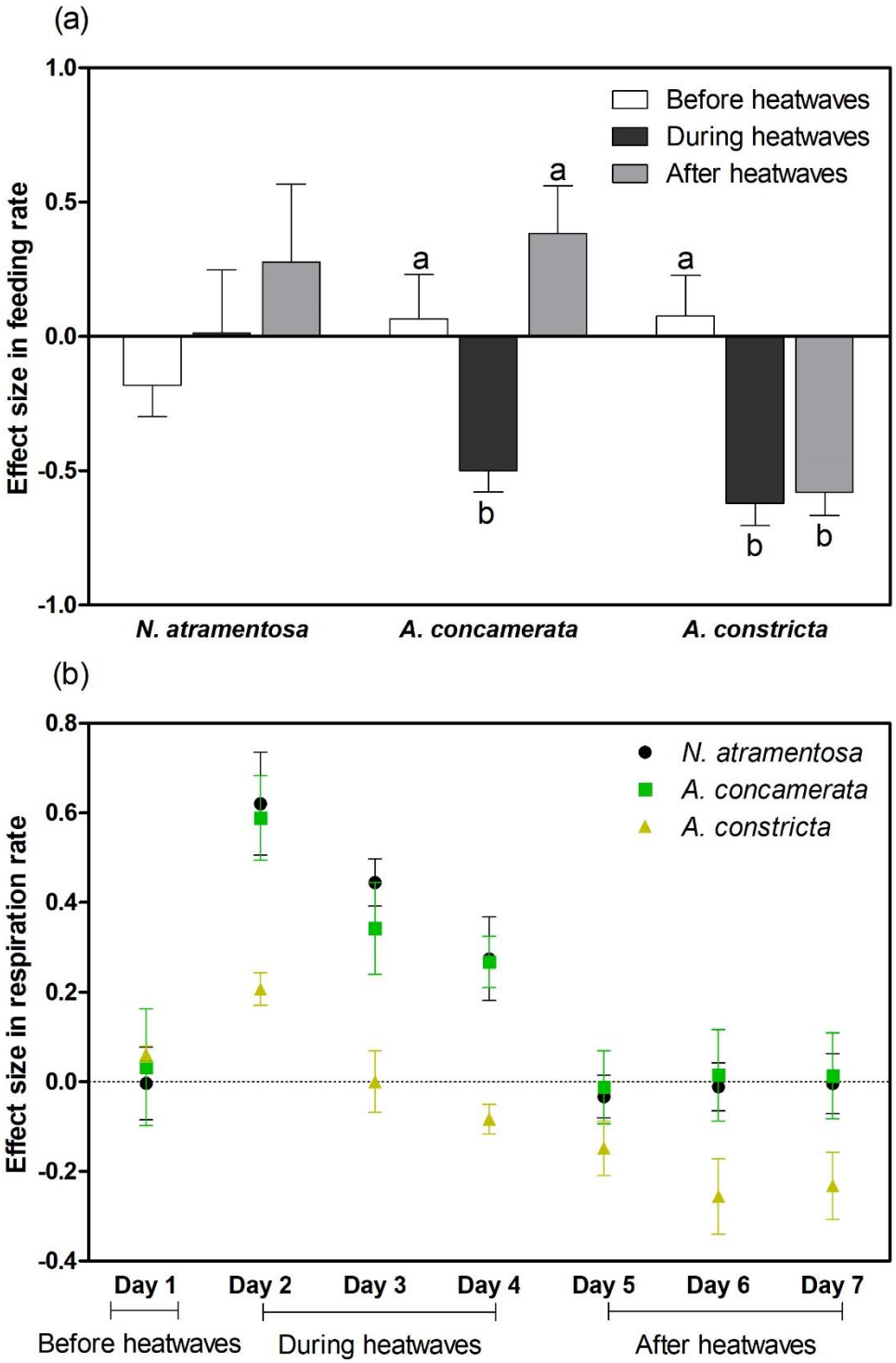

Fig. 3 The change in (a) feeding rate (mean + S.E., n = 6) and (b) respiration rate (mean ± S.E., n = 6) of *N. atramentosa*, *A. concamerata* and *A. constricta* throughout the exposure period, showing the effect of heatwaves and recoverability of gastropods. The different letters within each species indicate significant difference.

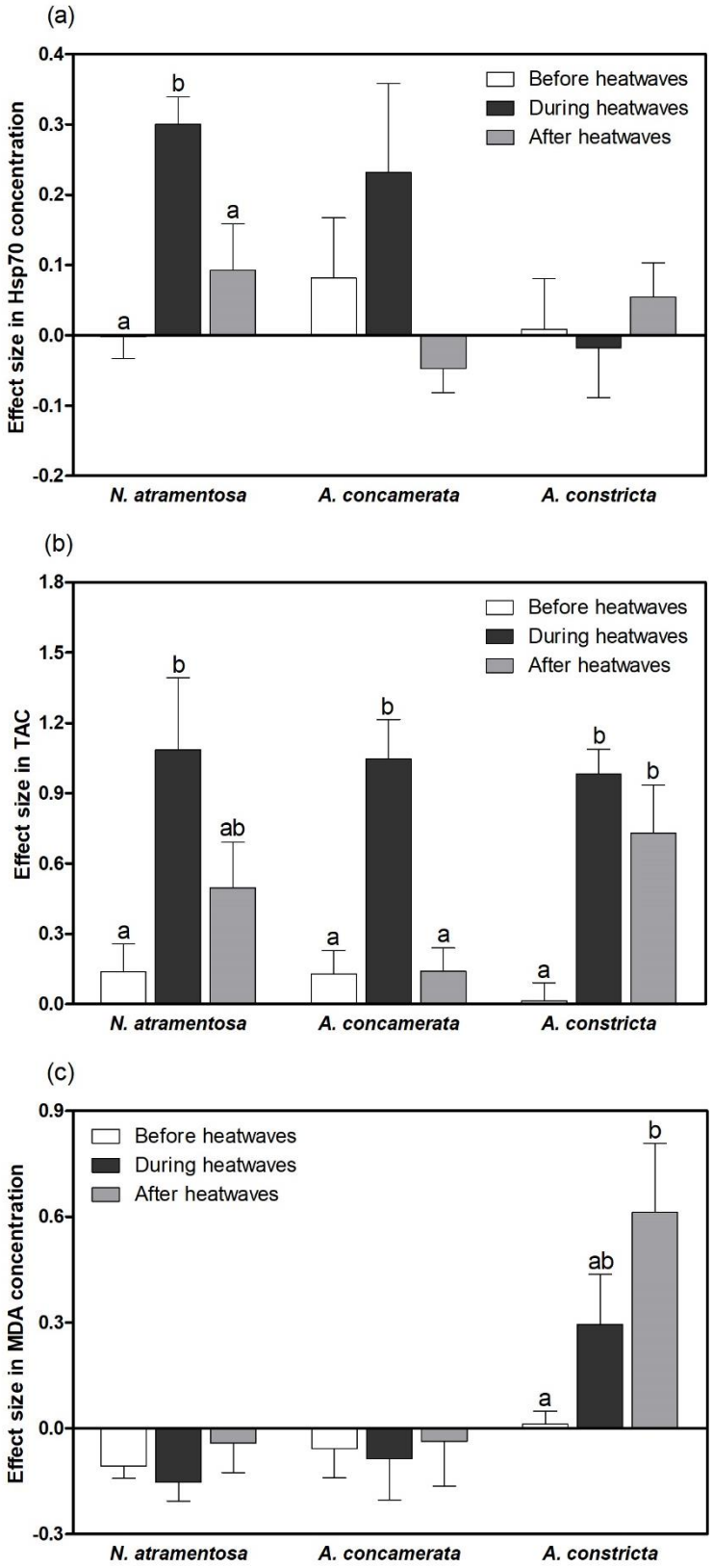

Fig. 4 The change in (a) Hsp70 concentration, (b) TAC and (c) MDA concentration in *N. atramentosa*, *A. concamerata* and *A. constricta* throughout the exposure period (mean + S.E., *n* = 6). The different letters within each species indicate significant difference.

Table 1 Temperature range of different environmental media and body temperature of gastropods at different air temperatures (mean ± S.E.). N.A.: Not available due to the absence of individuals.

	Air temperature				
	21°C	26°C	31°C	36°C	41°C
Environmental media					
Seawater	21.1 – 22.8	23.9 – 25.7	27.2 – 28.9	28.9 – 30.0	30.0 – 33.9
Rock surface	28.9 – 33.9	32.8 – 37.8	37.8 – 45.0	40.0 – 52.2	42.8 – 55.0
Substratum under rocks	21.1 – 23.9	23.9 – 26.7	25.0 – 28.9	26.1 – 30.0	28.9 – 32.2
Gastropods on rock surface					
N. atramentosa	29.7 ± 0.40	31.0 ± 0.46	34.6 ± 0.62	N.A.	N.A.
A. concamerata	28.7 ± 0.49	32.8 ± 0.59	N.A.	N.A.	N.A.
A. constricta	26.5 ± 0.33	28.5 ± 0.49	32.9 ± 0.37	N.A.	N.A.
Gastropods under rock surface					
N. atramentosa	23.6 ± 0.55	24.7 ± 0.50	28.5 ± 0.79	31.2 ± 0.55	34.6 ± 0.56
A. concamerata	24.0 ± 0.37	24.8 ± 0.66	27.7 ± 0.59	31.0 ± 0.55	33.2 ± 0.20
A. constricta	23.6 ± 0.78	25.8 ± 0.37	27.6 ± 0.44	30.5 ± 0.41	N.A.

4.5 DISCUSSION

Heatwaves are expected to threaten the survival of intertidal organisms, which are already living close to their thermal limit (Nguyen et al., 2009; Somero, 2010; Vinagre et al., 2016), but this prediction is chiefly based on their thermotolerance without considering their adaptability and recoverability. In this study, we showed that intertidal gastropods can adaptively modify their physiology and behaviour in response to a sudden increase in temperature, probably enabling them to overcome the transient impacts of heatwaves and hence persist in their habitat.

Seawater temperature in the intertidal zone can be greatly elevated by solar radiation, while the extreme air temperature of heatwaves reduces heat dissipation. As a result, intertidal organisms are subject to an abnormally high seawater temperature during heatwaves (Lima and Wethey,

2012). Here, we found that the shallow seawater in the rocky shore can be heated up to about 32°C during heatwaves, at which the less thermotolerant species (*A. concamerata* and *A. constricta*) would be impaired by acute thermal stress. For example, their feeding performance deteriorated at 32°C probably due to the weakened foot strength. In contrast, the resistant species (*N. atramentosa*) can still maintain foot strength and hence feeding performance during heatwaves in view of stronger thermotolerance. Physiologically, aerobic metabolism is the key process determining biological performance because it yields metabolic energy. In general, aerobic metabolism increases with temperature to meet the elevated energy demand for physiological functions, and thus biological performance would be undermined when aerobic metabolism fails to increase with temperature (Pörtner, 2002, 2012). We observed that all gastropods upregulate aerobic metabolism as a short-term response when seawater temperature increases abruptly from 26°C to 32°C (i.e. from Day 1 to Day 2). This response can generate more metabolic energy for various physiological processes, including molecular defence mechanisms (Dong et al., 2011). However, the sensitive species (*A. constricta*) was unable to maintain elevated aerobic metabolism over time, implying an impairment in aerobic respiration due to mitochondrial damage (Sokolova et al., 2012), which possibly explains why it was unable to recover after heatwaves.

Induction of oxidative stress by thermal stress, which is caused by the enhanced formation of reactive oxygen species (ROS) through aerobic respiration in mitochondria (Abele et al., 2002), could account for the vulnerability of organisms to heatwaves. To minimize oxidative stress, antioxidative enzymes are needed to scavenge excess ROS (Abele and Puntarulo, 2004), which can otherwise directly damage biomolecules and hence impair cellular functions (Lesser, 2006; Lushchak, 2011). As such, all tested gastropods increased their antioxidant capacity during heatwaves as a defence response against oxidative stress, which has also been shown in a variety of organisms (e.g. Madeira et al., 2013; Pimentel et al., 2015; Kamyab et al., 2017). Apart from antioxidative enzymes, thermal stress can induce the production of heat shock proteins, which are responsible for repairing damaged proteins and stabilizing denaturing proteins (Feder and Hofmann, 1999; Tomanek, 2010). Nevertheless, this defence response is species-specific and temperature-dependent (Dong et al., 2008; Prusina et al., 2014). In this study, only the resistant species was able to upregulate the production of heat shock proteins during heatwaves, which probably accounts for its stronger thermotolerance. On the contrary, the sensitive species failed to show this defence response during heatwaves probably due to insufficient energy budget for the

production of heat shock proteins, which is energy-demanding (Somero, 2002). As a result, it has higher susceptibility to heatwaves because only antioxidative defence may be inadequate to alleviate the impacts of acute thermal stress (Pimentel et al., 2015).

Nevertheless, heatwaves are usually transient, possibly allowing the less thermotolerant species to recuperate when temperature returns to the ambient level. We found that both respiration rate and feeding rate of *A. concamerata* (resilient species) return to the baseline level after heatwaves. Such recoverability is likely attributed to its capacity to eradicate the oxidative stress induced by heatwaves so that cellular damage can be averted. In contrast, *A. constricta* (sensitive species) was unable to restore respiration rate and feeding rate to the baseline level probably because of cellular damage by ROS, indicated by the elevated MDA concentration. Importantly, cellular damage was even exacerbated after heatwaves, implying elevated oxidative stress. This could be mediated by the production of ROS after re-oxygenation of tissues at lower temperature (Abele et al., 2002; Heise et al., 2006; Lushchak, 2011), because the impaired aerobic respiration of *A. constricta* at the heatwave temperature could lead to tissue hypoxia (Pörtner, 2002, 2012). Consequently, the antioxidative defence would be inadequate to counter the elevated oxidative stress, resulting in cellular damage and failure to recover from thermal stress (Heise et al., 2006; Madeira et al., 2013; Pimentel et al., 2015). The higher vulnerability of *A. constricta* to thermal stress likely stems from its higher metabolic rate than the others (Fig. S3), which would generate too much ROS to be sufficiently scavenged by antioxidative enzymes. In other words, our findings highlight the advantage of having low metabolic rate to resist thermal stress (Marshall and McQuaid, 2011).

