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The challenge of securing future food production for aquaculture species under environmental change: enhancing physiological performance under environmental stress

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The challenge of securing future food production for aquaculture species under environmental change: enhancing physiological performance under environmental stress

by

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Author's Declaration

At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Conferences

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Ahmed Salama Ali Abbas

Abstract

Rising sea surface temperatures and ocean acidification present profound challenges for many marine species, leading to cascading effects on ecosystem functionality and food security. Phenotypic plasticity is anticipated to play a key role in helping marine ectotherms maintain performance and acclimate to changes in these global environmental drivers. However, our understanding of phenotypic plasticity in the context of climate change is largely based on short-term studies, with limited ecological relevance, which often overlook some important response modifiers such as environmental context, exposure nature, reproductive modes, and potential trade-offs between fitness components. As a result, the full potential for marine ectotherms to exhibit physiological acclimation within and across generations remains not well understood. This thesis addresses this knowledge gap by investigating the extent of physiological plasticity in key intertidal species, which are crucial for both ecological and commercial purposes, considering such response modifiers. Different species are exposed to ocean warming (OW) and/or ocean acidification (OA) across various life-cycle stages. I first characterize metabolic plasticity in adult intertidal gastropods after exposure to OW and OA, exploring the effects of exposure length (over 12 months) and seasonal dynamics on metabolic responses (Chapter 2). I then assess the cost of thermal acclimation in adults of two gastropods with different reproductive modes, examining the potential trade-off between scope for growth (SfG), reproduction, and survival in relation to reproductive mode and the magnitude of temperature change (Chapter 3). I also compare the effects of two thermal regimes different in their nature (repeated heat shocks *vs* chronic warming) on thermal tolerance and performance in adults of a commercial gastropod, characterizing differences in condition index and thermal performance and tolerance traits (Chapter 4). Lastly, I evaluate the effects of exposing parents of a commercially important gastropod to these

thermal regimes (applied in Chapter 4) on offspring thermal performance developed under two temperatures (15 °C or 20 °C), exploring the potential for parental effects and/or developmental plasticity (Chapter 5). My findings reveal several important insights. Firstly, the interactive effects of OW and OA on metabolism of gastropods were observed only after 6 months of exposure under summer conditions, with species-specific responses. Metabolic changes were intricately linked to how species respond to seasonal environmental fluctuations, either exacerbating or mitigating the consequences of stressors. Secondly, a trade-off between SfG, reproduction, and survival during thermal acclimation was observed, where the pattern was dependent on temperature change magnitude and reproductive mode. Thirdly, the nature of the thermal regime influenced adult responses as, while both thermal regimes (repeated heat shocks or chronic warming) resulted in higher thermal tolerance compared with control, differences in the measured traits indicated different mechanisms were at play, and differences in the overall cost of exposure. Finally, chronically warmed parents showed an increase in maternal provisioning, however reduced hatching success, larval development, and overall performance regardless of developmental conditions. Conversely, heat-hardened parents produced eggs of a smaller average size, yet they exhibited comparable hatching success to control parents. When compared to offspring from control parents, those from heat-hardened parents demonstrated heightened overall physiological performance under warm developmental conditions across stages. Overall, my results provide valuable insights for understanding the capacity for physiological acclimation within and across generations of important mollusc species, with promising effects of heat hardening on thermal performance in both parents and their offspring. This knowledge is paramount for better conservation and commercial sustainability management of our marine resources under predicted climate change scenarios.

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Chapter 1

General Introduction: Physiological plasticity of marine ectotherms to climatic change stressors: the potential for acclimation

1.1 Introduction

The increased atmospheric CO_2 levels continue to drive climate change, leading to unprecedented rises in sea surface temperatures, known as ocean warming (OW), and a concurrent decrease in sea water pH, termed ocean acidification (OA) (IPCC, 2023). Climatic change stressors are causing altered physiological performance of marine organisms, with negative consequences to ecosystem functionality and food security (Masanja et al., 2023; Scanes & Byrne, 2023). Thus, understanding the responses of biological systems to climatic stressors is paramount (Huo et al., 2023). Indeed, whether marine populations will be able to cope with these changes, rapidly enough to avoid extinction (Visser, 2008), is a question that has been intensively investigated during last two decades (Harvey et al., 2013; Przeslawski et al., 2015; Figuerola et al., 2021; Venegas et al., 2023).

Marine animals can respond to climate change in a number of ways including adaptation, phenological changes and/or phenotypic plasticity. Genetic adaptation, which involves permanent alterations in the genotypes of a given population, is the main driver for the adaptive responses to environmental conditions, and the way by which a species can avoid global extinction (Visser, 2008). However, genetic changes are unlikely to occur sufficiently fast to allow populations to adapt to the rapid pace of environmental changes predicted under climate change scenarios (Merilä, 2012; Kristensen et al., 2018). Phenological plasticity has enabled species to alter their spatiotemporal distributions in order to follow their physiological niche (Parmesan and Yohe, 2003). Such phenological changes may allow marine animals to remain in an environment that enables optimal fitness (Poloczanska et al., 2016). However, this phenological plasticity is not an option for sessile, less locomotory, and not migratory species, such as many marine invertebrates. Finally, phenotypic plasticity, which refers to the ability of a genotype to create diverse phenotypes in

response to the environment (Schlichting & Smith, 2002; Schlichting & Wund, 2014), is an immediate response strategy to environmental changes that encompasses behavioural and physiological adjustments to maintain individual performance (Beaugrand & Kirby, 2018). Phenotypic plasticity is mediated by non-genetic mechanisms within and across generations (Bonduriansky et al., 2012; Putnam, 2021), and it can enable population and species' persistence and resilience under environmental stress (Putnam, 2021). Thus, the presence of such adaptive phenotypic plasticity can be critical in shaping the performance of marine species and their future generations under climatic change stressors (Munday et al., 2013; Beaman et al., 2016; Putnam, 2021). Understanding the extent of phenotipic plasticity of marine populations globally will allow us to identify winners and loosers in the context of climate change (Somero, 2010; Watson, 2018). Environmentally induced phenotypic plasticity is not only shaped by the environment experienced within a single generation but can also be influenced by environments experienced by the previous generations of marine organisms via transgenerational effects (Munday et al., 2013). Thus, in predicting marine animal responses to climatic change stressors, it is crucial to take into account the role of physiological plasticity within a generation, as well as how the environmental conditions experienced by previous generations can shape the responses to acclimation under the anticipated climate change scenarios. In this chapter, I aim to discuss our current understanding of the within-generation physiological plasticity of marine ectotherms to climatic stressors and the role of parental exposure to environmental stress in inducing transgenerational plasticity (TGP), with a focus on marine molluscs, when the extent of the literature allows, as they are more relevant to my thesis. I first discuss the within-generation reversible plasticity of marine ectotherms, summarising their responses to elevated temperature with focus on their thermal acclimation responses. After that, I shed light on the influence of some response modifiers, such as the interaction between multiple stressors (focusing on OW and OA), as well as the length of exposure and variation of environmental context, the nature of exposure, and the differences in mode of reproduction, on the extent of the physiological plasticity. I then summarise the role of developmental plasticity in shaping marine ectotherms' responses to OW. After that, I discuss the potential of the parental exposure to environmental stress in altering the offspring's phenotypes, focusing on the role of fitness trade-offs between

parents and their offspring in influencing the parental effects responses in the context of climate change. I end up with thesis aim, objectives, and model species.

1.2 Reversible plasticity under climate change

The impacts of environmental change on organisms ultimately hinge on their ability to adjust key physiological traits, thereby facilitating performance and long-term persistence. Phenotypic plasticity can lead to adaptive changes in physiological traits of marine animals within a generation, enhancing their resilience to environmental stressors (Dong et al., 2021; Collins et al., 2023). This physiological plasticity in response to environmental changes has been documented to result in short-lived positive effects on performance traits, such as metabolism (Seebacher et al., 2015). However, the long-term fitness changes associated with this plasticity are often inferred rather than directly measured (Huey and Berrigan 1996; Einum, 2019). Physiological plasticity demands an expenditure of energy to activate adjustment mechanisms under stress (Kühnhold et al., 2017, 2019). These adjustments result in energy reallocation (Sokolova et al., 2012), leading to potential trade-offs among various fitness components (Mardones et al., 2021). While physiological plasticity is crucial for short-term survival, understanding the associated fitness changes (benefits/costs) of an observed trait plasticity becomes pivotal for a more comprehensive estimation of an animal's capacity for acclimation in the long-term in response to different environmental stressors.