Although physiological adaptation appears ineffective for metabolically active species to counter acute thermal stress, behavioural adaptation may play an important role. For example, *A. constricta* were not even found under rocks during heatwaves probably because they actively moved to the shallow subtidal zone with lower temperature to minimize thermal stress. Apart from this behavioural response, the white ridged shell of *A. constricta* can reduce heat absorption rate from solar radiation and allow faster heat dissipation (Harley et al., 2009), so that its body temperature can be lower than the black-shelled *A. concamerata* and *N. atramentosa* when on the rock surface. The difference in morphology can also explain why the black-shelled gastropods prefer not to expose to sunlight even at lower air temperature. Indeed, hiding in shaded areas or

moving to thermally favourable locations is the simplest and most effective way to avoid excess heat absorption from solar radiation (Judge et al., 2009; Chapperon and Seuront, 2011). Yet, more gastropods appeared on the rock surface when air temperature decreases, indicating that they can adaptively alter their habitat preference to regulate body temperature to their suitable thermal range (Muñoz et al., 2005; Kearney et al., 2009). In short, we demonstrated that behavioural adaptation can effectively help intertidal organisms lower their body temperature so that the acute thermal stress caused by heatwaves during low tide can be averted.

To date, how heatwaves influence marine ecosystems is less studied due to their rarity in nature, but they usually have substantial impacts on marine organisms over a short period of time (Garrabou et al., 2009; Wernberg et al., 2013). Nevertheless, the adaptability and recoverability of organisms, which allow them to accommodate the impacts of acute thermal stress, have been overlooked. We revealed that intertidal organisms can adaptively modify their physiology, such as upregulation of aerobic metabolism and molecular defence mechanisms, not only to overcome the impacts of heatwaves, but also to recuperate when temperature returns to the ambient level. Although physiological adaptation may not be effective for sensitive species, behavioural adaptation (e.g. hiding) can be exhibited to avoid thermal stress in a short period of time. Our findings suggest that physiological and behavioural adaptations are fundamental to explaining why some marine organisms can accommodate the sudden (heatwaves) and long-term (ocean warming) increase in temperature and hence sustain their populations (Poloczanska et al., 2011; Smale et al., 2015). As such, their ecological functions in the community will likely be maintained despite the transient impacts of heatwaves.

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4.7 SUPPLEMENTARY INFORMATION

Fig. S1 The sampling points for investigating the habitat preference and body temperature of *N. atramentosa*, *A. concamerata* and *A. constricta* in the rocky shore at Marino Rocks. Image retrieved from Google Earth.

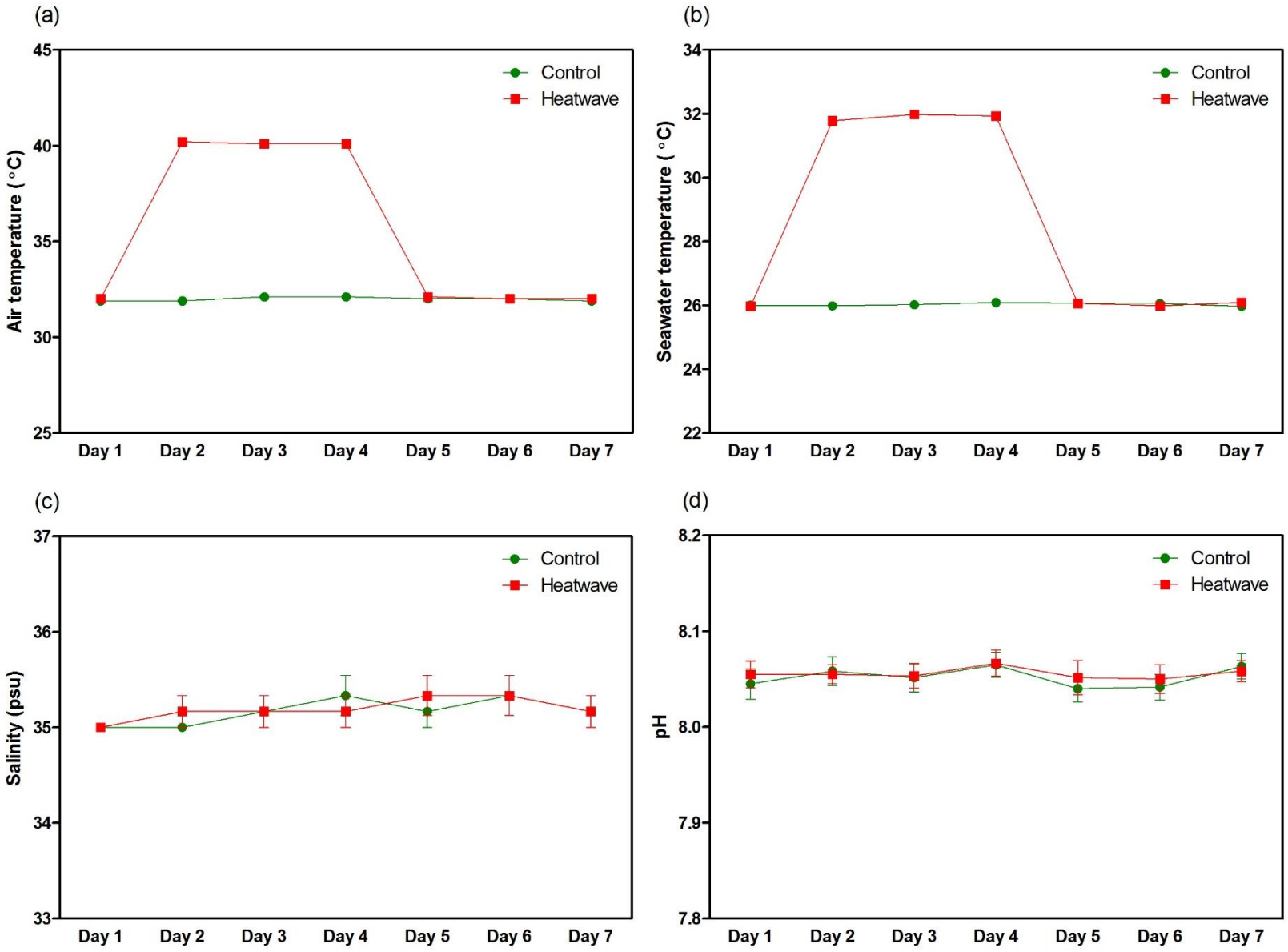

Fig. S2 (a) Air temperature, (b) seawater temperature, (c) salinity and (d) pH in different treatments from Day 1 to Day 7.

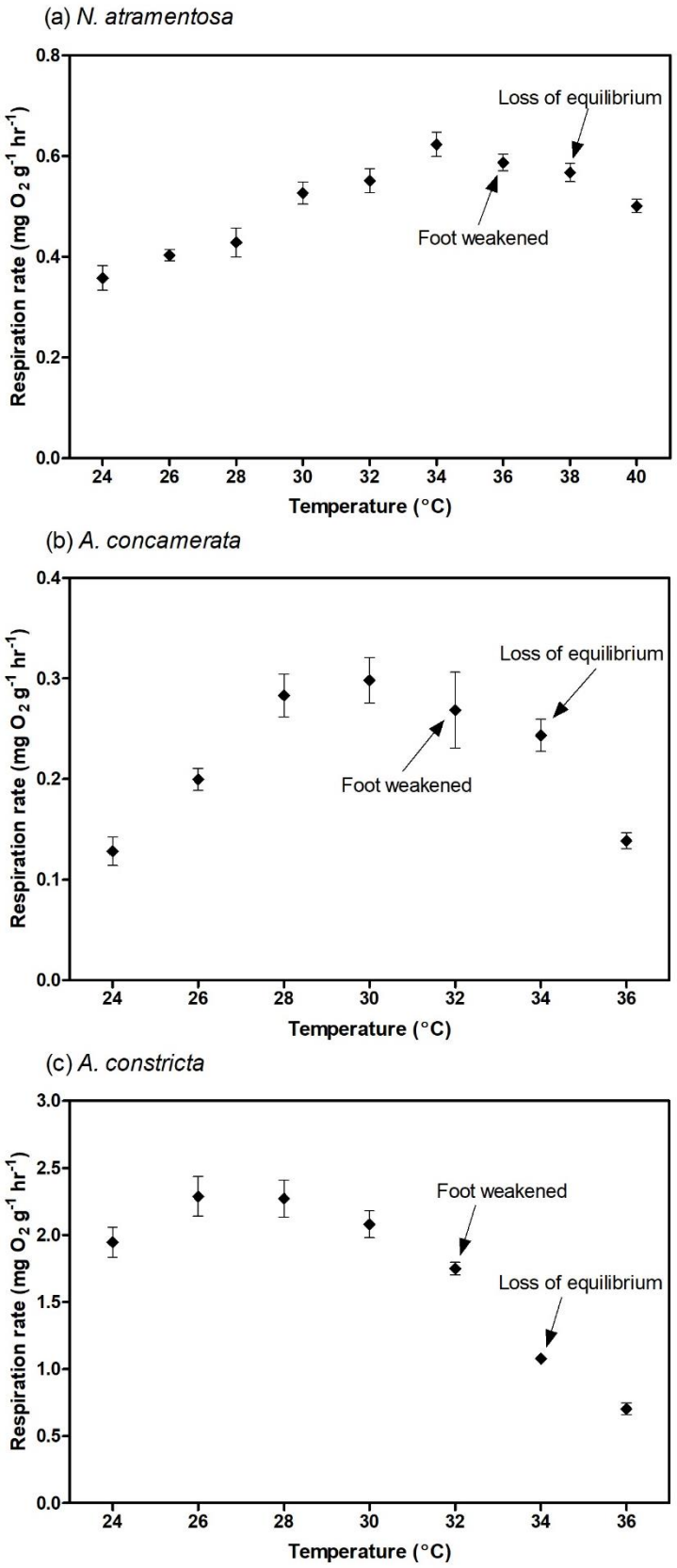

Fig. S3 The respiration rate of (a) *N. atramentosa*, (b) *A. concamerata* and (c) *A. constricta* along an increasing temperature ramp (mean ± S.E., $n = 5$).

Table S1 PERMANOVA table showing the temporal change in feeding rate, respiration rate, heat shock protein (Hsp70), total antioxidant capacity (TAC) and malondialdehyde (MDA), which indicates the effect of heatwaves on gastropods and their recoverability. Different superscript letters represent significant difference.

	df	MS	F	*p*	Comparison of means
Feeding rate					
N. atramentosa	2	0.320	1.04	0.377	
A. concamerata	2	1.20	9.23	**0.002**	Day1^{a} Day6^{a} Day3^{b}
A. constricta	2	0.921	12.5	< **0.001**	Day1^{a} Day6^{b} Day3^{b}
Respiration rate					
N. atramentosa	6	0.423	12.0	< **0.001**	Day1^{a} Day5^{a} Day6^{a} Day7^{a} Day4^{ab} Day3^{bc} Day2^{c}
A. concamerata	6	0.315	5.58	< **0.001**	Day1^{a} Day5^{a} Day6^{a} Day7^{a} Day4^{ab} Day3^{ab} Day2^{b}
A. constricta	6	0.166	8.14	< **0.001**	Day6^{a} Day7^{a} Day5^{ab} Day4^{ab} Day3^{abc} Day1^{bc} Day2^{c}
Hsp70					
N. atramentosa	2	0.119	10.3	**0.003**	Day1^{a} Day7^{a} Day4^{b}
A. concamerata	2	0.097	2.37	0.136	
A. constricta	2	0.007	0.320	0.732	
TAC					
N. atramentosa	2	1.37	4.62	**0.027**	Day1^{a} Day7^{ab} Day4^{b}
A. concamerata	2	1.67	17.2	< **0.001**	Day1^{a} Day7^{a} Day4^{b}
A. constricta	2	1.51	12.6	< **0.001**	Day1^{a} Day7^{b} Day4^{b}
MDA					
N. atramentosa	2	0.019	0.847	0.448	
A. concamerata	2	0.004	0.049	0.953	
A. constricta	2	0.541	4.56	**0.028**	Day1^{a} Day4^{ab} Day7^{b}

Statement of Authorship

Title of Paper	Impacts of near-future ocean acidification and warming on the shell mechanical and geochemical properties of gastropods from intertidal to subtidal zones
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Name of Principal Author (Candidate)	Jonathan Y.S. Leung		
Contribution to the Paper	Conceived, designed and conducted the experiment; analysed the data; wrote the manuscript.		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23/01/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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CHAPTER 5

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Article

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Impacts of Near-Future Ocean Acidification and Warming on the Shell Mechanical and Geochemical Properties of Gastropods from Intertidal to Subtidal Zones

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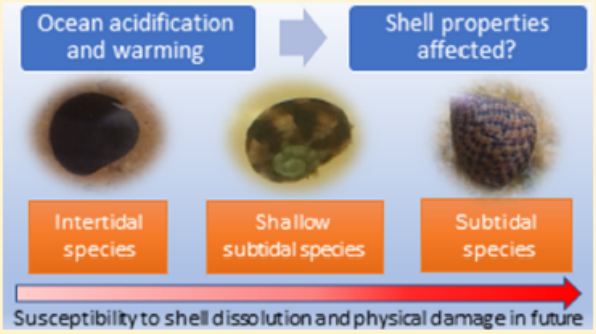
Supporting Information

ABSTRACT: Many marine organisms produce calcareous shells as the key structure for defense, but the functionality of shells may be compromised by ocean acidification and warming. Nevertheless, calcifying organisms may adaptively modify their shell properties in response to these impacts. Here, we examined how reduced pH and elevated temperature affect shell mechanical and geochemical properties of common grazing gastropods from intertidal to subtidal zones. Given the greater environmental fluctuations in the intertidal zone, we hypothesized that intertidal gastropods would exhibit more plastic responses in shell properties than subtidal gastropods. Overall, three out of five subtidal gastropods produced softer shells at elevated temperature, while intertidal gastropods maintained their shell hardness at both elevated pCO_2 (i.e., reduced pH) and temperature. Regardless of pH and temperature, degree of crystallization was maintained (except one subtidal gastropod) and carbonate polymorph remained unchanged in all tested species. One intertidal gastropod produced less soluble shells (e.g., higher calcite/aragonite) in response to reduced pH. In contrast, subtidal gastropods produced only aragonite which has higher solubility than calcite. Overall, subtidal gastropods are expected to be more susceptible than intertidal gastropods to shell dissolution and physical damage under future seawater conditions. The increased vulnerability to shell dissolution and predation could have serious repercussions for their survival and ecological contributions in the future subtidal environment.



INTRODUCTION

In view of the accelerated increase in atmospheric carbon dioxide (pCO_2) concentration, the future impacts of ocean acidification (OA) and warming on marine ecosystems have galvanized substantial concern over the past decade.[1,2] Calcifying organisms are considered to be particularly susceptible to OA based on the assumption that calcification is retarded by the reduced carbonate saturation state in seawater, leading to reduced shell growth.[1,3] Yet, recent evidence reveals that calcification can be maintained or even enhanced under OA conditions because this process is biologically regulated and not necessarily influenced by carbonate saturation state.[4−9] Nevertheless, the properties of calcareous structures (e.g., solubility and mechanical strength of shells and exoskeletons) may still be adversely affected by the reduced pH in seawater, thereby impairing their functionality and possibly diminishing the survival of calcifying organisms.[10−12] Indeed, secular oscillations of pCO_2 and temperature in seawater have caused mass mortality of calcifying organisms when production of their calcareous structures is not favored by the seawater chemistry.[13,14] Therefore, the current increase in pCO_2 concentration at an unprecedented rate may pose a strong selection pressure on calcifying organisms to modify their calcifying mechanism so that their calcareous structures can be functionally suitable in future marine ecosystems.