1.2.1 Responses to elevated temperatures

The thermal tolerance window of marine animals, defined as a performance range encompassing optimal and suboptimal zones, is commonly expressed as a performance breadth (Fry, 1971). Outside an optimal zone, physiological performance is negatively affected, with animals entering tolerance ranges, where stress resistance mechanisms are activated (Sokolova et al., 2012; Sokolova, 2021). Consequently, marine species under OW are prone to experiencing negative physiological consequences, exhibiting greater vulnerability compared to terrestrial species (Pinsky et al., 2019). A recent review, encompassing 421 studies conducted over three decades investigating the impact of OW

on marine organisms, revealed that 78.8 % of studies reported negative effects on physiology, life cycle, dispersal, reproduction, and/or survival, with invertebrates exhibiting the highest frequency of negative effects (Venegas et al., 2023).

The upper thermal tolerance limits of marine ectotherms exhibit a strong correlation with their habitat temperature. Populations in warmer environments generally possess greater thermal tolerance than those in colder regions (Sorte et al., 2011; Linares et al., 2013; Reed & Thatje, 2015). For example, tropical species, tend to be more heat-tolerant than temperate species (Somero, 2010; Gleason & Burton, 2013), suggesting thermal adaptation to habitat conditions among marine species. In the tropics, adaptation to stable thermal environments often leads to narrow thermal windows, along with limited capacity for thermal acclimation (Morley et al., 2017; Madeira et al., 2018 b; Armstrong et al., 2019). Consequently, many tropical marine species already live near their upper thermal limits (e.g., Mortensen & Dunphy, 2016) and may therefore be vulnerable to ocean warming (OW). This is also the case for some species inhabiting the upper shore in the high intertidal, where ectotherms regularly experience thermal extremes, and have narrow thermal safety margins (Stillman, 2003; Somero, 2010).

Marine animals with wide thermal windows, can also adjust their physiology in response to climate change stressors, demonstrating buffering plastic responses that enhance their resilience and survival (Pörtner and Farrell, 2008). This physiological plasticity enables them to acclimate to new thermal environments. Indeed, many marine ectotherms have demonstrated significant potential for thermal acclimation, leading to increased thermal tolerance and performance limits. These organisms can adjust their physiological responses, reducing sensitivity to thermal stress and increasing their critical thermal maximum (Dong et al., 2021; Leeuwis & Gamperl, 2022; Collins et al., 2023). This plasticity is reversible, aiding animals in quickly restoring performance at a certain temperature (Seebacher et al., 2015; Brahim et al., 2019). Additionally, acclimation to different temperatures has been shown to induce differential shifts in thermal performance curves, enhancing various thermal physiological traits (Seebacher & Little, 2021; Dwane et al., 2023). Nevertheless, the capacity for acclimation varies significantly among species and is contingent upon their unique physiological limits, ecological traits, and habitat conditions

(Dong et al., 2021; Collins et al., 2023), thus constraining the generalisability of the responses. Consequently, interspecific studies play a crucial role in expanding our understanding of responses to climatic stressors more broadly. Indeed, interspecific comparisons are commonly regarded as the primary level at which environmental adaptation is studied. This is because species serve as biologically distinct units (Powers 1987), and the physiological variation between species is generally more extensive than within species (Spicer and Gaston 1999). As a result, conducting interspecific comparisons of acclimation capacity among species inhabiting the same environment becomes imperative in understanding the repercussions of climatic stressors on that particular ecosystem.

1.2.2 Response modifiers

The responses of marine organisms to environmental stressors are intricately linked to their ability to sustain significant energy allocation, a factor defining physiological plasticity and, consequently, influencing their ecological success under climate change stressors (Sokolova et al., 2012; Sampaio & Rosa, 2020). Thus, it becomes paramount to consider the context of exposure conditions that influences the ability of animals to adjust their key physiological traits and hence promote or prevent their adaptive acclimatory responses when designing experiments investigating animals' capacity for acclimation. While the physiological responses of marine animals to increased temperatures exhibit species-specific variation (Lefevre, 2016; Venegas et al., 2023), various response modifiers, such as the interaction of multiple stressors, duration of exposure, seasonality of environmental context, nature of exposure, and difference in reproductive mode may play a crucial role in shaping these responses. In the subsequent subsections, I will delve into evidence shedding light on the potential influence of these response modifiers on the responses of marine animals to environmental stressors in the context of climate change.

1.2.2.1 Interaction of multiple stressors

Evaluating acclimation capacities to elevated temperatures across taxa is pivotal for predicting marine animals' future responses, yet it is crucial to acknowledge that

temperature rarely operates in isolation. Indeed, marine animals inhabit environments characterized by multiple stressors, where they face challenges that extend beyond elevated temperatures. Recognizing this complexity, studies on multiple stressors endeavour to capture the intricate interplay, striving for heightened ecological realism. This approach proves essential as the repercussions of a single stressor can trigger physiological adaptations that shape the organism's response to additional stressors (Todgham and Stillman, 2013; Gunderson et al., 2016). The effects of combinations of stressors have been widely studied under laboratory conditions, with less studies available in the field (Collins et al, 2023). In the marine environment, elevations in sea water temperatures are often associated with concomitant decreases in pH (OA) and oxygen, so that OW, hypoxia and OA have been referred to as the deadly trio (Sampaio & Rosa, 2020). Here, I focus on the combination of OA and OW. The physiological responses of marine ectotherms exhibit high variability to OW (Venegas et al., 2023) and OA (Figuerola et al., 2021) when studied in isolation. These stressors produce additive, antagonistic, or synergistic interactions across various taxa when co-occurring (Schalkhausser et al., 2014; Trnovsky et al., 2016; Ong et al., 2017), with a previous meta-analysis identifying synergistic interactions as the most common type (Harvey et al., 2013. The combined effects result in heightened negative consequences, including a significant reduction in condition index, increased oxidative stress, impacted metabolism, and lower survival rates (Matoo et al., 2013; Ong et al., 2017). Furthermore, there is a decrease in shell density and biomineralization reported in some studies (Ivanina et al., 2013; Chatzinikolaou et al., 2017). Contrastingly, other studies highlight an antagonistic effect of combined OW and OA. For instance, in the case of the flat oyster *Ostrea angas*i, the co-occurrence of OA alleviated the negative effects of OW on survival rates, and OW, in turn, decreased metabolic demands under OA alone (Pereira et al., 2019). Such effect of warming buffering metabolic demands of OA was also reported in other marine ectotherms, including molluscs (Melatunan et al., 2011; Quieros et al., 2015). This diversity in responses demonstrates the complexity of the interactions between OW and OA and emphasizes the need for experimental designs to properly unravel their combined impact on marine ecosystems.

1.2.2.2 Length of exposure and environmental context

Factors such as the length of exposure or the variations in environmental context, can also play critical roles as response modifiers, yet have often been overlooked in studies of the physiological effects of climatic stressors (Gunderson et al., 2016). Indeed, discrepancies in published literature findings arise from inconsistencies in methodological designs. Many studies on the physiological effects of OW and OA predominantly focus on short-term responses, with exposure times ranging from days to a few weeks (Melatunan et al., 2011; Cardoso et al., 2017; Harvey & Moore, 2017; Calosi et al., 2017; Madeira et al., 2018 a). These short-term experiments do not necessarily provide a comprehensive understanding of long-term physiological responses (Dupont et al., 2012; Form & Riebesell, 2012; Paganini et al., 2014; Suckling et al., 2015). Moreover, longer exposure durations have been demonstrated to trigger physiological compensatory mechanisms, potentially leading to physiological acclimation, compared to short-term exposures (Suckling et al., 2015; Sundin et al., 2019), emphasizing the need for adequate time for such adjustments to occur. Extending the duration of exposure is thus essential for a comprehensive assessment of the fitness benefits or costs associated with these physiological adjustments. For instance, Mardones et al. (2021) found that the short-term exposure (50 days) of the gastropod *Ocenebra erinaceus* to OW and OA led to decreased growth and metabolic rates, whereas a more extended exposure period (95 days) resulted in full compensation of metabolic rates. However, when the exposure was prolonged up to 10 months, it caused a complete cessation of reproduction. Conversely, extended exposure to OW and OA proved beneficial for the sea urchin *Sterechinus neumayeri*, allowing the retention of reproductive success after 17 months but not after 6 months (Suckling et al., 2015). This highlights the importance of considering the length of exposure in understanding the adaptive responses of marine organisms to single or multiple environmental stressors.

Seasonality in responses and context variability in exposure conditions can also influence marine animal responses (Christensen et al., 2011; Nardi et al., 2017, 2018; Suárez et al., 2020). Given the variation in environmental conditions and seasons, stressors may occur in or out of phase, contributing to further environmental complexity. Indeed, seasonal changes in exposure conditions influenced responses of the Pomacentrid coral reef fish

Abudefduf vaigiensis to OW and OA (Mitchell et al., 2023). Here future winters conditions reduced physiological performance compared with present-day summer and future summer conditions, despite a compensatory effect, via increased long-term energy storage. Furthermore, seasonal context variability in exposure conditions has been demonstrated to modify ecological interactive responses to OW and OA, with such interactions becoming evident only under long-term exposure (Godbold & Solan, 2013). The physiological responses to long-term exposure to OW, OA, and their combined effects under seasonally changing conditions remain largely undocumented, yet these responses are crucial determinants of how organisms might respond to environmental change. Hence, it is imperative to consider longer durations of ecologically relevant exposures when investigating acclimatory capacity to OW and/or OA.

1.2.2.3 Nature of exposure

The nature of the thermal regime also plays an important role in determining thermal acclimation potential. In recent years, there has been growing recognition that careful consideration of the thermal conditions experienced by an organism in its natural environment is essential for accurately predicting its ability to acclimate to environmental changes and anticipate future performance (Morash et al., 2018). Notably, many intertidal ectotherms do not encounter static temperatures naturally; instead, they face dynamic and fluctuating environmental conditions that may have diverse effects on thermal tolerance and performance (Dong et al., 2021; Leeuwis & Gamperl, 2022; Collins et al., 2023). Plastic responses under fluctuating and unpredicted conditions have been observed to potentially result in greater thermal performance or tolerance compared to static exposure conditions (Dong et al., 2006; Kern et al., 2015; Kang et al., 2019; Nancollas & Todgham, 2022). Therefore, a fluctuating thermal environment, characterized by intervals of elevated temperatures followed by periods of lower temperatures, may serve as a refuge, allowing time for recovery and reducing the costs associated with chronic warming, thereby promoting acclimation (Auld et al., 2010; Chevin & Hoffmann, 2017). Moreover, there is the potential for heat hardening to occur, a phenomenon where organisms experience a short-term increase in heat tolerance following sub-lethal extreme heat exposure (Bilyk et al., 2012). Heat hardening has been reported across various taxa (Hilker

et al., 2016; Hackerott et al., 2021). Its effects are associated with the activation of cellular stress responses, enhancing oxidative stress and mitochondrial respiration capacities, and inducing metabolic remodelling (Dunphy et al., 2018; Georgoulis et al., 2021, 2022; Collins et al., 2023). In marine molluscs, for instance, exposure of adults to single or multiple heat shocks induced heat hardening, resulting in positive short-term effects on survival and increased thermal tolerance (Moyen et al., 2020; Georgoulis et al., 2021, 2022; Zhang et al., 2021; Zhang et al., 2023). Despite its potential importance in acutely impacting thermal tolerance, the effects of heat hardening remain relatively understudied experimentally compared to the effects of chronic, static thermal exposures. Comparing the effects of repeated heat shocks (perhaps inducing heat hardening) with that of the long-term chronic and static warming on the thermal performance and upper thermal limits of the same species will help in understanding the role of thermal exposure nature in shaping physiological responses of marine ectotherms.

1.2.2.4 Mode of reproduction

Numerous studies propose that climate change will adversely impact the fitness of marine organisms, with potential cascading effects on populations and ecosystems. Such predictions frequently rely on short-term experiments and indirect measures of physiological performance, serving as proxies for fitness, which has been usually inferred (Huey and Berrigan 1996; Einum, 2019). Understanding the repercussions of environmental change on fitness components is crucial, as they ultimately determine the persistence of populations in the face of climate change. High temperatures exert negative influences on the reproduction of many marine species, with the extent of these impacts varying based on the duration and magnitude of the warming event. These impacts range from shifts in spawning phases to the complete suppression of reproduction (Alix et al., 2020; Gallo et al., 2020; Boni, 2019; Collin & Salazar, 2010; Delorme & Sewell, 2016; Múgica et al., 2015; Pankhurst & Munday, 2011; Petes et al., 2007). These responses indicate the adverse effects of OW on the reproductive success of marine species and populations. Therefore, direct measures of fitness such as reproductive success (as well as growth and survival) should be incorporated into studies that evaluate for a species' thermal acclimation potential, to provide a more comprehensive understanding of species

susceptibility or resilience to OW and to determine the potential long-term ecological success associated with different acclimation strategies. To comprehend the fitness changes associated with acclimation, it is essential to consider the complex life cycles and diverse reproductive modes characteristic of marine invertebrates, particularly marine molluscs (Reid, 1996). For marine ecosystems to remain healthy and diverse in the changing conditions, it will be key for organisms with diverse modes of reproduction to persist. The reproductive and developmental modes exhibited by marine molluscs significantly impact their distribution and abundance, showcasing a spectrum of complexity (Fortunato, 2004; Foggo et al., 2007; Lee & Boulding, 2009; Hoffman et al., 2011). Notably, direct developers have the capacity to generate phenotypic variation through local adaptation, while planktonic dispersers demonstrate greater physiological flexibility (Yamada, 1987; Hollander, 2008; Sotka, 2012). Moreover, marine gastropods with distinct reproductive modes exhibit differential energy allocation to reproduction (Hughes & Roberts, 1980; Gibson & Chia, 1991; Perron, 1986; Chaparro et al., 2012). It is plausible to hypothesize that the phylogenetic divergence in reproductive strategies may lead to varied energy allocations for physiological adjustments during thermal acclimation, subsequently influencing the types of trade-offs that may occur in fitness components. However, this hypothesis remains largely unexplored. Delving into the interplay between reproductive modes and fitness costs of thermal acclimation in marine molluscs will contribute significantly to unravelling the complex interplay between physiological plasticity and fitness, providing a more comprehensive understanding of the adaptive strategies employed by these organisms and assessing the broader ecological implications of such plasticity.

Increasing complexity of experimental designs, considering such aforementioned response modifiers, is needed to enhance our predictive abilities and deepen our understanding of species-specific responses to environmental stressors and to provide better insights into the capacity of marine species for acclimation under OW and/or OA.

1.3 Developmental plasticity

While a substantial body of literature exists on within generation plasticity, with a primary focus on adult responses, it is imperative to broaden our considerations to encompass responses of other life stages and subsequent generations, which are less well documented. The phenotypic plasticity in response to OW is anticipated to vary among the life stages of marine animals due to differences in their sensitivity to thermal stress (Truebano et al., 2018; Gleason et al., 2018). Specifically, larvae of marine ectotherms are predicted to exhibit higher susceptibility to climatic stressors compared to adults (Byrne and Przeslawski, 2013; Przeslawski et al., 2015), resulting in impacted survival, growth, and an increased incidence of developmental abnormalities. In this context, relying solely on adult responses may fall short in predicting the responses of marine ectotherms to climatic stressors on the long-term. Therefore, studies that incorporate stress exposures across different life stages will offer more accurate predictions of their future performance, considering the varying sensitivities and responses at different life history stages. While adult thermal acclimation is reversible, developmental plasticity can lead to more permanent responses, especially when established during a critical window of development (Burggren, 2020). For example, a correlation was observed between exposing larvae of the quagga mussel *Dreissena bugensis*, to varying temperature conditions and the subsequent development of diverse shell morphotypes in adults (Peyer et al., 2010). In marine ectotherms under thermal stress, developmental plasticity can play a pivotal role in shaping thermal performance (Pottier et al., 2022; Shi et al., 2020). For example, in the copepod, *Tigriopus californicus*, developmental exposure to 25 °C resulted in higher plasticity in critical thermal maxima in adults compared with when they developed under 20 °C (Healy et al., 2019). However, a meta-analysis encompassing 150 studies and involving 138 ectothermic species indicated that significant heterogeneity in developmental plasticity and its carry over effects is present across taxa (Pottier et al., 2022). Phenotypic plasticity is influenced not only by the environmental conditions an organism may encounter during its own lifetime but also extends to the conditions experienced by preceding generations (Burgess & Marshall, 2014). A growing consensus suggests that parental environments can significantly impact the performance of their offspring, with emerging evidence supporting transgenerational effects (Byrne et al.,

2020). In the following section, I discuss the effects of parental environments during the reproductive phase on their offspring performance.