Most calcifying organisms (e.g., gastropods and bivalves) produce shells as the primary structure for defense against predators, harmful substances, and adverse environmental conditions; therefore, their survival may be diminished if the mechanical strength of their shells is weakened. It is noteworthy that the mechanical strength of shells can be subject to environmental conditions which affect the production of organic matrix occluded in the shell.[15,16] Since pH and temperature are the key factors governing physiological processes, including calcification,[5,17] the mechanical strength of shells may be altered by warmer and more acidic seawater. From the mineralogical perspective, shells are primarily composed of crystalline calcium carbonate formed after crystallization of amorphous calcium

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carbonate (ACC), which is the most soluble and unstable form.[15] This process may also be hampered by reduced pH through physiological pathways,[18,19] possibly compromising shell integrity and mechanical strength.[12,20] Furthermore, shell dissolution during calcification may be intensified under OA conditions.[3] One of the potential adaptive strategies to minimize shell dissolution is to produce less soluble shells, possibly by changing carbonate polymorphs (i.e., calcite and aragonite) or regulating the incorporation of magnesium ions in calcite.[9,13,14,21,22] Yet, whether calcifying organisms can alter their shell geochemical properties in response to OA conditions remains less understood, especially when ocean warming may further modify these properties.[23,24] In this regard, how reduced pH and elevated temperature influence the mechanical strength, crystallization, and solubility of shells deserves further investigation to shed light on the acclimation ability of calcifying organisms to future seawater conditions.

In this study, we examined the impacts of near-future ocean acidification and warming on the shell mechanical (shell hardness and elastic modulus) and geochemical (relative ACC content, calcite/aragonite, and Mg/Ca in calcite) properties to assess how the mechanical strength and solubility of shells are affected by the future seawater conditions. Then we evaluated whether calcifying organisms can alter these properties to accommodate the future seawater conditions. Grazing gastropods from intertidal to subtidal zones were selected because of their important roles in maintenance of ecosystem health and trophic dynamics in coastal and marine ecosystems.[25] Since intertidal species are exposed to large fluctuations in pH and temperature, we hypothesized that intertidal gastropods have a greater capability to modify their shell properties to accommodate the future seawater conditions (e.g., elevated calcite/aragonite or reduced Mg/Ca in calcite) and hence make their shells less susceptible to dissolution than those of subtidal gastropods. In contrast, species which cannot exhibit plasticity in their calcifying mechanism will likely produce shells that are less functionally suitable in future marine ecosystems, possibly leading to profound ecological consequences (e.g., reduced survival due to higher susceptibility to shell dissolution or predator attack).

MATERIALS AND METHODS

Collection of Specimens and Experimental Conditions. Seven species of common grazing gastropods were collected from intertidal to subtidal zones in South Australia (35°02′−35°36′ S, 138°30′−138°06′ E). They were categorized into three groups based on their position in the tidal range: (1) intertidal (*Nerita atramentosa* and *Austrocochlea constricta*), (2) shallow subtidal (*Austrocochlea odontis* and *Turbo undulatus*), and (3) subtidal (*Thalotia conica*, *Phasianella australis*, and *Bulla quoyii*). Upon return to the laboratory, individuals were exposed to one of the crossed combinations of pCO_2 and temperature in experimental aquaria for 4 months (see Supporting Information for the rearing conditions). Two levels of pCO_2 (400 vs 1000 ppm) and temperature (26 vs 30 °C for intertidal species; 21 vs 24 °C for shallow subtidal and subtidal species) were chosen in this study, where the ambient temperatures were based on in situ measurement of seawater at the collection site in summer. The elevated pCO_2 and temperature levels simulate the predicted RCP8.5 scenario for the year 2100.[26]

Shell Analyses. After the exposure to experimental conditions, the newly produced shell at the growing edge (i.e., outer lip) was collected, washed with deionized water, and air-dried at room temperature for analyses of mechanical and geochemical properties. Given the limited shell growth of some species, shells from three to four individuals from the same treatment were used to make a composite sample, except the test for mechanical properties.

Vickers hardness and elastic modulus (i.e., a measure of resistance to elastic deformation) of shells were determined using a microhardness tester (Fischerscope HM2000, Fischer, Germany). A shell fragment was mounted firmly onto a metal disc using cyanoacrylate adhesives (n = 4 fragments from four individuals per treatment). Then the shell surface was indented by a Vickers four-sided diamond pyramid indenter for 10 s in the loading phase (peak load, 300 mN; creep, 2 s). In the unloading phase, the load decreased at the same rate as the loading phase until the loading force became zero. At least five random locations on each shell fragment were indented. Vickers hardness and elastic modulus were calculated based on the load−displacement curve using software WIN-HCU (Fischer, Germany).

Relative amorphous calcium carbonate (ACC) content was determined using a Fourier transform infrared spectrometer (Spectrum 100, PerkinElmer, USA). A small quantity of shell powder was transferred onto the sample holder to obtain the infrared absorption spectrum, ranging from 400 to 1800 cm^{-1} with background correction (n = 4 replicates per treatment). The relative ACC content was estimated as the intensity ratio of the peak at 856 cm^{-1} to that at 713 cm^{-1}.[27]

Carbonate polymorphs were analyzed using an X-ray diffractometer (D4 ENDEAVOR, Bruker, Germany). A small quantity of shell powder was transferred onto a tailor-made sample holder and then scanned by Co Kα radiation (35 kV and 30 mA) from 20° to 70° 2θ with step size of 0.018° and step time of 1 s (n = 4 replicates per treatment). Carbonate polymorphs in the shell powder were identified based on the X-ray diffraction spectrum using the EVA XRD analysis software (Bruker, Germany). Calcite to aragonite ratio was estimated according to the following equation, with a correlation coefficient of 0.9999:[28]

$$\frac{I_C^{104}}{I_A^{221}} = 3.157 \times \frac{X_C}{X_A}$$

where I_C^{104} and I_A^{221} are intensity of the calcite 104 peak (34.4° 2θ) and aragonite 221 peak (54.0° 2θ), respectively; X_C/X_A is the calcite to aragonite ratio.

Magnesium to calcium ratio was determined by energy dispersive X-ray spectroscopy under the Philips XL 30 field emission scanning electron microscope. A small quantity of shell powder was transferred onto a stub and coated by carbon (n = 4 replicates per treatment; three trials per replicate). Then the shell powder was irradiated by an electron beam with an accelerating voltage of 12 kV to obtain the energy spectrum with background correction. The elements in the shell powder were identified and magnesium to calcium ratio was calculated using software Genesis Spectrum SEM Quant ZAF (EDAX, USA).

Statistical Analyses. Two-way permutational analysis of variance (PEMANOVA) was applied to test the effects of pCO_2 and temperature on all the aforementioned shell properties using software PRIMER 6 with PERMANOVA+ add-on.

RESULTS

The shell hardness of *N. atramentosa*, *A. odontis*, and *T. conica* was unaffected by elevated pCO_2 and temperature (Figure 1, Table

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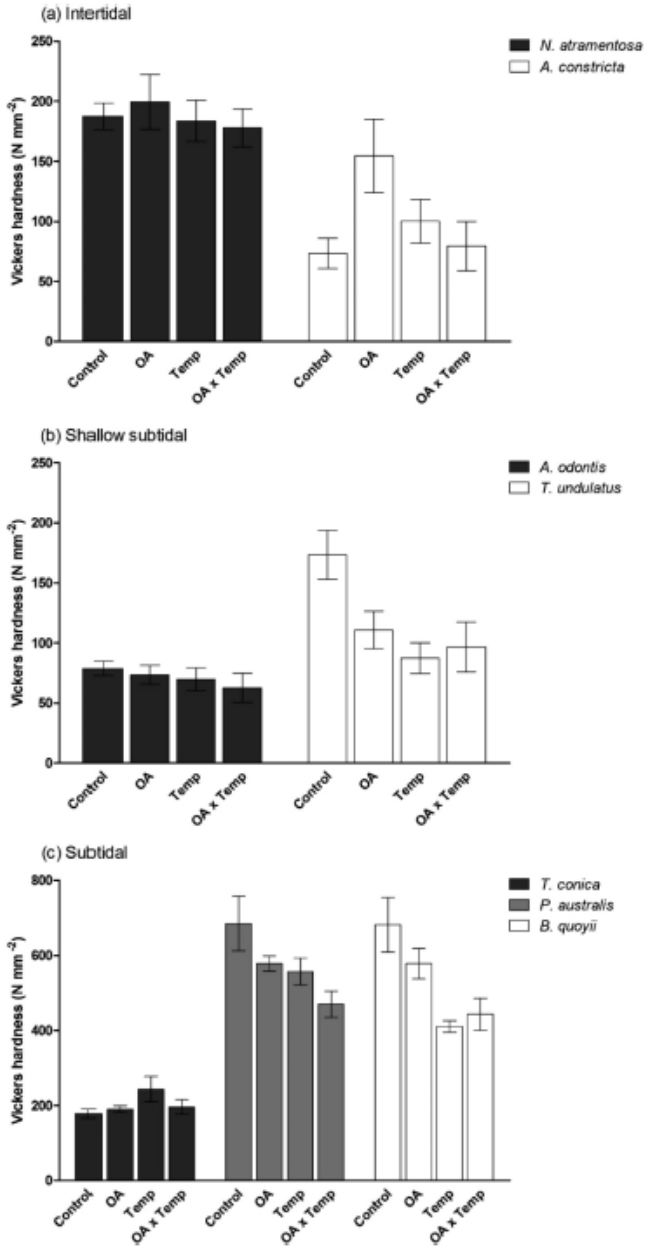

Figure 1. Vickers hardness of gastropod shells produced under different treatment conditions (mean ± S.E.). The gastropods are categorized according to their tidal position: (a) intertidal, (b) shallow subtidal, and (c) subtidal. Control: ambient pCO_2 × ambient temperature. OA: elevated pCO_2 × ambient temperature. Temperature (Temp): ambient pCO_2 × elevated temperature. OA × Temp: elevated pCO_2 × elevated temperature.

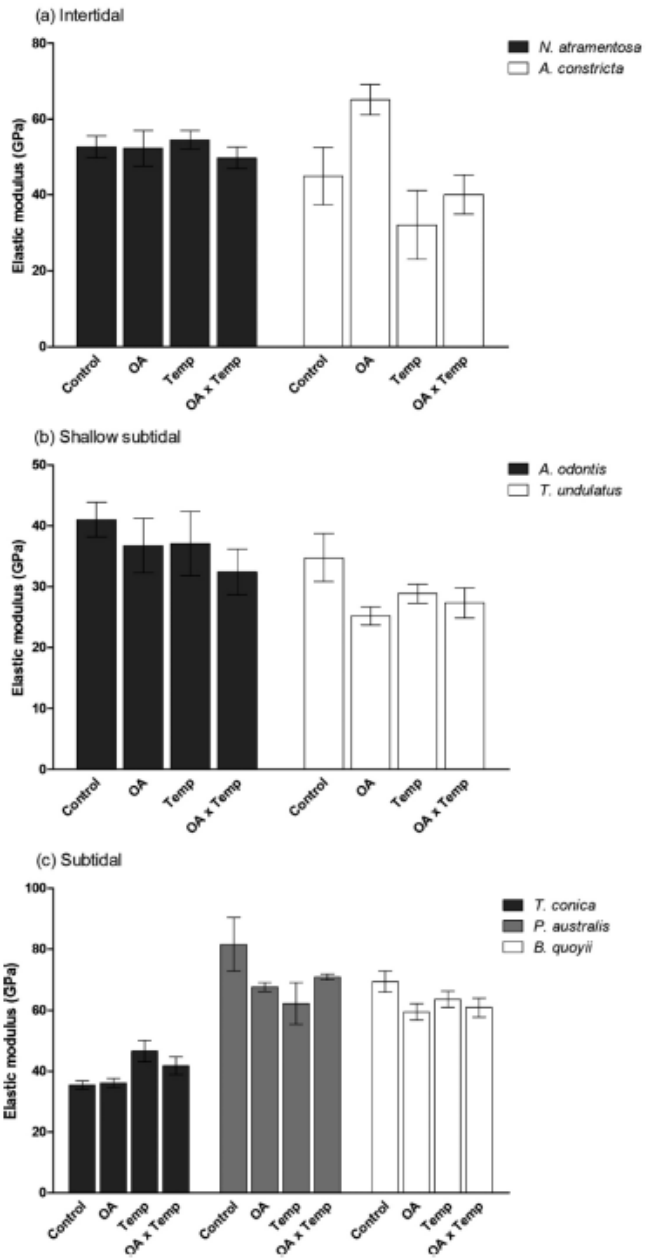

Figure 2. Elastic modulus of gastropod shells produced under different treatment conditions (mean ± S.E.). The gastropods are categorized according to their tidal position: (a) intertidal, (b) shallow subtidal, and (c) subtidal.

S1). Under OA conditions, *A. constricta* produced harder shells at ambient temperature, but not at elevated temperature. The shell hardness of *T. undulatus*, *P. australis*, and *B. quoyii* was reduced at elevated temperature. Overall, three out of five subtidal gastropods produced softer shells at elevated temperature, while intertidal gastropods maintained their shell hardness at both elevated pCO_2 (i.e., reduced pH) and temperature. The elastic modulus of shells was neither affected by pCO_2 nor temperature for all the tested species, except *A. constricta* and *T. conica* (Figure 2, Table S1). *A. constricta* produced stiffer shells (i.e., higher elastic modulus)at elevated pCO_2, but more elastic shells at elevated temperature. *T. conica* produced stiffer shells at elevated temperature.

Concerning the geochemical properties, the relative ACC content was neither affected by pCO_2 nor temperature for all the tested species, except *T. conica* which had slightly higher relative ACC content under OA conditions (i.e., less crystalline shells) (Table 1, Table S1). Regardless of treatment conditions, six out of seven tested species were monomineralic, where only *N. atramentosa* produced calcite (Table 1). *A. constricta* was the only tested species that can produce both calcite and aragonite (i.e., bimineralic). Although aragonite was the predominant carbonate polymorph in the shell of *A. constricta*, the proportion of calcite increased under OA conditions (i.e., higher calcite to aragonite ratio) (Figure 3, Table S1). Elevated pCO_2 and temperature led

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Table 1. Carbonate Polymorph and Relative Amorphous Calcium Carbonate Content in the Gastropod Shells Produced under Different Treatment Conditions (Mean ± S.E.)[a]

tidal level	species	carbonate polymorph	relative amorphous calcium carbonate content			
			control	OA	Temp	OA × Temp
intertidal	*N. atramentosa*	calcite	1.91 ± 0.05	1.94 ± 0.08	1.85 ± 0.01	1.91 ± 0.08
	A. constricta	aragonite + calcite	3.15 ± 0.12	3.06 ± 0.04	3.23 ± 0.10	3.18 ± 0.07
shallow subtidal	*A. odontis*	aragonite	2.15 ± 0.02	2.15 ± 0.03	2.12 ± 0.05	2.18 ± 0.04
	T. undulatus	aragonite	2.12 ± 0.05	2.11 ± 0.07	2.15 ± 0.07	2.08 ± 0.08
subtidal	*T. conica*	aragonite	2.02 ± 0.06	2.15 ± 0.04	2.11 ± 0.05	2.17 ± 0.00
	P. australis	aragonite	2.32 ± 0.03	2.34 ± 0.03	2.33 ± 0.02	2.31 ± 0.05
	B. quoyii	aragonite	2.00 ± 0.02	1.96 ± 0.01	2.00 ± 0.05	2.04 ± 0.02

[a]Control: ambient pCO_2 × ambient temperature. OA: elevated pCO_2 × ambient temperature. Temp: ambient pCO_2 × elevated temperature. OA × Temp: elevated pCO_2 × elevated temperature.

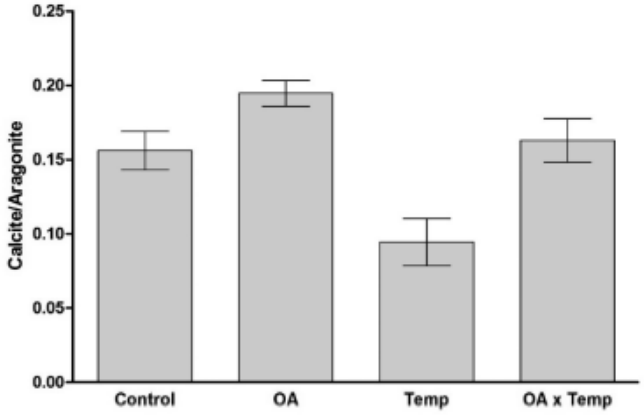

Figure 3. Calcite to aragonite ratio in the shell of intertidal *Austrocochlea constricta* produced under different treatment conditions (mean ± S.E.).

to a higher Mg/Ca in the calcitic shell of *N. atramentosa*, but Mg/Ca in the calcite of *A. constricta* was reduced at elevated temperature (Figure 4, Table S1). In short, intertidal gastropods showed more plastic responses in the shell geochemical properties than subtidal gastropods.

■ DISCUSSION

Shells are the vital structure for defense against predators, and thus the mechanical strength of shells is strongly related to the survival of calcifying organisms. Here, we demonstrated that the ability of gastropods to maintain such defense under future seawater conditions is species-specific. Yet, we found that the shell hardness of intertidal gastropods is unaffected by reduced pH and elevated temperature, whereas some subtidal gastropods produce softer shells at elevated temperature, which are less resistant to compressive force (e.g., crushing by carnivorous crabs). Because shell hardness is chiefly contributed by the quantity of organic matrix occluded in the shell rather than the pure calcium carbonate minerals,[15,16] the adverse effect of elevated temperature could be attributed to the thermal stress posed on physiological processes (e.g., production of organic matrix).[12,29] In fact, many subtidal species are vulnerable to the predicted elevated temperature in the near future.[2,30] Under thermal stress, less energy can be allocated to calcification due to the higher energetic cost of cellular maintenance,[31] possibly resulting in the formation of softer shells. Interestingly, the intertidal gastropod *A. constricta* produced harder and stiffer shells under OA conditions, meaning that the shells are stronger. However, softer and more elastic shells were produced when combined with elevated temperature, meaning that mechanical

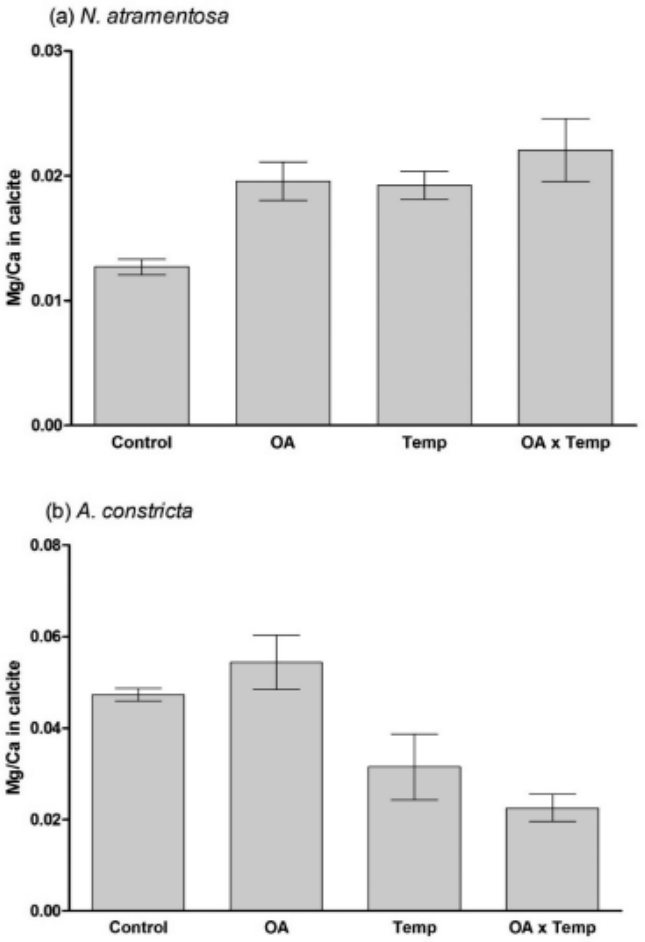

Figure 4. Magnesium to calcium ratio in the calcite of intertidal (a) *Nerita atramentosa* and (b) *Austrocochlea constricta* produced under different treatment conditions (mean ± S.E.).

strength can be maintained under future seawater conditions (c.f. control). Indeed, temperature was shown to be the key factor in modulating the impact of OA on the mechanical properties of shells via physiological processes.[12,32]

Ocean acidification is expected to retard calcification and possibly jeopardize the fitness and survival of calcifying organisms.[3,13,14] The effect of reduced pH on calcification may first appear during crystallization of ACC. Since ACC is the transient phase of crystalline calcite or aragonite, its quantity in the shell can reflect the degree of crystallization. In this study, crystallization of ACC appeared to be unaffected by reduced pH

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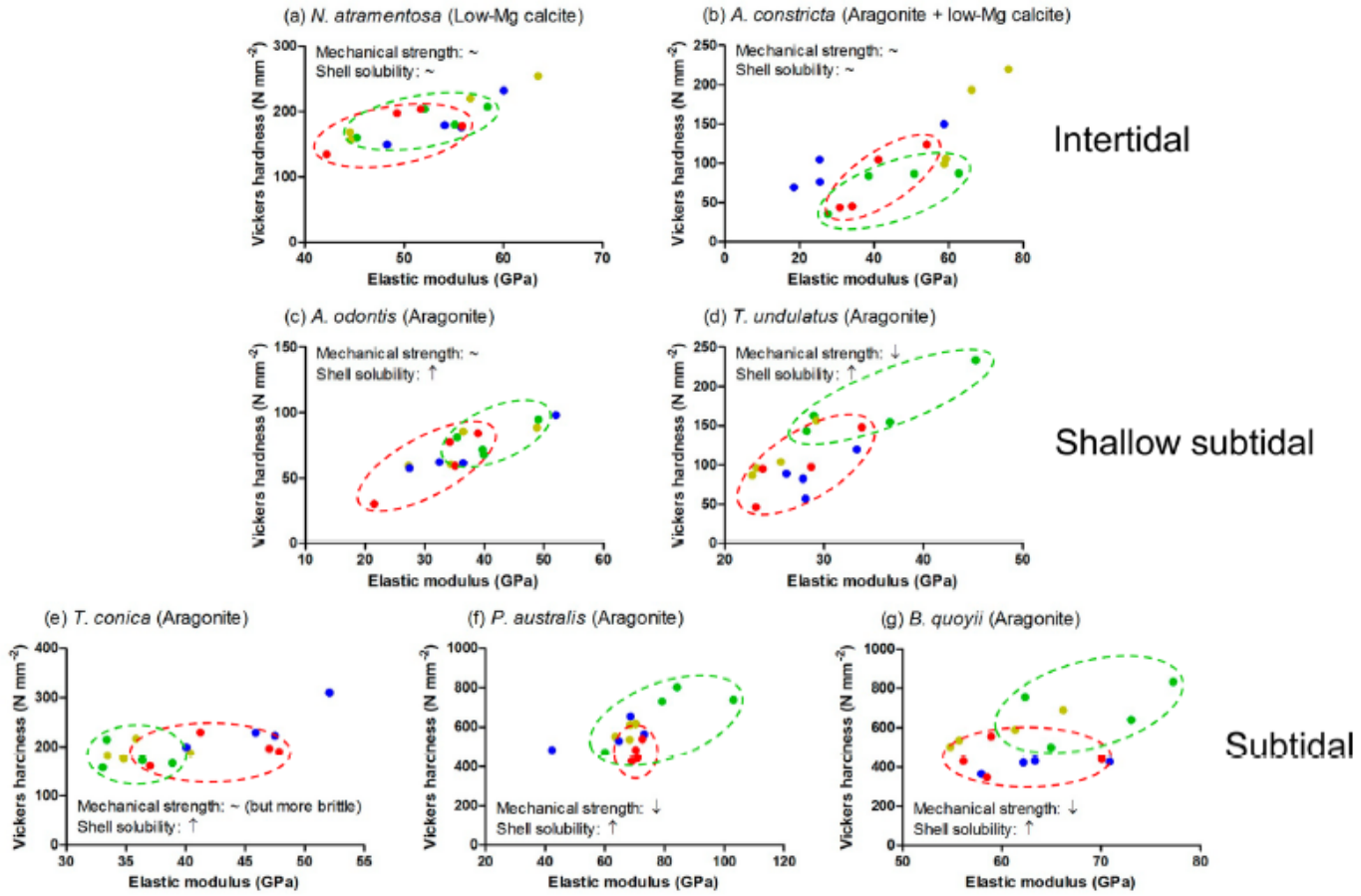

Figure 5. Predicted impacts of future seawater conditions (i.e., combined elevated pCO_2 and temperature) on the solubility and mechanical strength of gastropod shells. The susceptibility of aragonitic shells to dissolution is predicted to increase under ocean acidification, while the susceptibility of calcitic shells remains low when low-Mg calcite is produced. The mechanical strength of shells is reduced when shell hardness decreases. The dashed ellipses are drawn to help visualize whether the mechanical properties of shells are different in samples between the current (green) and future (red) seawater conditions (green dot, control; yellow dot, OA; blue dot, temperature; red dot, OA × temperature).

for all the tested species (except the subtidal gastropod *T. conica*), probably due to biological regulation of intracellular pH (e.g., proton regulation).[5] The slightly less crystalline shell of *T. conica* produced under OA conditions could be attributed to disruption in crystallographic control,[20] intracellular ion homeostasis,[19] or synthesis of macromolecules (e.g., proteins) for stabilization of ACC.[18] Regardless of the underlying mechanism, the less crystalline shell indicates reduced shell integrity, which is not favorable.[11,20]

Even though crystallization of ACC can be maintained, as demonstrated here, reduced pH in seawater may intensify shell dissolution during calcification.[3] To minimize shell dissolution, precipitating calcite rather than aragonite is regarded as an adaptive strategy due to lower solubility of the former.[9,13,21] Some corals, for example, have been shown to change the carbonate polymorph of their skeletons from aragonite to calcite when the seawater chemistry favors precipitation of calcite.[34] Indeed, secular oscillations of pCO_2 have posed a strong selection pressure on calcifying organisms to modify the carbonate polymorph of their shells.[13,33] In this study, however, the carbonate polymorph was species-specific and all the monomineralic aragonite-producing gastropods showed no sign of producing calcite under OA conditions. The inability to alter the carbonate polymorph of shells in response to reduced pH may pose adverse effects on calcification. For example, obligate aragonite-producing corals suffer from delayed initiation of calcification and skeletal growth under OA conditions.[36,37] Although the monomineralic gastropods in this study did not change the carbonate polymorph of their shells, the effect of OA may be manifested across generations. For example, juvenile mussels (*Mytilus edulis*) whose parents were exposed to elevated pCO_2 (1000 μatm) only produce calcite, while both calcite and aragonite can be produced by those spawn and grown at ambient pCO_2 level.[11] The transgenerational effect of OA on shell mineralogy remains largely unexplored and warrants further investigation.

For the only bimineralic gastropod (i.e., *A. constricta*) in this study, the effects of reduced pH and elevated temperature on calcite to aragonite ratio were discernible. On the basis of kinetics and thermodynamics, calcite is less soluble than aragonite but its stability decreases with increasing temperature,[23] suggesting an antagonistic effect between reduced pH and elevated temperature on precipitation of calcite. This theoretical prediction is partially substantiated by our findings, where a greater proportion of calcite was precipitated at reduced pH, but the elevated temperature insignificantly lowered the proportion of calcite. The increased precipitation of calcite under OA conditions could be mediated by preferential dissolution of aragonite.[35] For example, Fitzer et al. showed that the calcitic layer of mussel shells continues to grow under OA conditions, while the growth of aragonitic layer ceases.[11] Because of the higher solubility of aragonite, an increase in precipitation of

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calcite under OA conditions can also minimize the energetic cost of regulating the pH of calcifying fluid at the calcification site,[13,33] and thus allow more flexible energy allocation to other physiological processes. Nevertheless, the increased precipitation of calcite under OA conditions was offset by elevated temperature; consequently, the proportion of calcite under future seawater conditions was similar to that under contemporary conditions (i.e., control).