1.4 Transgenerational plasticity

Parental effects, a phenomenon observed when the environment experienced by parents influences the reaction norm of their offspring's' phenotypes, represent a distinctive form of phenotypic plasticity extending across generations through the transmission of non-genetic factors (Mousseau & Fox, 1998; Marshall & Uller, 2007; Marshall et al., 2008; Uller, 2008; Ho & Burggren, 2010; Torda et al., 2017; Chirgwin et al., 2018). Various terms in the literature, such as transgenerational plasticity (TGP), non-genetic inheritance, anticipatory parental effects, carry-over effects, and intergenerational effects, have been used interchangeably to describe this type of phenotypic plasticity (Bonduriansky et al., 2012; Uller et al., 2013; Byrne et al., 2020). For the sake of this thesis, the terms TGP and parental effects will be used interchangeably to encompass the influences of parental stress exposure on offspring performance, and better align with the literature reviewed. No specific underlying mechanism is inferred.

TGP is proposed to enhance the persistence and resilience of populations and species when faced with environmental stress. This suggests that TGP provides populations with the necessary time for genetic changes to occur through accommodation and assimilation processes. As a result, adaptation rates are accelerated, fostering diversification, and contributing to the overall resilience of the population and species (Schlichting & Wund, 2014; Torda et al., 2017; Fox et al., 2019; Kelly, 2019; Eirin-Lopez & Putnam, 2019; Yin et al., 2019; Putnam, 2021). Many studies have demonstrated beneficial effects of parental exposure to environmental stress on offspring performance, while others report neutral or negative responses (Ross et al., 2016; Torda et al., 2017; Donelson et al., 2018; Byrne et al., 2020). The underlying reasons for these discrepancies in parental effect responses, as well as whether and when they might be adaptive, remain unclear (Donelson et al., 2018).

Parental effects should be adaptive when the environment of the offspring can be predicted by that of the parents (Marshall & Uller, 2007; Fischer et al., 2011; Donelan et

al., 2020). For example, juvenile damselfish demonstrated the ability to fully compensate for the adverse impacts of elevated water temperature on metabolic rate and aerobic scope, when their parents were also raised under elevated temperatures (Donelson et al., 2012). Similarly, in the context of OA, a study on *Mytilus edulis* demonstrated that subjecting parents to a pCO_2 level of 1,000 µatm over a period of six months had a notable impact. The offspring produced by these conditioned parents developed calcitic shells, which are the less soluble form of calcium carbonate under OA, in contrast to the more soluble aragonitic shells typically formed during metamorphosis into juveniles under normal conditions (Fitzer et al., 2014). This is an intriguing calcification plasticity for those mussels to cope with OA. Lack of, or even negative parental effects have also been reported across taxa and stressors. For instance, in the case of the scallop *Argopecten irradians* and the clam *Mercenaria mercenaria*, conditioning parents under OA did not confer benefits to the offspring. In both species, the larvae produced by parents exposed to OA conditions were either equally or more sensitive to OA compared to larvae from adults exposed to control conditions (Griffith & Gobler, 2017). Therefore, these heterogenous responses indicate that marine animals use a range of strategies that influence their offspring performance, posing challenges in comprehending the optimal conditions for triggering adaptive parental effects and understanding its potential in mitigating the impacts of future climatic stressors.

One potential cause for the variability in parental effect responses could be associated with trade-offs between parental and offspring fitness in different environments (Burgess & Marshall, 2014; Waite & Sorte, 2022). Modifications in gamete phenotypes and physiological mechanisms associated with parental effects can be energetically expensive for parents (Uller, 2008). Therefore, parents may prioritize investing in their own survival, even if it comes at the cost of the fitness of their offspring (Burgess & Marshall, 2014; Jensen et al., 2014; Marshall & Uller, 2007). Consequently, not all parental exposure conditions can promote the investment in such energetically exhausting offspring fitness. To overcome this challenge, we should consider exposure conditions that may reduce or prevent such trade-off between parents and offspring.

It is noteworthy that many studies on TGP related to climate change subject adult parents to stress magnitudes they have not encountered before during short-term exposures. This approach may not favour within generation plasticity in parents due to the extremely high costs associated with it, reducing the capacity to trigger TGP. In contrast, longer exposure periods during the reproductive phase have been observed to lead to more pronounced positive parental effects (Dupont et al., 2012; Suckling et al., 2015). For example, exposing the sea urchin *Psammechinus miliaris* and the endangered sea cucumber *Apostichopus japonicus* to parental conditioning involving OW and OA yielded distinct outcomes in the performance of their offspring. The augmented performance and heightened tolerance were particularly notable when the duration of parental exposure was extended (Suckling et al., 2014; Wang et al., 2015). Therefore, prolonged periods of exposure may be necessary to induce parental acclimation to chronic warming, as elaborated in section 2.2.2. This extended timeframe enhances the potential for investments in offspring fitness, fostering adaptive parental effects. Moreover, the examination of the nature of thermal regime's role, as discussed in section 2.2.3, particularly focusing on the potential of repeated exposure to heat shocks to induce heat hardening in parents, provides an avenue for assessing approaches that may trigger adaptive parental effects. This comparative analysis becomes particularly valuable when compared with the effects of parental exposure to long-term chronic warming. Both repeated heat shock exposures and prolonged chronic warming may facilitate parents' acclimation, or they may impose distinct costs associated with parental acclimation due to their different nature. Consequently, this may give rise to differential parental effects on their offspring performance.

1.5 Conclusions

Climatic change stressors pose a significant threat to the biology of marine species, jeopardizing ecosystem functionality and food security. Phenotypic plasticity emerges as an immediate response strategy to environmental change, offering animals the potential to maintain performance within and across generations amid climatic stressors. While marine ectotherms exhibit the ability to acclimate to elevated temperatures, this capacity is species-specific and may be constrained by the ability to maintain energy supply for

physiological adjustments, which is modulated by the exposure context as well as the animal's biological characteristics. Predicting the physiological responses of marine ectotherms in the wild goes beyond the effects observed upon short-term exposures. The modifying effects of physiological plasticity require more rigorous testing before attempting to forecast the consequences of climate change on marine ecosystems, to properly investigate and understand the adaptive capabilities of marine species. Furthermore, to comprehensively assess the extent and consequences of environmentally induced phenotypic plasticity in marine organisms, studies that take into account the influence of environmental conditions experienced within and across generations are required. These studies are crucial for understanding how marine species and populations can alter their responses under climate change stressors (Bernal et al., 2022; Munday et al., 2013). Moreover, investigating optimal exposure conditions that result in adaptive parental effects is paramount to get this knowledge, which not only illuminates the susceptibility or resilience of marine organisms to climate change but also aids in the development of effective conservation and resources sustainability strategies (Donelson et al., 2023).

1.6 Thesis aim and objectives

The aim of this thesis was to assess the extent and cost of physiological plasticity of key marine molluscs to warming across life history stages. This aim was achieved through the following main objectives:

1- To investigate within-generation, long-term physiological plasticity of three species of adult intertidal gastropods to warming, alone and in combination with decreased pH. The effects of seasonal variation (temperature, photoperiod, and tidal levels) and length of exposure (3, 6, and 12 months) on such plasticity was also assessed (**Chapter 2**). The beginning of the experimental phase of my PhD was significantly delayed by Covid-imposed restrictions. As a result, this chapter is based on an existing dataset, previously generated as part of the UK Ocean Acidification Research Programme (UKOARP, grant no. NE/H017445/1). I carried out all formal analyses, results presentation, interpretation and writing.

2- To characterise the cost of physiological plasticity and any potential trade-offs between fitness components. This was done by characterizing the cost of thermal acclimation in terms of fitness components (scope for growth, survival, and reproduction) in two congeneric intertidal gastropods with different reproductive modes (**Chapter 3**).

3- To compare the effects of different thermal regimes on adult performance, evaluating the differences between acclimation to chronic (6 month) elevated temperatures, and exposure to repeated heat shocks (heat hardening) in a commercially important intertidal gastropod (**Chapter 4**).

4- To compare the effects of different parental thermal regimes (i.e. those applied in chapter 4) on offspring performance during development under different temperatures. Here, I investigated the potential for positive parental effects and/or developmental plasticity (**Chapter 5**).