For calcite-producing organisms, magnesium ions in seawater are incorporated into calcite during calcification, increasing shell solubility; however, calcifying organisms may regulate the incorporation of magnesium ions to minimize the impact.[35,38] Theoretically, low-Mg calcite (Mg/Ca < 0.04) is preferentially precipitated under OA conditions due to lower solubility, which provides a positive feedback mechanism to counter shell dissolution.[22] Indeed, calcite-producing organisms were shown to produce shells of lower Mg/Ca under OA conditions, making the shell more chemically stable.[35,39] Contrary to our prediction, a higher Mg/Ca was found in *N. atramentosa* under OA conditions, which may be due to the change in calcification rate or calcium metabolism.[40] Temperature can also affect the incorporation of magnesium ions into calcite, where a positive correlation is mostly observed in marine organisms.[41] For example, Kamenos et al. showed that the Mg/Ca of coralline algae increases at a rate of ~0.02 $°C^{-1}$.[42] In this study, increase in Mg/Ca with temperature was observed only in *N. atramentosa*, while an opposite trend was found in *A. constricta*. The mechanism underlying this contrasting trend requires further investigation as how temperature affects magnesium incorporation into calcite is still not fully understood.[38] Nevertheless, both *N. atramentosa* and *A. constricta* still produced low-Mg calcite (Mg/Ca < 0.03) at elevated pCO_2 and temperature, indicating that their shells are resistant to dissolution under future seawater conditions.[22,35]

As oceans acidify and warm at an unprecedented rate, calcifying organisms will be under selection pressure to alter their shell properties to maintain shell functions, or their survival may be diminished as observed in the past mass extinction events.[13,14] On the basis of the foregoing account, we conclude that crystallization of ACC can be maintained under future seawater conditions, but the monomineralic gastropods are unable to alter the carbonate polymorph of their shells. Since the subtidal gastropods produce only aragonite, which is more soluble than calcite, it implies that more energy will be required to maintain calcification in future marine ecosystems, which leads to energy trade-offs against other physiological processes (e.g., production of organic matrix). In addition, the weaker mechanical strength and higher susceptibility to dissolution of their shells indicate higher vulnerability to predator attack, such as crushing by carnivorous crabs or boring by molluscivores (e.g., muricid gastropods). In contrast, the shells of intertidal gastropods appeared to be robust to future seawater conditions. In particular, the plastic responses exhibited by *A. constricta* (e.g., changes in calcite/aragonite and Mg/Ca) can make the shell less susceptible to dissolution. Our findings imply that the future impacts of OA and warming on the fitness of subtidal gastropods would be more significant than that of intertidal gastropods (Figure 5), possibly affecting the ecological functions in the future subtidal environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b02359.

Rearing conditions, PERMANOVA table, and carbonate system parameters of seawater (PDF)

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Notes

The authors declare no competing financial interest.

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Contribution to the Paper	Conceived, designed and conducted the experiment; analysed the data; wrote the manuscript.		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Helped develop and revise the manuscript; provided funding.		
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CHAPTER 6

Environmental Science & Technology

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Mineralogical Plasticity Acts as a Compensatory Mechanism to the Impacts of Ocean Acidification

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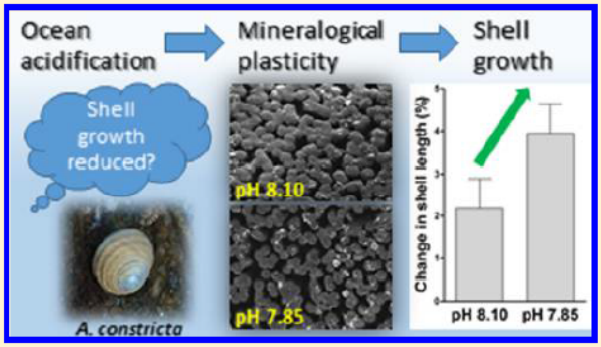
Supporting Information

ABSTRACT: Calcifying organisms are considered particularly susceptible to the future impacts of ocean acidification (OA), but recent evidence suggests that they may be able to maintain calcification and overall fitness. The underlying mechanism remains unclear but may be attributed to mineralogical plasticity, which modifies the energetic cost of calcification. To test the hypothesis that mineralogical plasticity enables the maintenance of shell growth and functionality under OA conditions, we assessed the biological performance of a gastropod (respiration rate, feeding rate, somatic growth, and shell growth of *Austrocochlea constricta*) and analyzed its shell mechanical and geochemical properties (shell hardness, elastic modulus, amorphous calcium carbonate, calcite to aragonite ratio, and magnesium to calcium ratio). Despite minor metabolic depression and no increase in feeding rate, shell growth was faster under OA conditions, probably due to increased precipitation of calcite and trade-offs against inner shell density. In addition, the resulting shell was functionally suitable for increasingly "corrosive" oceans, i.e., harder and less soluble shells. We conclude that mineralogical plasticity may act as a compensatory mechanism to maintain overall performance of calcifying organisms under OA conditions and could be a cornerstone of calcifying organisms to acclimate to and maintain their ecological functions in acidifying oceans.



INTRODUCTION

The future impacts of ocean acidification (OA) on marine ecosystems, primarily driven by elevated atmospheric carbon dioxide concentration (pCO_2), have drawn substantial attention over the past decade.[1] It is now well-established that OA can worsen the overall performance of many marine organisms through impaired metabolism, feeding, growth, and calcification,[2] implying that OA will bring penetrating changes to future marine ecosystems.[3] In recent years, however, some studies showed that marine organisms are able to acclimate to elevated pCO_2 (or reduced pH) conditions after a prolonged exposure period so that their overall performance and fitness can be maintained or even enhanced.[4−6] This indicates that homeostasis can be maintained under OA conditions,[7] but the underlying mechanisms remain less understood.

Under OA conditions, an additional metabolic cost is incurred to maintain acid−base balance or homeostasis will be disrupted by metabolic depression due to the uncompensated extracellular pH.[8] To offset this metabolic cost, the metabolic rate can be upregulated,[9,10] which determines the amount of metabolic energy produced for various physiological and biochemical processes. This strategy can provide temporary relief but may be maladaptive in the long term because of the elevated use of energy budget or energy trade-offs against other physiological processes.[11] In this regard, homeostasis is more likely achieved by regulating energy budget or energy demand.[12] For example, marine organisms can partition their metabolic energy among somatic growth, calcification, maintenance, defense, locomotion, and reproduction to maximize fitness.[13,14] Yet, how marine organisms reallocate their metabolic energy or modify their energy demand to maintain homeostasis, and hence fitness, under OA conditions still warrants further investigation.

Calcification is one of the most energy-demanding processes,[15] and shell growth is enabled when surplus energy is available. Given the reduced saturation state of carbonate in acidified seawater, the energetic cost of calcification will be elevated, possibly leading to slower shell growth and weaker shell strength.[16−18] Nevertheless, shell growth is obligate to increase shell strength for defenses against predators and shell size for continuing somatic growth. It is noteworthy that the energetic cost of calcification depends not only on the quantity of shell produced but also on the quality as related to the

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biological control over shell mineralogy, such as proportion of carbonate polymorphs (i.e., calcite and aragonite), magnesium content in calcite, and quantity of organic matrix produced to increase shell strength or crystallize amorphous calcium carbonate.[19−21] Such mineralogical plasticity, which modifies the energetic cost of calcification and physicochemical properties of shells, may be fundamental to maintain shell growth and integrity (e.g., shell density and strength) under OA conditions and account for the inconsistent responses of calcifying organisms to OA.[22]

Here, we examined the impacts of near-future OA on the performance of a gastropod *Austrocochlea constricta* by quantifying feeding rate, respiration rate, somatic growth, and shell growth. Given the theoretical elevated energetic cost and inconsistent results of calcification under OA conditions, we tested the hypothesis that this gastropod can exhibit mineralogical plasticity to maintain shell growth and integrity by measuring shell mechanical (hardness and elastic modulus) and geochemical properties (amorphous calcium carbonate, calcite to aragonite ratio, and magnesium to calcium ratio). If calcifying organisms can exhibit mineralogical plasticity as a compensatory response to OA, it may be the key acclimation mechanism that not only allows more flexible energy allocation and maintenance of homeostasis but also renders their shells more functionally suitable for the future "corrosive" ocean.

MATERIALS AND METHODS

Collection of Gastropods. Adult *A. constricta* (shell length: 13−17 mm) were collected from the rocky shore at Marino Rocks (35°20′39″S, 138°30′30″E), South Australia. The individuals were allowed to acclimate to ambient conditions in a plastic holding aquarium (1 m × 40 cm × 30 cm) filled with natural seawater (pH 8.05 ± 0.10, temp 24 ± 1 °C, salinity 35 ± 1 ppt, dissolved oxygen 6.5 ± 0.1 mg O_2 L^{-1}) in a greenhouse under natural day/night cycles for 2 weeks. Food was provided as epiphytes on rocks from the collection site. Seawater (~50%) in the aquarium was renewed weekly.

Experimental Setup. Two levels of pCO_2 (400 and 1000 ppm), which correspond to pH ~8.10 and ~7.85 respectively, were chosen in this study. The elevated pCO_2 level simulates the predicted RCP8.5 scenario for the year 2100.[23] Following acclimation, three individuals (shell length: 15.2 ± 1.0 mm) were transferred into a plastic aquarium (30 cm × 20 cm × 18 cm) filled with ~5 L natural seawater adjusted to one of the experimental pCO_2 levels for 10 weeks (n = 5 replicate aquaria per pCO_2). Elevated pCO_2 (or reduced pH) was achieved by aerating the seawater with CO_2-enriched atmospheric air using a gas mixer (Pegas 4000 MF, Columbus Instruments, Columbus, OH). Ambient temperature of seawater was maintained by placing the aquaria in a water bath that was maintained at 24 °C using a heater/chiller unit (TC60, TECO, Italy). Food was provided as epiphytes on rocks from the collection site and replenished weekly. Seawater (~50%) was replaced weekly to prevent accumulation of metabolic waste.

Temperature and pH of seawater were measured twice a day using a pH/temperature meter (HI 98128, HANNA Instruments, Vöhringen, Germany), calibrated using NBS buffers. Salinity was measured weekly using a hand-held refractometer. Total alkalinity was also measured weekly using a potentiometric titrator (888 Titrando, Metrohm, Herisau, Switzerland) calibrated using buffers purchased from the same manufacturer (Metrohm, Herisau, Switzerland). The pCO_2, dissolved inorganic carbon (DIC), and saturation states of calcite (Ω_{cal}) and aragonite (Ω_{ara}) were calculated using the CO2SYS program,[24] with dissociation constants from Mehrbach et al.[25] refitted by Dickson and Millero.[26] In addition, all the carbonate system parameters of seawater in the study site were measured (n = 10 replicate seawater samples), where the pH of seawater was measured once every 2 h to examine the diel variation (Figure S1). The carbonate system parameters of seawater in the study site were similar to those in the pH 8.10 treatment (Table 1), meaning that this treatment provides a good representation for the current natural conditions.

Table 1. Carbonate System Parameters of Seawater in the Study Site and Aquaria Throughout the Experimental Period (Mean ± S.E.)

	natural seawater	pH 8.10	pH 7.85
measured parameters			
salinity (ppt)	34.8 ± 0.13	34.6 ± 0.10	34.5 ± 0.17
temp (°C)	23.5 ± 0.24	23.6 ± 0.03	23.8 ± 0.02
total alkalinity (μmol kg^{-1})	2085 ± 41.0	2038 ± 29.6	2099 ± 27.8
pH (NBS scale)	8.09 ± 0.009	8.08 ± 0.007	7.86 ± 0.009
pH range	7.96−8.22	7.98−8.18	7.72−8.00
calculated parameters			
pCO_2 (ppm)	466 ± 9.09	467 ± 11.2	881 ± 27.6
DIC (μmol kg^{-1})	1865 ± 38.7	1824 ± 25.6	1974 ± 27.0
Ω_{cal}	3.73 ± 0.08	3.64 ± 0.11	2.43 ± 0.06
Ω_{ara}	2.45 ± 0.05	2.39 ± 0.07	1.60 ± 0.04

Somatic and Shell Growth. To quantify growth, the shell length and total weight of all individuals were measured in weeks 0, 5, and 10 of the experimental period using a digital calliper and an electronic balance, respectively (n = 3 individuals per aquarium per pCO_2). Following the feeding and respiration experiments (see the next section), all individuals were dissected to obtain the flesh weight which was further separated into organ weight and foot weight. In addition, the total weight, flesh weight, and organ weight of individuals newly collected from the rocky shore were measured to estimate the change in the ratios of flesh weight to shell weight and organ weight to flesh weight (n = 15 individuals). All of the weights were measured on a fresh weight basis.

Feeding and Respiration. The feeding rate and respiration rate of all individuals were measured following the 10-week exposure period (n = 3 individuals per aquarium per pCO_2). For feeding rate, the individuals, which had been starved for 2 days before experimentation to standardize their hunger level, were allowed to feed on the epiphytes in their aquarium for 1 h. The amount of food consumed was determined by the change in total weight before and after feeding trials. The initial and final total weights were measured to the nearest 0.0001 g after blotting the shell and removing the seawater inside the shell by gently tapping the operculum. The feeding rate is calculated according to the following formula:

$$\text{feeding rate} = \frac{\text{final total weight} - \text{initial total weight}}{\text{feeding time}}$$

For respiration rate, one individual was transferred into a 73 mL airtight chamber filled with experimental seawater (i.e., pCO_2 manipulated) and allowed to acclimate for 1 h. Then, the seawater in the chamber was fully replaced with oxygen-

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saturated experimental seawater, followed by sealing the chamber for 1 h. The initial and final dissolved oxygen concentrations of the chamber water were measured using an optical dissolved oxygen probe (LDO101, HACH, USA). Blank samples without individuals were used to correct the background change in oxygen concentration.

Shell Properties. Prior to shell analyses, the shells were washed with deionized water and air-dried at room temperature. Only the shell at the growing edge (i.e., outer lip) was collected and used. Given the limited amount of newly formed shell, shells from three to four individuals from the same treatment were used to make a composite sample.

Shell hardness and elastic modulus were determined using a microhardness tester (Fischerscope HM2000, Fischer, Germany) with load resolution of 40 μN and displacement resolution of 0.1 nm. A flat shell fragment was mounted firmly onto a metal disk using cyanoacrylate adhesives (n = 4 fragments from 4 individuals per pCO_2). Then, the shell surface was indented by a Vickers 4-sided diamond pyramid indenter for 10 s in the loading phase (peak load, 300 mN; creep, 2 s). In the unloading phase, the load decreased at the same rate as the loading phase until the loading force became zero. At least six random locations on each shell fragment were indented. Vickers hardness and elastic modulus were calculated based on the load−displacement curve using software WIN-HCU (Fischer, Germany).

Amorphous calcium carbonate (ACC) was determined using a Fourier transform infrared spectrometer (Spectrum 100, PerkinElmer, USA). A small quantity of shell powder was added onto the sample holder to obtain the infrared absorption spectrum, ranging from 400 to 1800 cm^{-1} with background correction (n = 4 replicates per pCO_2). The relative ACC content was estimated as the intensity ratio of the peak at 856 cm^{-1} to that at 713 cm^{-1}.[27]

Carbonate polymorphs were analyzed using an X-ray diffractometer (D4 ENDEAVOR, Bruker, Germany). A small quantity of shell powder was transferred onto a tailor-made sample holder and then scanned by Co Kα radiation (35 kV and 30 mA; λ = 1.790 Å) from 20° to 70° 2θ with step size of 0.018° and step time of 1 s (n = 4 replicates per pCO_2). The carbonate polymorphs in the shell powder were identified based on the X-ray diffraction spectrum using the EVA XRD analysis software (Bruker, Germany). Calcite to aragonite ratio was estimated according to the following equation with a correlation coefficient of 0.9999[28]

$$\frac{I_C^{104}}{I_A^{221}} = 3.157 \times \frac{X_C}{X_A}$$

where I_C^{104} and I_A^{221} are intensity of the calcite 104 peak (34.4° 2θ) and aragonite 221 peak (54.0° 2θ), respectively; X_C/X_A is the calcite to aragonite ratio.

Magnesium to calcium ratio was determined by energy-dispersive X-ray spectroscopy under the Philips XL 30 field emission scanning electron microscope. A small quantity of shell powder was transferred onto a stub and coated by carbon (n = 4 replicates per pCO_2; 3 trials per replicate). The shell powder was irradiated by an electron beam with an accelerating voltage of 12 kV to obtain the energy spectrum with background correction. The elements in the shell powder were identified, and the magnesium to calcium ratio was calculated using software Genesis Spectrum SEM Quant ZAF (EDAX, USA).

Statistical Analysis. A two-tailed t-test was used to examine the effect of pH on growth, feeding rate, respiration rate, and shell properties. The assumptions of normality and homoscedasticity were tested by Shapiro-Wilk test and Levene's test, respectively. Logarithmic transformation was applied if either one of the assumptions was violated. Statistical analyses were performed using software SPSS 20.0 for Windows.

RESULTS

The shell length of *A. constricta* continued to increase throughout the 10-week exposure period, and the increase was enhanced by ~80% at pH 7.85 (t-test, t = −2.58, p = 0.015) (Figure 1a). Similarly, the total weight continued to increase, but the increase was unaffected by pH (t-test, t = −0.589, p = 0.560) (Figure 1b). Flesh weight to shell weight ratio was reduced at both pH levels, indicating faster growth in the shell than the flesh (by weight) (Figure 2a). Despite the seemingly greater decrease at pH 8.10, pH effect was insignificant (t-test, t = −0.943, p = 0.354). Flesh weight was strongly positively correlated with shell weight at pH 7.85 (Pearson correlation, R^2 = 0.621, p = 0.0005), but weakly at pH 8.10 (Pearson correlation, R^2 = 0.205, p = 0.091). Organ weight to flesh weight ratio increased at both pH levels, indicating the

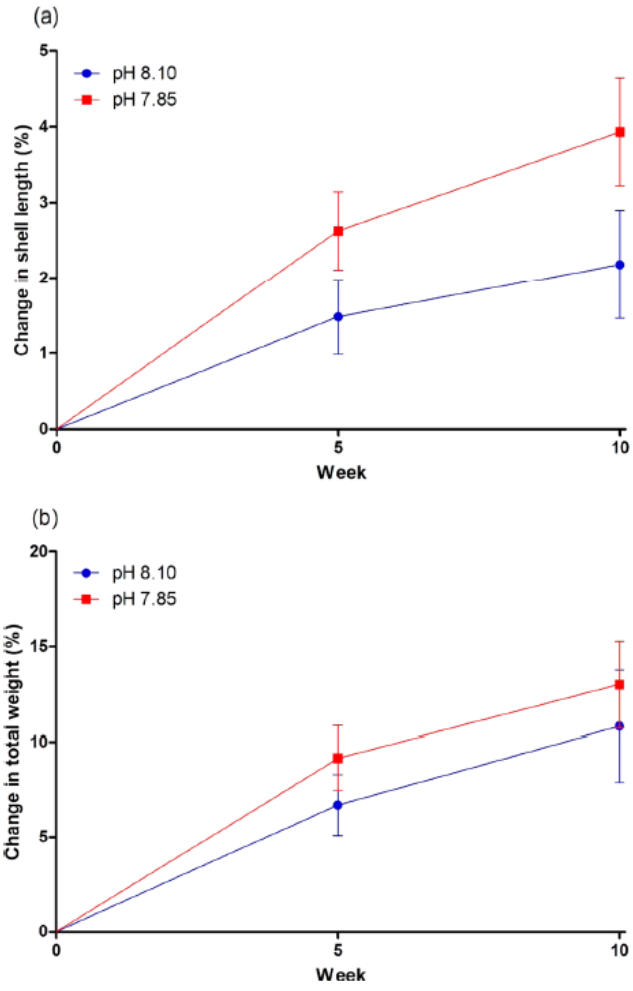

Figure 1. Percentage change in (a) shell length and (b) total weight of *A. constricta* across the 10-week exposure period at different pH levels (mean ± S.E., n = 15).

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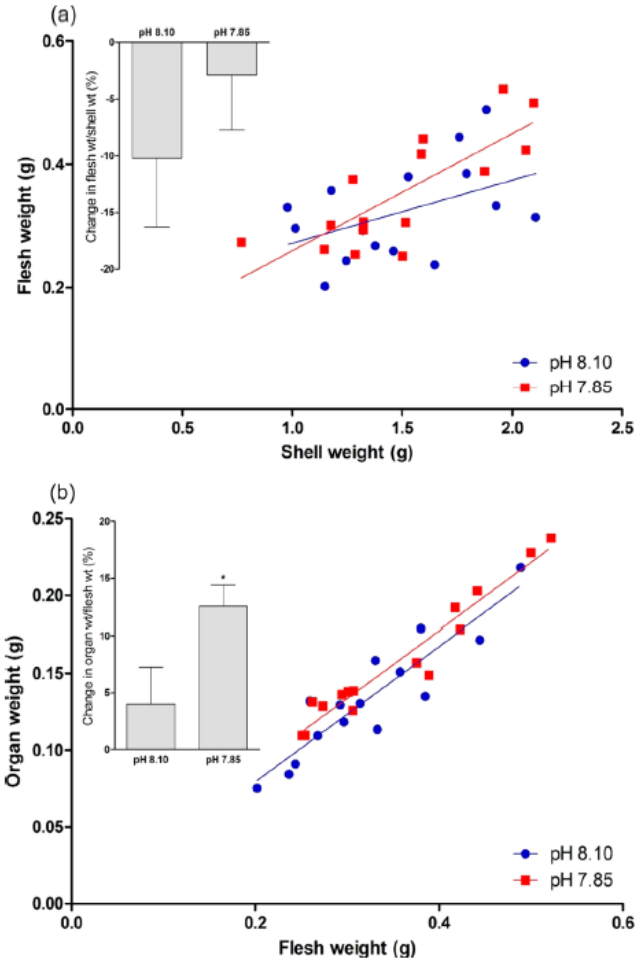

Figure 2. Percentage change in (a) flesh weight to shell weight ratio and (b) organ weight to flesh weight ratio of *A. constricta* (mean + S.E., n = 15) and their relationship following the 10-week exposure period at different pH levels.

gain of energy reserves, but the increase was significantly greater at pH 7.85 (t-test, t = −2.27, p = 0.031) (Figure 2b). Organ weight was positively correlated with flesh weight at both pH levels (pH 8.10: Pearson correlation, R^2 = 0.829, p < 0.0001; pH 7.85: Pearson correlation, R^2 = 0.942, p < 0.0001).

The feeding rate of *A. constricta* was unaffected by pH (t-test, t = −0.123, p = 0.903), but respiration rate was reduced by ~25% at pH 7.85 (t-test, t = 2.08, p = 0.047) (Table 2). The shell produced at pH 7.85 was ~140% harder than that at pH 8.10 in Vickers scale (t-test, t = −3.59, p = 0.011), but pH did not significantly alter the elastic modulus (t-test, t = −0.721, p = 0.498). The relative ACC content in the shell was unaffected by pH (t-test, t = 0.469, p = 0.655) (Table 2). Both calcite and aragonite were produced by *A. constricta* (i.e., bimineralic; Figure 3). Regardless of pH level, aragonite was the predominant carbonate polymorph in the shell, but the proportion of calcite was elevated at pH 7.85 (t-test, t = −3.11, p = 0.021). The magnesium to calcium ratio in calcite ranged from 0.027 to 0.028 and was not influenced by pH (t-test, t = 0.219, p = 0.834).

DISCUSSION

Ocean acidification is regarded as a threat to future marine ecosystems by diminishing the fitness and survival of marine

Table 2. Feeding Rate, Respiration Rate, and Shell Properties (Vickers Hardness, Elastic Modulus, Relative Amorphous Calcium Carbonate (ACC) Content, Calcite/Aragonite, and Mg/Ca in Calcite) of *A. constricta* Following the 10-Week Exposure Period at Different pH Levels (Mean ± S.E, n = 15 for Feeding Rate and Respiration Rate; n = 4 for Shell Properties)[a]

	pH 8.10	pH 7.85
feeding rate (mg ind^{-1} h^{-1})	11.3 ± 1.19	11.5 ± 0.68
respiration rate (μg O_2 ind^{-1} h^{-1})	44.0 ± 3.63*	33.7 ± 3.46
Vickers hardness (N mm^{-2})	82.1 ± 5.71	196.9 ± 39.6*
elastic modulus (GPa)	41.7 ± 3.34	45.5 ± 4.14
relative ACC content	2.00 ± 0.02	1.99 ± 0.02
calcite/aragonite	0.147 ± 0.011	0.198 ± 0.012*
Mg/Ca in calcite	0.028 ± 0.003	0.027 ± 0.006

[a]The asterisks indicate significant difference (p < 0.05).

organisms.[1] Yet, they may be able to acclimate to OA by altering metabolism, reallocating energy budget or modifying energy demand to maintain homeostasis.[7,11,12] We demonstrate that near-future OA may not compromise the overall performance of a calcifying gastropod, suggesting that its homeostasis is maintained. Although metabolic depression was observed (i.e., reduced respiration rate), which is commonly observed in marine mollusks and probably elicited by uncompensated extracellular pH,[8,29] a mild degree of metabolic depression is not necessarily deleterious because optional physiological processes can be minimized to maintain energy balance.[29,30] In contrast, increasing the metabolic rate to compensate for the metabolic cost of hypercapnia is maladaptive, especially when energy resources are limited.[9,10] While mild metabolic depression allows marine organisms to persist in OA environment, it often compromises their growth and fitness in the long term (e.g., reduced body size) due to the reallocation of energy budget to somatic maintenance.[11] We found, however, that both somatic growth and shell growth of *A. constricta* were maintained under OA conditions, meaning that they were supported by adequate metabolic energy.

Instead, our findings imply that growth is more influenced by the energy gain through feeding[7] rather than the metabolic energy produced by respiration as the latter is generated using energy reserves. This proposition is derived from previous studies where pH-induced metabolic depression fails to reduce somatic growth and shell growth when feeding rate is unaffected (e.g., blue mussels in naturally CO_2-enriched waters[4]). Energy gain through feeding can offset the elevated metabolic cost of hypercapnia,[5,16] indicating that feeding performance is fundamental to overall fitness. In the present study, the unaffected feeding rate plus the reduced respiration rate under OA conditions resulted in greater energy reserves (i.e., higher organ weight to flesh weight ratio). The gain of energy reserves is favorable as they can be used to provide temporary relief when energy demand is elevated (e.g., under stressful conditions) or food availability in the environment is reduced.

How OA impacts calcification has been an overriding concern for the function of future marine ecosystems because of the disproportionally large ecological roles of calcifying organisms in driving ecosystem stability.[2,31] If calcification is hindered, the fitness and survival of calcifying organisms may be diminished,[32] possibly causing profound ecological consequences on marine ecosystems.[3] Under OA conditions, calcification

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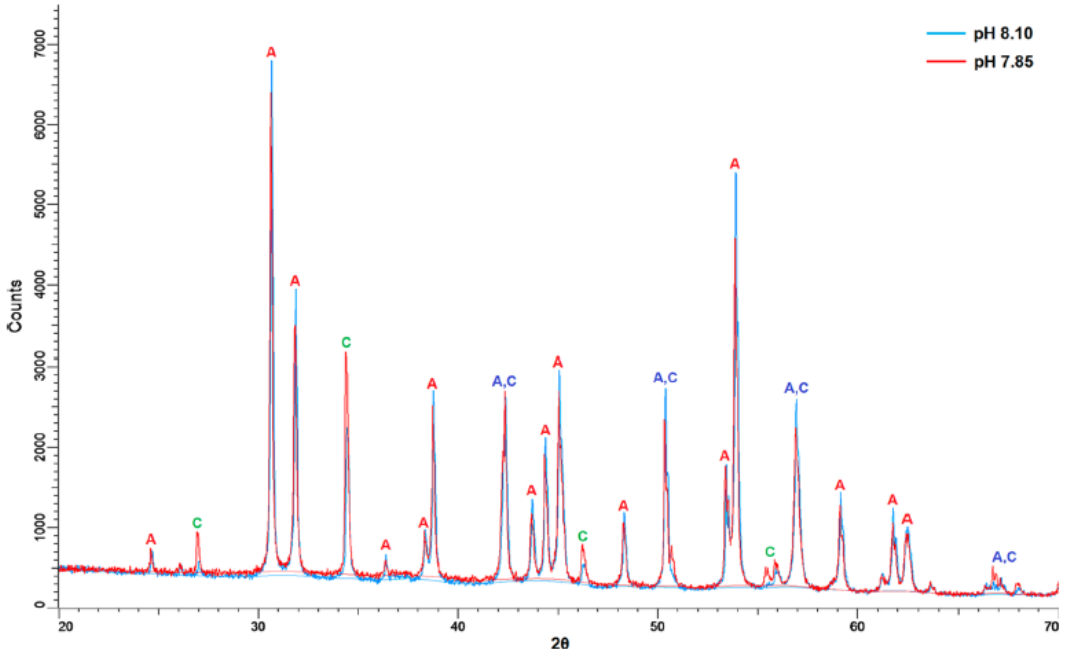

Figure 3. XRD spectrum showing the presence of both calcite and aragonite in the shell of *A. constricta*. The calcite to aragonite ratio is indicated by relative intensity of the calcite 104 peak (34.4° 2θ) to aragonite 221 peak (54.0° 2θ). At pH 7.85, the intensity of calcite peaks increases, but intensity of aragonite peaks drops, meaning that the proportion of calcite increases. A: aragonite. C: calcite.

can be retarded kinetically and thermodynamically by the reduced saturation state of carbonate,[18,22,33] or biologically by the disruption of biochemical and physiological processes involved (e.g., extracellular acid−base equilibrium).[34−36] Although marine organisms may maintain calcification, energy trade-offs against other processes are inevitable, reducing overall fitness in the long term.[9,10] Based on the current understanding, therefore, it is counterintuitive that *A. constricta* not only had faster shell growth, but also increased energy reserves under OA conditions. We reason that the faster shell growth is attributed, at least partly, to increased precipitation of calcite, which confers two major benefits. First, calcite is more thermodynamically stable than aragonite due to lower solubility of the former, thereby reducing the potential for shell dissolution during precipitation.[19,37] Second, constructing aragonitic shells is more energy-demanding than calcitic shells as more organic materials are required to precipitate aragonite and higher proton pumping efficiency is needed to make the calcifying fluid supersaturated for precipitation of aragonite.[38,39] From an energetic perspective, therefore, precipitation of calcite is preferred under OA conditions, especially when the metabolic energy is reduced.[40] Morphologically, the increased precipitation of calcite allows faster shell growth due to lower density and solubility than aragonite.[19] Nevertheless, calcification is also determined by the density of carbonate crystals in the shell, which can be influenced by OA.[16,36,41] Based on our qualitative examination of the ultrastructure, the carbonate crystals precipitated under OA conditions appeared less compact (Figure 4). This observation suggests that there may be a trade-off between shell density and shell extension. Regardless, our findings demonstrated that the net calcification and hence shell growth of *A. constricta* can be maintained, or even slightly enhanced, under OA conditions by exhibiting mineralogical plasticity.