The intertidal zone, characterized by a steep gradient in biotic and abiotic stressors, provides an ideal environment to study physiological adaptation across populations and species (Bartholomew, 1987; Somero 2002; Stenseng et al., 2005; Dong et al., 2006). Intertidal organisms are valuable models in ecophysiology due to their unique morphological, biological, and life history traits (Rolán-Alvarez et al., 2015). Their distribution is largely dependent on their ability to cope with daily fluctuations in their thermal environment (Stillman and Somero 1996). Consequently, this thesis focuses on a range of intertidal models to address the objectives outlined above. As I have used different model organisms to address each of the objectives, a justification of the suitability of each model to address the question is given in each chapter.

Chapter 2

Seasonal and species-specific physiological responses to Long-term warming and ocean acidification in marine gastropods

2.1 Abstract

The serious effects of rising sea surface temperatures (OW) and ocean acidification (OA), on many marine species is clear. Yet, the potential for seasonal dynamics to ameliorate or intensify species' physiological responses to these stressors has not received the same attention. In this 12-month long study, I investigated the impact of OW and OA on three ecologically important intertidal gastropods (*Nucella lapillus, Osilinus lineatus,* and *Littorina littorea*). Snails were exposed to two temperature regimes: historical averages (ambient) or a warming scenario (ambient + 4 °C), three atmospheric CO_2 levels in the ambient (380, 750, 1000 ppm) and two CO_2 levels in the warming regime (380, 750 ppm) under the influence of changing environmental conditions. Rates of oxygen consumption, as a proxy for metabolism, were measured at 3, 6, and 12 months intervals. I found that the interactive effects of OW and OA become significantly evident under simulated summer conditions after 6 months exposure. Moreover, metabolic changes are intricately entwined with how species respond to the fluctuations in environmental conditions associated with the changing seasonal conditions, in some cases exacerbating, and in other mitigating the consequences of these stressors. In *N. lapillus*, oxygen consumption increased in summer conditions irrespective of temperature regime. Elevated OA resulted in a reduction in metabolism under ambient temperature in winter conditions initially, but this was not sustained over a more protracted time, with no main effect or interaction effect under OW. In *O. lineatus*, OA had no effect under ambient temperature, but oxygen consumption increased in summer conditions for both ambient and warming regimes, OW increased oxygen consumption in winter conditions, while synergistic interaction was observed in summer conditions. In *L. littorea*, under ambient temperature, OA had no significant effect in summer, but reduced its oxygen consumption during the winter, while under warming, oxygen consumption decreased in winter but not summer conditions. OW alone increased metabolism in summer, with antagonistic interaction with OA. This

temporal perspective of environmental variability is crucial when attempting to predict the physiological and, resultant, ecological implications of climatic stressors, for accurate forecasting species responses. This study lends no support to a unifying effect of climatic stressors on physiological responses, and that the seasonal responses and the species-specific variations can greatly complicate any general conclusions.

2.2 Introduction

It is now indisputable that the warming of our ocean and atmosphere is being driven by anthropogenic CO_2 input as result of, amongst other things, burning fossil fuels (IPCC, 2021). The unprecedented rate of warming as a result of climate change is leading to increased sea surface temperatures (i.e. ocean warming, OW), which are predicted to increase by 2 – 4 ˚C by the end of the century (IPCC, 2023). This OW accompanied by changes in the carbonate chemistry of sea water as a result of CO_2 first dissolving in and then reacting with, resulting in, amongst other things a reduction in sea water pH of 0.3 – 0.4 pH units (i.e. ocean acidification, OA) (IPCC, 2023). OW and OA both affect physiological processes of aquatic species (Mayor et al., 2015; Goncalves et al., 2017. 2018; Oliveira et al., 2020), limiting their performance, and ultimately impacting the functioning of marine ecosystems (Bindoff et al., 2019; Doney et al., 2020). Our understanding of what the physiological responses to climatic stressors are, how they interact with other natural changes, and how all of this affects the ecological success of species and populations is still incomplete.

The species of marine ectotherms that have been investigated including molluscs, tend to show highly variable physiological responses to OW (Venegas et al., 2023) and OA (Figuerola et al., 2021) in isolation, but when the two stressors interact, additive, antagonistic, or synergistic effects are observed (Schalkhausser et al., 2014; Trnovsky et al., 2016; Ong et al., 2017; Zhang et al., 2015; Hoshijima et al., 2017). Initially synergistic interactions were thought to be the most prevalent response to combined stressors, based on previous meta-analysis (Harvey et al. 2013). However, this is not invariant. Some studies have reported antagonistic effects of combined OW and OA in marine molluscs (Melatunan

et al., 2011; Pereira et al., 2019; Quieros et al., 2015). However, a major limitation is that most investigations of OW and OA focus on short-term physiological responses, with exposure ranging from days to a few weeks (Melatunan et al., 2011; Cardoso et al., 2017; Harvey & Moore, 2017; Ong et al., 2017; Wang et al., 2018; Johnson & Hofmann, 2020). This is a limitation as short-term exposures are not always predictive of long-term physiological responses (Dupont et al., 2012; Form & Riebesell, 2012; Suckling et al., 2015). Moreover, longer exposure times have been shown to elicit physiological compensatory mechanisms that may lead to acclimation (Suckling et al., 2015; Sundin et al., 2019; Maboloc & Chan, 2021), as time is required for physiological adjustments to take place. In addition, studies accounting for other response modifiers, such as the seasonality and the natural variability of habitat conditions (e.g. temperature, photoperiod, and tidal cycles) are needed in order to increase our predictive power, enabling extrapolation of individual to ecosystem level responses (Gunderson et al., 2016; Pörtner et al., 2017; Wahl et al., 2015). Indeed, seasonal status of blue mussels, *Mytilus galloprovincialis*, have been shown to modify its physiological responses to OW and OA (Nardi et al., 2017, 2018). Moreover, seasonal changes in exposure conditions influenced responses of the Pomacentrid coral reef fish *Abudefduf vaigiensis* to OW and OA (Mitchell et al., 2023). Here future winters conditions reduced physiological performance compared with present-day summer and future summer conditions, despite a compensatory effect, *via* increased long-term energy storage. Tidal fluctuations were also found to influence the stress intensity of OA and OW (Christensen et al., 2011). Therefore, predicting the ecological impacts of OA and OW requires long-term, ecologically realistic studies that allow us to characterise the susceptibility of individuals and populations.

In their long-term (542 days) experiment, Godbold and Solan (2013) investigated the effect of OW and OA on growth, bioturbation/ bioirrigation behaviour and associated levels of ecosystem functioning (nutrient generation) on a functionally important intertidal polychaete, *Alitta virens*. They found that effects of OW and OA and their interaction were not detectable over the short term (7 days), but appeared only after several months. Moreover, the responses observed were greatly impacted by seasonal changes, e.g. under ambient temperature regime, the bioturbation depth under elevated pCO_2 conditions was greater in warmer months (6 and 18) compared to colder months (3 and 12). However, this

pattern did not hold under warming regime when there were lower bioturbation depths during the first 12 months, although similar depths to those under ambient were observed after 18 months. Unfortunately, there are few long-term multi-stressor studies, such as that carried out by Godbold and Solan (2013), with no such studies incorporating tidal and photoperiods context variations.

Therefore, the aim of this study was using the same experimental design as Godbold and Solan (2013), to determine whether physiological responses of other intertidal species follow similar patterns to the ecological responses they observed. This was achieved investigating the effects of long-term exposure (12 months) to hypercapnic sea water (pCO_2 = 380, 750, and 1000 ppm) and warming on the rate of oxygen uptake (as a measure of metabolism) of three intertidal marine gastropod species under seasonally changing conditions, exposed to realistic tidal emersion periods. The capacity of organisms to acquire the energy needed to support physiological adjustments controls individual's persistence under changing ocean conditions (Calosi et al., 2017; Hemraj et al., 2020). This physiological adaptability depends, to a great extent, on the regulation of energy homeostasis (Sokolova et al., 2012), and energy reallocation (Goncalves et al., 2018). Energy metabolism therefore links organismal physiology and ecology (Sokolova, 2013), and so whole-organism metabolic rate is considered a key physiological trait of wide-ranging ecological importance (Burton et al., 2011).

Across all hypercapnic treatments employed, temperature was adjusted monthly following a seasonal cycle using two different temperature regimes: 1) matching historical mean monthly temperatures at the study site (ambient cycle), or 2) a warming scenario (ambient cycle + 4 ˚C). Levels of atmospheric [CO_2] were 380, 750 and 1000 ppm in the ambient treatment, and 380 and 750 ppm in the ambient + 4 ˚C treatment. Rates of oxygen consumption were measured for each species after 3, 6, and then 12 months.