Interestingly, OA led to the production of harder shells, while the elastic modulus (i.e., stiffness) was maintained, meaning that the shells are more resistant to compressive force. Shell hardness is primarily subject to the amount of organic matrix (e.g., chitin, glycoproteins, silk proteins, or acidic proteins) occluded in the shell, which can provide exceptional strength.[19,42,43] However, production of organic matrix is energy-demanding. For example, organic matrix, typically comprising <5% in the shell, can account for ∼50% of total energetic cost of shell production.[15] Our results imply that more energy was allocated for the production of organic matrix to enhance shell strength under OA conditions. The underlying mechanism is still enigmatic, but it may be related to an upregulation of enzymatic activity, protein synthesis, or organic matrix gene expression induced by OA.[36,41,44] These mechanisms may also explain why somatic growth and shell growth can be maintained under OA conditions.[35,45]

Although calcification is a biological process, the reduced saturation state of carbonate inevitably retards this process because of altered kinetics and thermodynamics. Consequently, the energetic cost of calcification will increase with decreasing pH of seawater, possibly diminishing shell growth, fitness, and ultimately survival of calcifying organisms.[16−18] In this regard, these organisms will be under selection pressure to modify the mineralogy of their shells. We illustrate the adaptive values of mineralogical plasticity to maintain shell growth and, hence, fitness under OA conditions. For example, increased precipitation of calcite minimizes the energy used for calcification and allows faster shell growth and, hence, somatic growth. In contrast, obligate aragonite-producing organisms will probably suffer more in OA environment due to the elevated energetic cost and slower rate of calcification.[34] Despite the trade-off against inner shell density, the energy conserved can be allocated for the production of organic matrix to augment shell strength as the compensatory mechanism. Producing harder shells without increasing stiffness is favorable for survival in OA environment because the inner calcified layers (i.e., prismatic and nacreous layers) of shells are susceptible to dissolution

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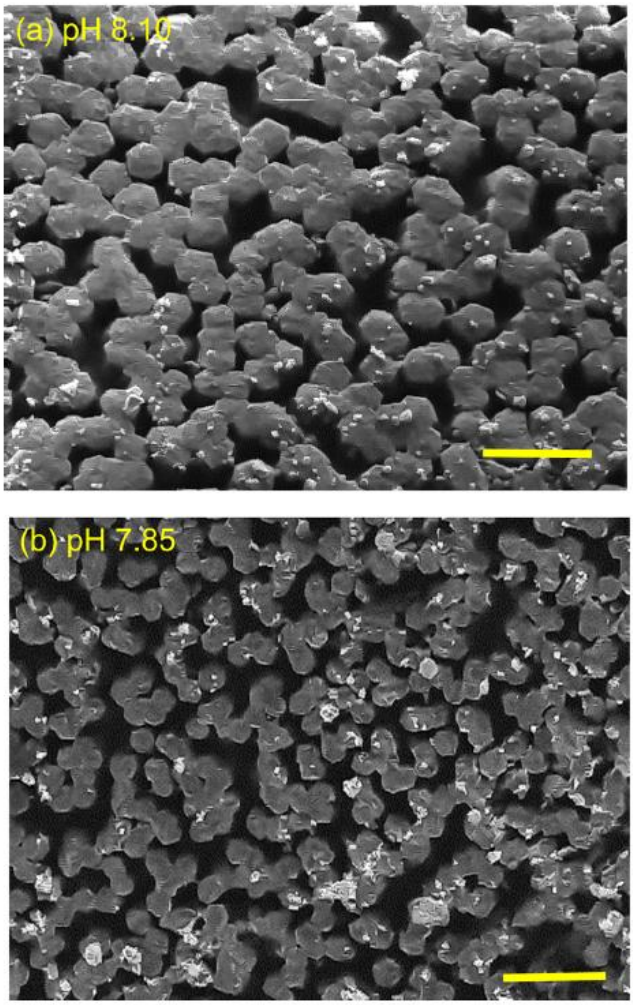

Figure 4. Scanning electron micrographs of the nacreous layer of shells, showing a slightly greater density of carbonate crystals precipitated at (a) pH 8.10 than (b) pH 7.85. Scale bar: 20 μm.

upon shell damage,[46] especially as shell repair is a slow and energy-demanding process.

Magnesium is incorporated into the crystal lattice of calcite during calcification, affecting the solubility and rate of precipitation of calcite,[39,47] but incorporation of magnesium in calcite can be biologically controlled.[48,49] In general, the reduced saturation state of carbonate favors precipitation of low-Mg calcite (Mg/Ca < 0.04) kinetically and thermodynamically due to the higher solubility of high-Mg calcite.[22,37,50,51] In this study, however, Mg/Ca in calcite did not significantly decrease with pH probably because low-Mg calcite (Mg/Ca < 0.03) has been precipitated under contemporary conditions.

The adaptive value of changing shell mineralogy has been recognized on the geological time scale when the physicochemical conditions shifted between "aragonite sea" and "calcite sea" due to the change in temperature, pCO_2 concentration, or Mg/Ca of seawater.[38,52] In general, it is more favorable to precipitate calcite than aragonite in the "calcite sea" and vice versa. The survival of calcifying organisms can be severely impacted if their shell mineralogy fails to match the physicochemical conditions of seawater.[38] Since the onset of Industrial Revolution, pCO_2 concentration has been increasing at an unprecedented rate and is predicted to reach ca. 1000 ppm by the next century if no policy is implemented to reduce global CO_2 emissions (i.e., RCP8.5 scenario).[23] While this time period is relatively short compared to the geological time scale, it is relatively long compared to the life cycle of many marine organisms. Thus, we suggest that calcifying organisms may be able to acclimate to the near-future OA, provided they can show mineralogy plasticity and have sufficient energy resources to maximize their fitness and survival.

The present study demonstrates that a small, but measurable, metabolic depression does not adversely affect somatic growth and shell growth under OA conditions when energy gain is maintained through feeding. Although calcifying organisms appear particularly vulnerable to OA, which elevates the energetic cost of calcification, we unravel the adaptive values of mineralogical plasticity (e.g., increased precipitation of calcite) to enhance shell growth under OA conditions. More importantly, the shells produced are functionally adjustable to the future "corrosive" ocean (e.g., harder shells with lower solubility). If calcifying organisms can exhibit this mineralogical plasticity to maintain overall fitness, not only may they be more robust to OA but also their functional roles in the community will be more stable than previously thought.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04709.

Diel variation of seawater pH in the study site (PDF)

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CHAPTER 7

GENERAL DISCUSSION

As ocean acidification and warming are regarded as the imminent climate change stressors in marine ecosystems (Fabry et al., 2008; Doney et al., 2009), a plethora of studies have been conducted to predict the fitness and survival of marine organisms in future (Byrne, 2011; Kroeker et al., 2013, Nagelkerken and Connell, 2015), which offer important insights into their populations and ecosystem functioning. Despite the scientific merits, these studies may have overestimated the impacts of ocean acidification and warming because the exposure period is usually short and the experimental design is oversimplified. Thus, the acclimation capacity of marine organisms and the interaction between trophic levels are often overlooked. This thesis aims to address these issues by studying the fitness and survival of marine gastropods in a well-simulated environment. Bioenergetics and calcification are the key aspects in this thesis because they are highly associated with the long-term survival of calcifying organisms (Zhuravlev and Wood, 2009; Sokolova, 2013).

Results showed that prolonged exposure to elevated temperature undermined the fitness of subtidal gastropods via energy deficit and energy depletion, even though the temperature was below their thermal tolerance (Chapters 2 and 3). Despite the elevated energy demand under ocean acidification (Pörtner, 2008), the physiological performance, growth and survival of subtidal gastropods appeared to be unaffected (Chapters 2 and 3). This could be mediated by the boosted nutritional quality of primary producers through CO_2 enrichment, resulting in greater feeding rate and energy gain of gastropods (Chapter 3). However, such boosting effect was outweighed by the combined negative effects of ocean acidification and warming, which caused impaired aerobic metabolism, energy depletion and mass mortality of gastropods (Chapter 3). Compared to subtidal gastropods, intertidal gastropods appeared to be robust to thermal stress because of their adaptive physiological and behavioural responses, which allow them to counter or avoid the acute thermal stress posed by heatwaves and thus persist in their habitat (Chapter 4). Regarding calcification, ocean acidification in isolation had limited effects on both shell growth and shell properties of gastropods (Chapters 2, 3, 5 and 6), which contradict the paradigm that calcification is hindered by the reduced pH and carbonate saturation state of seawater (Orr et al., 2005). In contrast, ocean

warming posed adverse effects on shell growth and shell strength of some gastropods (Chapters 2 and 5). Intertidal gastropods appeared to have the ability to modify their shell properties (i.e. mineralogical plasticity) in response to the altered seawater conditions, but this adaptive response was not observed in subtidal gastropods (Chapters 5 and 6). Overall, results suggest that subtidal gastropods would be jeopardised by climate change stressors, possibly leading to runaway primary production and phase shift of habitat structures in the future subtidal environment.

7.1 IMPACTS OF OCEAN ACIDIFICATION AND WARMING ON FITNESS AND SURVIVAL

Ocean acidification is expected to pose adverse effects on marine organisms, especially calcifying organisms, in view of the increased energy demand for acid-base regulation and calcification (Fabry et al., 2008; Pörtner, 2008). Based on the results in previous chapters, however, ocean acidification had indiscernible effects on both intertidal and subtidal gastropods even though they had limited time to acclimate, suggesting that they are able to maintain homeostasis physiologically and energetically under ocean acidification. The limited influence of ocean acidification is likely because the gastropods have adapted to the reduced pH in their natural environment. For example, pH fluctuation in the intertidal environment is usually large due to various reasons, such as rainfall, freshwater input and respiration of organisms (Howland et al., 2000; Hu and Cai, 2013; Leung et al., 2015). Therefore, the relatively minor pH reduction under ocean acidification is probably insufficient to cause significant impacts on the physiological and behavioural performance of intertidal organisms (e.g. Amaral et al., 2011; Leung et al., 2015; Cross et al., 2016). Although pH in the subtidal environment is relatively stable, it can vary greatly near the substratum due to photosynthesis and respiration of primary producers (e.g. turf algae and seagrasses), which can create a diffusion boundary layer with a greater pH range than the mainstream seawater (Hurd et al., 2011; Cornwall et al., 2014). Since gastropods are benthic animals, they are subject to the pH range in this layer and may have already acclimated to the reduced pH under ocean acidification. Furthermore, CO_2 can act as a resource for primary producers so that herbivores may indirectly gain benefit from CO_2 enrichment through trophic transfer (Connell et al., 2013). Here, the turf algae growing under ocean acidification had lower C:N ratio, which drives greater feeding rate and hence boosts energy gain of gastropods (Chapter

3). This indirect positive effect through trophic transfer can even outweigh the direct negative effect of ocean acidification and translate into better fitness of herbivores (Kamya et al., 2017). Importantly, the temporal pH change caused by anthropogenic CO_2 emission is extreme slow so that marine organisms are likely able to accommodate the gradual pH reduction over time. Since most of the previous studies did not consider natural pH fluctuation, interaction between trophic levels and rate of pH change, the predicted impacts of ocean acidification on marine organisms may have been overestimated.

Instead, the fitness and survival of subtidal gastropods were tremendously influenced by ocean warming (Chapters 2 and 3). How the elevated temperature under ocean warming impacts marine organisms depends on the severity of thermal stress (Sokolova et al, 2012). Acute thermal stress is induced when the temperature exceeds the critical thermal maximum of organisms, which leads to instant loss of motor coordination (Lutterschmidt and Hutchison, 1997) and substantial reduction in aerobic metabolism (Pörtner, 2002). Subtidal organisms in temperate regions, including South Australia, are probably not subject to acute thermal stress under ocean warming because most of them are living far below their thermal tolerance and have greater acclimation capacity to elevated temperature (Vinagre et al., 2016). Nevertheless, results in the previous chapters revealed that the fitness of subtidal gastropods was still impacted by elevated temperature even below their thermal tolerance after a long-term exposure period. This suggests that subtidal gastropods can already be threatened by long-term sublethal thermal stress due to energy deficit and energy depletion. Such energy-based mechanism possibly explains the devastating impacts of persistent heatwaves on subtidal organisms in temperate regions (Wernberg et al., 2013), and substantiates the proposition that maintaining energy homeostasis is the key for long-term survival under stressful conditions (Sokolova et al., 2012, Sokolova, 2013).

Although ocean acidification did not cause significant impacts on subtidal gastropods, it could amplify the adverse effects of sublethal thermal stress under ocean warming. For instance, the survival of gastropods can be maintained under ocean acidification and warming in isolation probably because of the greater energy gain either through enhanced feeding rate on the more nutritious food or increased energy gain per feeding effort due to the higher energy content of food (Chapter 3). Yet, this indirect benefit via trophic transfer was probably overwhelmed by the combined effects of ocean acidification and warming on energy budget (e.g. acid-base regulation and calcification under ocean acidification; somatic maintenance under ocean warming), leading

to impaired aerobic metabolism, energy depletion and ultimately mortality. Therefore, these results imply that compensatory mechanisms (e.g. through trophic transfer or phenotypic plasticity of organisms) have a limit beyond which they become insufficient to buffer the direct negative impacts of climate change stressors.