Based on the previously described ecological changes resulting from exactly the same exposure regime as employed by Godbold and Solan (2013), I hypothesised that the interactive effect of OW and OA would only be detectable after prolonged, but not short-term, exposure and would be modulated by seasonal changes. I used three intertidal gastropods as models for this study. All are common on the temperate rocky shores of NW

Europe: the dogwhelk *Nucella lapillus*, a carnivorous gastropod inhabiting the mid-low shore (Crothers 1985), the common periwinkle *Littorina littorea*, an abundant grazer that ranges from the high intertidal to sublittoral zone (Fretter & Graham 1962), and *Osilinus lineatus*, the most abundant grazing species occurring on these shores. As these three species are abundant and have key roles in intertidal ecosystems, any effects climate change may have on their physiological responses could affect wider ecosystem functions.

2.3 Materials and methods

2.3.1 Animal collection, mesocosm design, and husbandry

Individuals of all three species were collected by hand during the low tide period from low intertidal and subtidal areas of the rocky shoreline at Mount Batten, Plymouth, UK (50°21'30"N 4°07'50"W). They were transported to the Plymouth Marine Laboratory Intertidal Mesocosm Acidification System (PML-IMAS) within 1 h of collection. Here they were initially allowed to acclimatise to laboratory conditions in ambient sea water, pH, and temperature, for approximately 3 weeks. The experimental exposures described below commenced in late winter and lasted for 12 months.

The design of the mesocosm system, together with a detail of temperature, salinity, pH, total alkalinity, inorganic nutrients, and components of the carbonate system are all exactly as described in Findlay et al. (2013). Briefly, the PML-IMAS consists of 20 X 1 m^3 mesocosm tanks (700 L of sea water and 300 L of overlying atmosphere) arranged in four rows of five tanks within different controlled temperature environments. Five experimental treatments were randomized among the 20 tanks, with four replicates per treatment. Across all treatments; temperature, day-night light cycles, and a semi-diurnal tidal cycle were adjusted monthly following a seasonal local conditions cycle under two temperature regimes: 1) matching historical mean monthly temperatures at the study site (ambient), or 2) a warming scenario (ambient cycle + 4 ˚C). Levels of atmospheric [CO_2] were 380, 750 and 1000 ppm for the ambient treatment, and 380 and 750 ppm for the ambient + 4 ˚C treatment.

The experimental design adopted followed very closely that described by Godbold & Solan (2013). Once the experimental conditions stabilized, individuals (N = 20 – 30) of each species were randomly assigned to each tank. Temperatures were adjusted by controlling the controlled air temperatures room, warm treatments were further regulated by use of immersion heaters (300 W) in tanks. The level of atmospheric CO_2-driven acidification in each tank was regulated by injecting a mixture of pure CO_2 gas with CO_2-free air into the water. Flow meters and mixing vessels were used to obtain the desired concentration in each tank, and this was monitored using a closed-path CO_2 analyser (820, Li-Cor) exactly as described by Findlay et al. (2008). Thick PVC covers were used to minimize loss of CO_2 from the tank atmosphere. The pH in the control treatments was altered to match the yearly mean pH, i.e. pH = 8.08 ± 0.07 (mean ± S.D.). The semi-diurnal tidal cycle was stimulated using a pump and ballast system (Findlay et al., 2008). During the emersion periods, snails were exposed to the same nominal CO_2 concentration as during immersion. Throughout the experiment *N. lapillus* were supplied with mussel pieces *ad libitium*, whereas *L. littorina* and *O. lineatus* were supplied the green seaweed *Ulva lactuca* once a week for food.

2.3.2 Measuring rates of oxygen consumption

Rates of oxygen consumption (MO_2) were measured after 3 months, then 6, and 12 months of exposure as described below. However, the first time point was missed for *L. littorea* due to logistic issues. High mortality of *O. lineatus* in more than one treatment prevented measurements being made at the third time point.

All snails were starved *in situ* for 24 h before any measurements were made. MO_2 was measured on individuals using stop-flow respirometry. Each respirometer consisted of a glass container, with a gas tight lid (Vol. = 707 ml), submerged in a water bath containing sea water identical to that the snail was being kept in. Each container was fitted with an oxygen-sensitive dot (Oxy2Dot™) a magnetic flea, separated by a perforated platform, and glass beads to mimic a substrate and minimise activity levels. The magnetic flea was powered by an underwater magnetic stirrer (Thermo Scientific Multi Position Stirrer, USA) in each case. Eighteen respirometers in total were run simultaneously. Temperature was monitored using a K type thermocouple within each set of chambers connected to a

temperature logger (Omega, HH806AU, Manchester, UK). Two blank chambers were run used to account for the possible build-up of microbial activity in the respirometers. Disturbance was minimized by reducing light and noise levels in the room as much as possible.

Measurements of MO_2 were made as follows. An individual was placed in each respirometer and allowed to settle to for 1 h. After this time the lid was secured, the time noted, and the decline in PO_2 measured at regular intervals by taking readings from the oxydot in the respirometer using an OxySense GEN III 5000 series oxygen analyser system (OxySense, Dallas, TX) modified from Rastrick and Whiteley (2011). In each case the decline in pO_2 was linear over the measurement period but never fell below a pO_2 = 17 kPa, so the snails were never exposed to hypoxic conditions. After the MO_2 of the snails was measured, they were carefully blotted dry and weighed using a precision balance (Cubis Semi-Micro Balance, Sartorius, Germany). MO_2 was expressed as μmol h^{-1} g^{-1}.

2.3.3 Statistical analysis

Analysis of variance (ANOVA) models were developed to determine the effects of OA and OW on mass-specific MO_2. For each species, under ambient or ambient + 4 ˚C regimes, tow-way ANOVA (pCO_2 X time point) was used to test the effect of elevated pCO_2 concentrations. In addition, two-way ANOVA was used to test for any interaction between temperature regime and pCO_2, as well as for the temperature regime effect (temperature regime X time point). All data were tested for normality and variance homogeneity by visually inspection of residuals, and general linear mixed model with restricted maximum-likelihood (REML) was used where homogeneity of variance was violated. 'Tank' was included as a random factor and whole-body mass as a covariate. The optimal model structure was determined using AIC and visualization of model residuals. There was no tank effect for any of the responses analysed and so this was eliminated from further tests. Statistical significance was assigned at $P < 0.05$. All analysis were conducted using SPSS v.25 software.

2.4 Results

2.4.1 MO_2 of *Nucella lapillus*

Presented in Figure 2.1 are the mean mass-specificMO_2s for *Nucella lapillus* under ambient (Fig. 2.1A) and warming (ambient + 4 ˚C (Fig. 2.1B) temperature regimes, under different pCO_2 treatments, and upon different lengths of exposure (time point x pCO_2). The length of exposure (seasonal conditions) significantly influenced the MO_2 of *Nucella lapillus* at both ambient and warming temperature regimes ($F_{2,98}$ = 11.95, P < 0.00; $F_{2,62}$ = 3.95, P = 0.024, respectively). While pCO_2 treatment had a significant effect on MO_2 at ambient ($F_{2,98}$ = 7.77, P < 0.001), there was no significant effect of warming ($F_{1,62}$ = 2.15, P = 0.14), and there was no significant interaction between length of exposure and pCO_2 at both temperature regimes ($F_{4,98}$ = 2.2, P = 0.072; $F_{2,62}$ = 0.142, p = 0.86, respectively). Across the seasons, under ambient and warming conditions, MO_2 rates were significantly greater at summer conditions (6 months) compared with winter conditions after both 3 (P = 0.036 and P = 0.004, respectively) and 12 (P < 0.001 and P = 0.009, respectively) months of exposure, irrespectively of pCO_2. Under ambient conditions, MO_2 decreased with increasing pCO_2 level, being significantly lower in winter (3 months) at 1,000 ppm, compared to the other two concentrations (P = 0.024 and P = 0.04 for 380 and 750 ppm respectively). However, this difference disappeared in the next two time points (6 and 12 months) (P > 0.05) (Fig. 2.1A and B).

There was no interactive effects of pCO_2 (380 and 750 ppm) and temperature regimes (pCO_2 x temperature regime) on MO_2 of *N. lapillus* either as overall interaction ($F_{1,135}$= 1.73, p = 0.18) nor at any of the three seasons: in winter conditions after 3 ($F_{1,41}$ = 0.35, P = 0.55) and 12 ($F_{1,43}$ = 0.57, P = 0.45) months of exposure, or in summer (6 months)($F_{1,43}$ = 0.001, P = 0.96) (Fig. 2.1C). Moreover, there was no significant differences in MO_2 between the two temperature regimes at any time point.

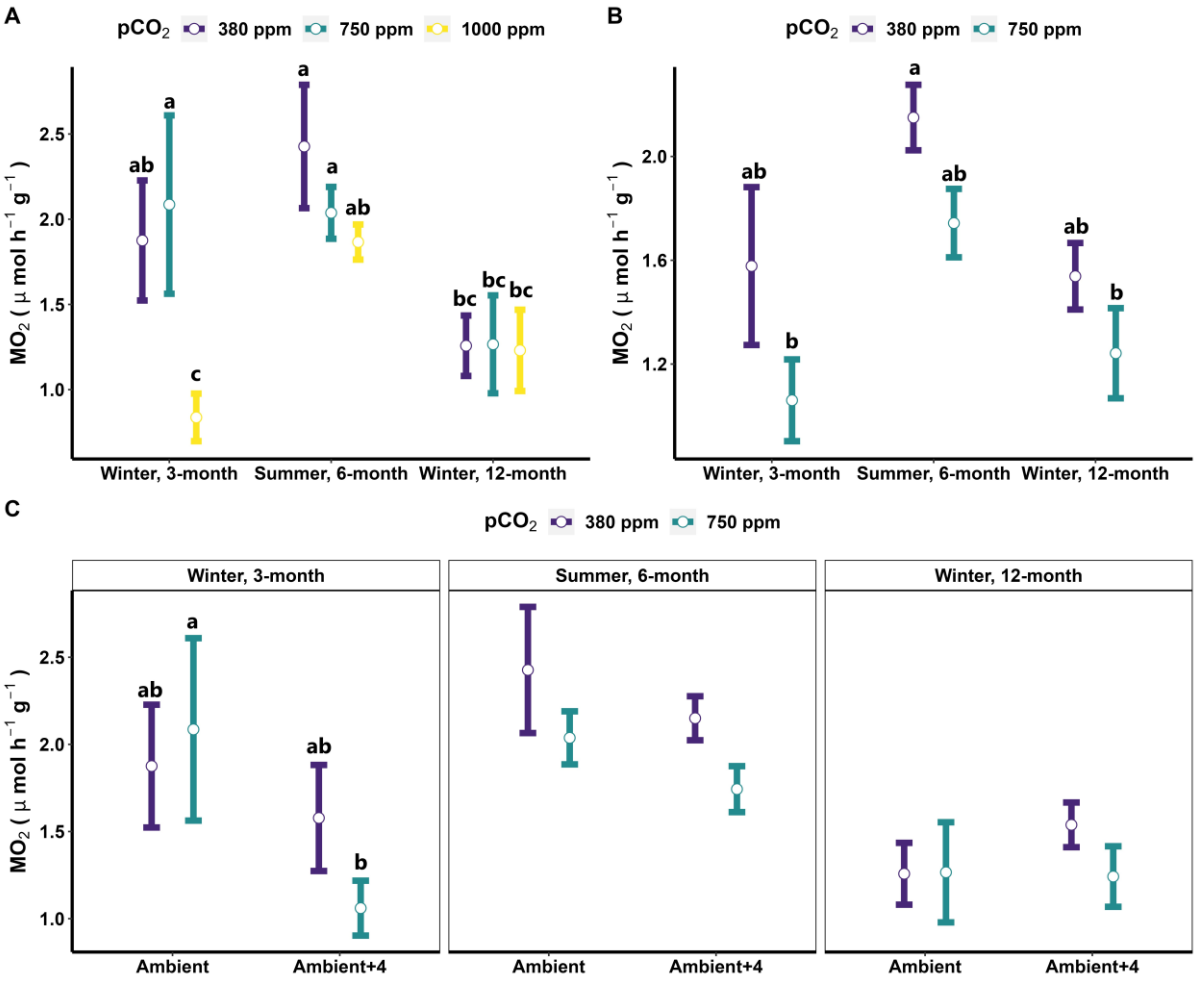


Figure 2.1. Mean (± S.E.) mass-specific rate of oxygen consumption for *Nucella lapillus* under A) ambient temperature at three different pCO_2 values, B) warming (ambient + 4 ˚C) temperatures at two different pCO_2 values, C) ambient and elevated (ambient + 4 ˚C) temperature at two different pCO_2 values, and upon different lengths of exposure. Lowercase letters indicate significant differences between treatments at $P < 0.05$ (N = 10 – 12 individuals per pCO_2 treatment, temperature regime, and time point). Values are expressed as means ± 1 S.E.

2.4.2 MO_2 of *Oscilinus lineatus*

For *Oscilinus lineatus*, under ambient (Fig. 2.2A) and warming (Fig. 2.2B) temperature regimes, there was a significant interaction between pCO_2 and length of exposure (time point) on MO_2 ($F_{2,65} = 6.52$, $P = 0.002$; $F_{1,44} = 8.48$, $P = 0.005$, respectively), as well as a significant main effect of length of exposure ($F_{2,65} = 30.86$, $P < 0.001$; $F_{1,44} = 9.72$, $P = 0.003$,

respectively), although not pCO_2 ($F_{2,65}$ = 0.67, P = 0.51; $F_{1,44}$ = 1, P = 0.32, respectively). Under winter conditions, (3 months), *O. lineatus* from both temperature regimes under elevated pCO_2 appeared to show a pattern of decreasing MO2, compared with the control (380 ppm) but the difference was not statistically significant (P = 0.1 and 0.3 for ambient and warming, respectively). Under summer conditions, (6 months) of exposure, *O. lineatus* at ambient regime and exposed to pCO_2 1,000 ppm and those at warming regime and exposed to pCO_2 750 ppm exhibited a significantly greater MO_2 compared with their counterparts at winter conditions (P < 0.001 and 0.002, respectively). While this increase in MO_2 was not significantly different from other pCO_2 treatments for individuals under the ambient regime (P > 0.05), at the same length of exposure, it was significantly different from control at warming regime (P = 0.028).

There was an interaction between temperature regime and pCO_2 in summer conditions, (6 months) ($F_{1,44}$ = 6.69, P = 0.013), but not in winter conditions (3 months) ($F_{1,44}$ = 0.37, P = 0.54). Exposure to warming regime increased MO_2 in winter (3 months) ($F_{1,43}$ = 11.12, p = 0.002) and summer conditions, (6 months) ($F_{1,43}$ = 14.77, P < 0.001), when compared to ambient regime (Fig. 2.2C). While responses under summer conditions were driven solely by a significant effect of OW, under winter conditions it was driven by a significant combined effect of OW and OA.