While ocean warming possibly poses negligible effects on intertidal organisms which have adapted to large temperature fluctuation, heatwaves may cause devastating impacts on their fitness and survival due to the drastic increase in temperature to an extreme level. Nevertheless, the temperature profile in the intertidal environment is highly heterogenous at small spatial scales (Helmuth et al., 2011), implying that intertidal organisms can move to thermally-favourable locations (e.g. crevices) during low tide to avoid the thermal stress caused by heatwaves (Chapperon and Seuront, 2011). Such behavioural response allows intertidal gastropods to maintain body temperature within their survivable range during heatwaves (Chapter 4). In addition, molecular defence mechanisms, such as upregulation of heat shock proteins and antioxidative enzymes, can help alleviate the impacts of thermal stress (Lesser, 2006; Tomanek, 2010). These defence mechanisms could play a critical role in determining the thermal tolerance of intertidal gastropods and their recoverability from thermal stress. Since heatwaves are mostly transient, intertidal gastropods are likely able to persist in their habitat and maintain their ecological functions because of their adaptive behavioural and physiological responses.

7.2 IMPACTS OF OCEAN ACIDIFICATION AND WARMING ON CALCIFICATION

Calcifying organisms are expected to be threatened by ocean acidification owing to the reduced pH and carbonate saturation state of seawater (Fabry et al., 2008; Doney et al., 2009), which hinder the construction of calcareous shells (Orr et al., 2005). Aragonite-producing organisms are considered particularly susceptible to ocean acidification in view of the higher solubility of aragonite than calcite (Feely et al., 2004). However, growing evidence, including the present findings, demonstrates that calcification can be maintained or even promoted under ocean acidification (Findlay et al., 2011; Garilli et al., 2015; Ramajo et al., 2016), suggesting that this process is not simply driven by pH and carbonate saturation state. In fact, most calcifying organisms do not directly utilize carbonate ions, but bicarbonate ions, as the substrate for calcification, meaning that formation of calcareous shells is not a chemical reaction between calcium and carbonate ions (Pörtner, 2008; Roleda et al., 2012). Yet, ocean acidification may still

dampen shell growth by increasing the energy cost of calcification because extra metabolic energy is needed to regulate the acid-base balance of extracellular fluid so that an alkaline condition is maintained in the fluid compartment for calcification (Waldbusser et al., 2013; Thomsen et al., 2015). Here, the negligible effects of ocean acidification on both shell growth and shell properties of gastropods imply that the energy cost of acid-base regulation on calcification is insignificant (see also McCulloch et al., 2012). In contrast, shell growth and shell properties were impacted by ocean warming, indicating that calcification is mainly driven by energy budget. Indeed, calcification is an energy-demanding biological process, involving multiple steps (Palmer, 1992; Addadi et al., 2006). For gastropods, bicarbonate ions are used as the substrate for calcification, which can be obtained directly from seawater or indirectly from metabolic CO_2 after conversion by carbonic anhydrase (Roleda et al., 2012). The bicarbonate ions are then converted into carbonate ions in the fluid compartment, which deposit as calcium carbonate with the aid of matrix proteins (Greenaway, 1971; Addadi et al., 2006). This calcifying process is feasible as long as adequate metabolic energy is provided. Given the substantial reduction in energy budget under ocean warming, metabolic energy would be preferentially allocated to somatic maintenance rather than calcification, thereby affecting shell growth and shell properties. This energy-based concept would be more accurate and relevant than the kinetics-based concept to decipher the impacts of ocean acidification and warming on calcification.

In view of the reduced pH under ocean acidification, it is more adaptive to produce shells of lower solubility to minimize shell dissolution. In fact, the survival of calcifying organisms can be reduced if their shell properties are not favoured by the seawater chemistry (Hautmann, 2006; Zhuravlev and Wood, 2009). While shell surface is covered by periostracum which can protect the shell from dissolution, the inner shell layers become susceptible to dissolution when the shell is broken (e.g. by predators) (Tunnicliffe et al., 2009). Changing mineralogical properties, such as constructing calcitic shells or reducing Mg content in calcite, is a potential mechanism to reduce shell solubility (Ries, 2011). It may also reduce the energy cost of calcification and hence alleviate the impacts of climate change stressors on shell growth. Based on the present findings, however, most of the gastropods did not modify their mineralogical properties in response to ocean acidification, implying that their calcifying mechanism remains unchanged. The production of aragonitic shells by the subtidal gastropods under ocean acidification is not favourable in light of the higher solubility of aragonite than calcite. As such, the energy cost of calcification would be

elevated because aragonite is less thermodynamically stable (Mackenzie et al., 1983; Waldbusser et al., 2013), possibly leading to energy trade-offs against other processes (e.g. shell growth). On the contrary, intertidal gastropods were able to alter mineralogical properties in response to the climate change stressors. For example, a greater proportion of calcite was precipitated under ocean acidification to reduce shell solubility. The production of low-Mg calcite by intertidal gastropods is also favourable to resisting shell dissolution (Ries, 2011). The large fluctuation of pH and temperature in the intertidal environment is probably the major reason why the shells of intertidal gastropods are more plastic than those of subtidal gastropods (Boyd et al., 2016). Therefore, the shell functions of intertidal gastropods are likely more robust than those of subtidal gastropods to future seawater conditions.

7.3 IMPLICATIONS FOR FUTURE MARINE ECOSYSTEMS

Based on the findings in this thesis, the survival of subtidal gastropods would be diminished in future marine ecosystems if they cannot acclimate to the slow rate of change in seawater pH and temperature caused by anthropogenic CO_2 emission. It is important to highlight that ocean warming poses greater impacts than ocean acidification because sudden increase in temperature by 3 – 4°C can already be triggered by heatwaves, where subtidal organisms have limited time to respond and acclimate (Wernberg et al., 2013). Although it has been suggested that subtidal organisms in temperate regions are living below their thermal tolerance and have greater acclimation capacity (Vinagre et al., 2016), prolonged exposure to sublethal thermal stress can already undermine their fitness and survival through energy depletion. Therefore, persistent heatwaves can result in mass mortality of subtidal organisms with little sign of recovery, leading to concomitant changes in community structures and ecosystem functioning (Wernberg et al., 2013). Since heatwaves are forecasted to be more frequent and persistent in future due to global warming (Meehl and Tebaldi, 2004), this extreme climatic event, rather than the climatic trend (i.e. ocean warming), would be the main driver governing the integrity of both contemporary and future marine ecosystems (Jentsch et al., 2007; Thompson et al., 2013). While only marine organisms in temperate regions were studied in this thesis, it is surmised that marine organisms in tropical regions would suffer more from elevated temperature that may even trigger acute thermal stress because many of them have been living close to their thermal tolerance and have limited

acclimation capacity (Nguyen et al., 2009; Vinagre et al., 2016). Ocean acidification has been studied extensively for more than a decade, but its impacts on marine organisms are still elusive in view of the inconsistent results in the literature. Given the slow rate of change in seawater pH, marine organisms are likely able to accommodate the impacts of ocean acidification and sustain their populations (Suckling et al., 2015). Indeed, growing evidence shows that some marine organisms can be better off under ocean acidification (Kamya et al., 2017) and even boost their populations in natural CO_2 vents (Connell et al., 2017), indicating that the impacts of ocean acidification may not be deleterious as previously thought.

As grazing gastropods have substantial contributions to herbivory in their community, they play important roles in trophic transfer and maintenance of ecosystem health by regulating the biomass of epiphytes (Jernakoff and Nielsen, 1997; Poore et al., 2012; McSkimming et al., 2015). Indeed, herbivorous gastropods have an innate capacity to counter the accelerated growth of primary producers through trophic compensation (Ghedini et al., 2015). For example, Falkenberg et al. (2014) showed that the top-down control by grazing gastropods can prevent the expansion of turf algae caused by CO_2 and nutrient enrichment. Nevertheless, this compensatory mechanism has a limit beyond which the feeding performance of herbivores is worsened by the impacts of climate change stressors (Mertens et al., 2015). Since CO_2 enrichment and elevated temperature can probably facilitate the growth of primary producers (Harley et al., 2012; Connell et al., 2013), runaway primary production and phase shift of habitat structures will likely ensue due to reduced herbivory. For example, CO_2 enrichment was shown to cause an expansion of turf algae and inhibit kelp recruitment, resulting in phase shift from a kelp-dominated to turf-dominated community (Connell and Russell, 2010). On the other hand, propagations of energy and nutrients to the consumers in the upper trophic levels will be dampened if herbivore populations are reduced (Vizzini et al., 2017). Eventually, the community structure, trophic transfer and functioning of marine ecosystems would be significantly modified in future.

7.4 SUGGESTIONS FOR FUTURE STUDIES

1. Potential buffering effect of diffusion boundary layers

To date, manipulative experiments for ocean acidification research have focused mostly on the average value of predicted seawater pH, which is achieved by aerating seawater with CO_2-enriched air. Although stable seawater pH can be obtained by this method, pH fluctuation is not taken into consideration, which occurs in the natural environment. Therefore, the experimental results would underestimate the acclimation capacity of marine organisms and have limited predictability for the impacts of ocean acidification on marine ecosystems (Boyd et al., 2016). Indeed, pH fluctuation can be huge, especially in the benthic zone, due to the diffusion boundary layer formed by respiration and photosynthesis of primary producers (Hurd et al., 2011). The seawater chemistry in this layer can be very different from that in the mainstream seawater (Hurd et al., 2011; Cornwall et al., 2014), and may buffer the impacts of ocean acidification on benthic organisms. Whether diffusion boundary layers can help alleviate the impacts of ocean acidification or even provide a favourable environment for benthic organisms remains poorly known (Cornwall et al., 2014). More field and laboratory studies are required to examine the influence of diffusion boundary layers on benthic organisms.

2. Positive effect of ocean acidification on calcification through trophic transfer

Since growing evidence reveals that calcification is primarily driven by energy budget rather than seawater carbonate chemistry, it is possible that herbivorous calcifying organisms can produce shells of better quality under ocean acidification because the nutritional quality of primary producers can be enhanced by CO_2 enrichment (Falkenberg et al., 2014; Vizzini et al., 2017). This potential mechanism through trophic transfer would provide valuable insights into how some calcifying organisms can be better off in the CO_2-enriched environment. To test this hypothesis, the relationship between nutritional quality (e.g. proteins, carbohydrates and energy content) of primary producers and shell properties (e.g. thickness, crystallinity and mechanical strength) of calcifying organisms can be examined.

3. Predator-prey relationship under future seawater conditions

Calcifying organisms produce shells as the primary structure for protection against physical damage, but the mechanical strength of shells can be weakened by ocean acidification and warming. In this regard, calcifying organisms are generally predicted to be more vulnerable to predator attack. Nevertheless, it is noteworthy that ocean acidification and warming can also worsen the foraging performance of predators (e.g. weakened muscular strength or mechanical

strength of feeding organs) and thus lower their energy gain per unit effort. As a result, predator-prey relationship can be modified and climate change stressors may favour preys over predators, depending on their responses. If predators suffer more than their preys, the top-down control by predators would be compromised and thus food web structure and trophic transfer in a community would be altered (Vizzini et al., 2017). Both plant-herbivore and predator-prey interactions can be studied simultaneously to examine the energy flow across multiple trophic levels in a community.

4. Transgenerational effect of ocean acidification and warming

To date, climate change research is mostly conducted using organisms in one generation. Despite the scientific merits, it is still insufficient to predict the consequences of marine organisms in future because they may be able to accommodate the impacts of climate change stressors over multiple generations (Ross et al., 2016). Study on this transgenerational plasticity is scant, but recent evidence shows that some marine organisms (e.g. molluscs and echinoderms) exposed to climate change stressors can produce offspring of greater acclimation capacity and fitness probably through increased maternal provisioning or epigenetic modifications (Ross et al., 2016; Thomsen et al., 2017). Therefore, further study is needed to test whether acclimation to ocean acidification and warming can be acquired over multiple generations.

7.5 CONCLUSION

The accelerated anthropogenic CO_2 emission will lead to ocean acidification and warming in future, which are expected to compromise the fitness and survival of marine organisms. The results in this thesis showed that ocean acidification had limited impacts on the physiological performance and growth of both intertidal and subtidal gastropods even though they had limited time to acclimate. In contrast, ocean warming markedly reduced the energy budget of subtidal gastropods following a prolonged exposure period, which was manifested at temperature below their thermal tolerance. This suggests that long-term exposure to sublethal thermal stress can already diminish the survival of subtidal organisms through energy depletion, which is probably associated with their mass mortality in the past heatwave events. Ocean acidification and warming were shown to boost the nutritional quality of primary producers, which can in turn compensate for the elevated energy demand of subtidal gastropods by increasing either feeding rate or energy

gain per feeding effort. Such boosting effect was, however, outweighed by the combined negative effects of ocean acidification and warming, resulting in energy depletion and mortality of gastropods. This suggests that the top-down control by gastropods would be weakened in the future subtidal environment, possibly culminating in runaway primary production. Although intertidal organisms have adapted to the large temperature fluctuation in their habitat, the extreme thermal stress posed by heatwaves may be strong enough to threaten their survival. Yet, intertidal gastropods were shown to be able to counter this thermal stress through adaptive behaviour (e.g. hiding) and molecular defence mechanisms (e.g. upregulation of antioxidative capacity), which enable them to survive during heatwaves and sustain their ecological functions in the intertidal environment.

Contrary to the prediction based on seawater carbonate chemistry, ocean acidification did not adversely affect shell growth and shell properties, implying that calcification is not chiefly governed by the pH and carbonate saturation state of seawater. Instead, ocean warming retarded shell growth and weakened shell strength of some subtidal gastropods. This substantiates that calcification is an energy-demanding biological process, while shell growth is subject mainly to energy budget. When pH is reduced, production of less soluble shells is favourable functionally and energetically. However, subtidal gastropods still produced aragonitic shells under ocean acidification, which are more susceptible to dissolution. In contrast, intertidal gastropods were able to adaptively modify their mineralogical properties, which can render their shells functionally suitable for the future seawater conditions and even promote shell growth by reducing the energy cost of calcification. Overall, the results in this thesis indicate that the fitness and survival of subtidal gastropods would be undermined by ocean acidification and warming and therefore habitat structures and trophic transfer along food chain in the subtidal community would be altered. Nevertheless, this pessimistic prediction is based on the assumptions that anthropogenic CO_2 emission is not regulated (i.e. business-as-usual emission scenario) and marine organisms fail to acclimate to the slow rate of change in pH and temperature. As such, anthropogenic CO_2 emission should be stringently regulated as a precautionary principle to slow down the climatic trends and minimize the occurrence of extreme climatic events. In this situation, it is optimistic that the community structure, integrity and functioning of marine ecosystems can be sustained in future.

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