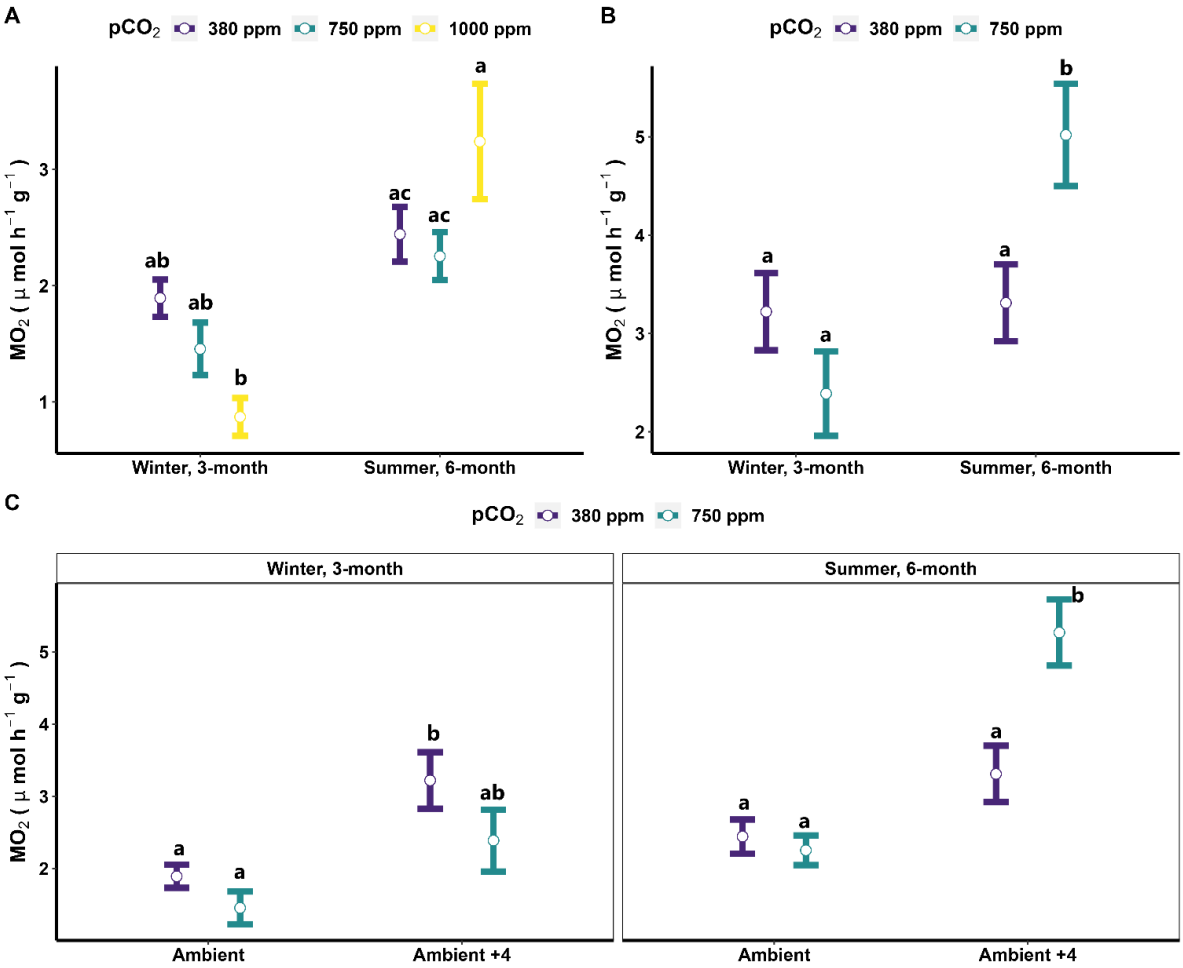


Figure 2.2. Mean (± S.E.) mass-specific rate of oxygen consumption for *Osilinus lineatus* under A) ambient temperature at three different pCO_2 values, B) warming (ambient + 4 ˚C) temperatures at two different pCO_2 values, C) ambient and elevated (ambient + 4 ˚C) temperature at two different pCO_2 values, and upon different lengths of exposure. Lowercase letters indicate significant differences between treatments at $P < 0.05$ (N = 10 – 12 individuals per pCO_2 treatment, temperature regime, and time point). Values are expressed as means ± 1 S.E.

2.4.3 MO_2 of *Littorina littorea*

For *Littorina littorea*, under ambient (Fig. 2.3A) and warming (ambient + 4 ˚C) regimes, there was a significant interaction between pCO_2 treatment and length of exposure on MO_2 ($F_{2,65} = 6.92$, $P = 0.002$, $F_{1,65} = 4.36$, $P = 0.042$, respectively), with no significant effect of length of exposure ($F_{2,65} = 0.26$, $P = 0.61$, $F_{1,65} = 0.21$, $P = 0.64$, respectively) or pCO_2 ($F_{2,65} =$

1.15, P = 0.32, $F_{1,65}$ = 2.45, P = 0.12, respectively). For *L. littorea* snails kept at ambient temperature regime, MO_2 was similar across pCO_2 treatments (P > 0.05), under summer conditions (6 months). However, under winter conditions (12 months), MO_2 decreased steadily with increasing pCO_2 levels where 1,000 ppm resulted in the lowest MO_2 compared to the control (380) ppm (P = 0.001) while 750 ppm was intermediate between them with no significance differences (P > 0.05) detected. Moreover, exposure to 1,000 ppm was associated with lower MO_2 compared at each pCO_2 level in summer conditions (6 months) (P < 0.001). While under the warming regime*, L. littorea* in summer conditions had a significantly lower MO_2 when exposed to pCO_2 750 ppm compared to 350 ppm (P = 0.036) (Fig. 2.3B). However, this difference disappeared at winter conditions (12 months) (P > 0.05).

Furthermore*,* there was a significant interaction between temperature regime and pCO_2 on MO2 in the summer conditions (6 months) ($F_{1,43}$ = 4.78, p = 0.008), mainly as a result of a significant increase in MO_2 under OW in isolation compared with ambient regime (p = 0.014), as well as greater than that of combined OW and OA (p = 0.008) (Figure 4A). There was no interaction effect detected in winter conditions after 12 months ($F_{1,44}$ = 1.91, P= 0.17), although *L. littorea* in the warming regime showed a significantly greater MO_2 than ambient ($F_{1,44}$ = 9.65, P= 0.003), while in summer conditions there was no significant difference between temperature regimes ($F_{1,44}$ = 2.99, P= 0.09) (Fig. 2.3C).

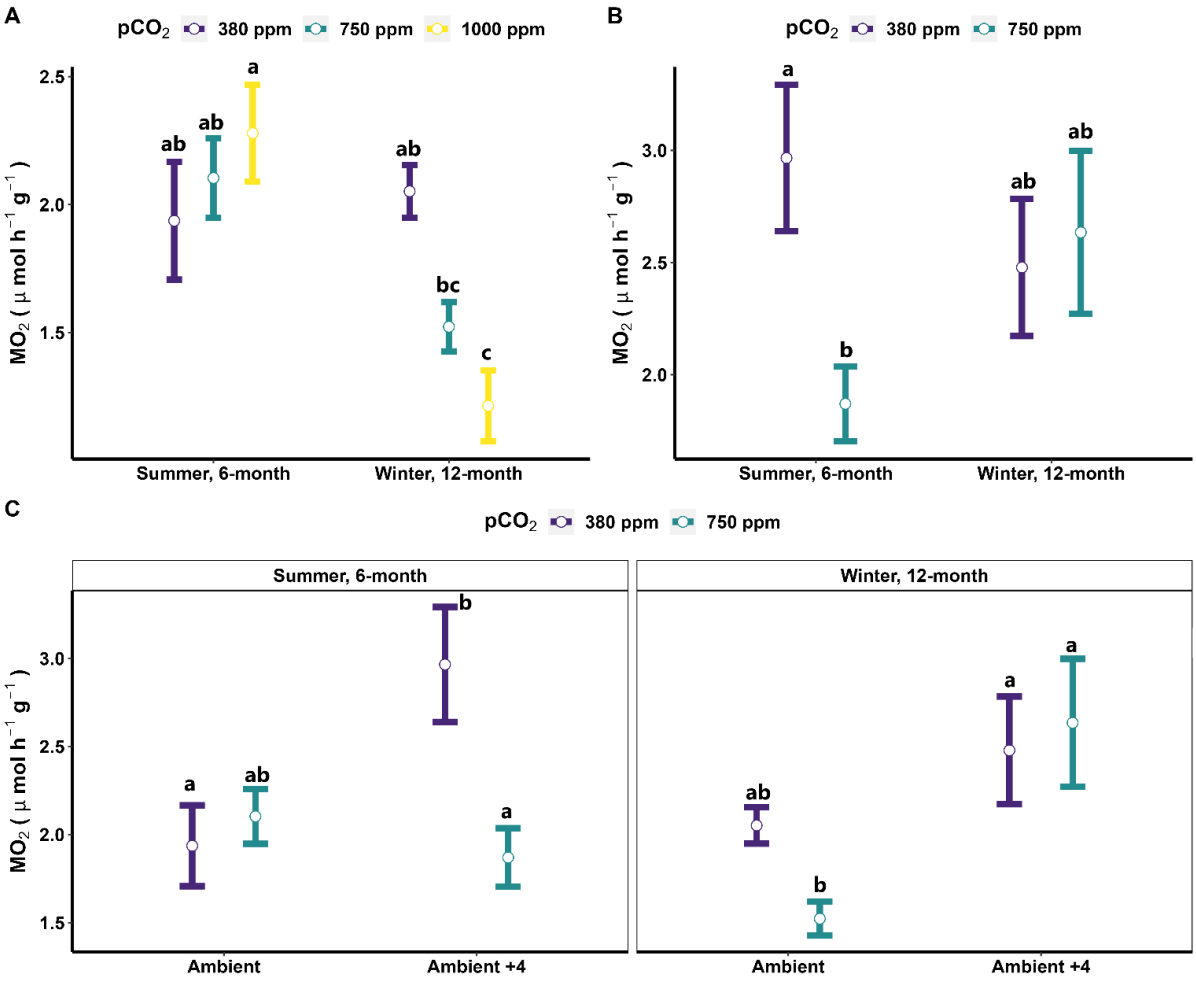


Figure 2.3. Mean (± S.E.) mass-specific rate of oxygen consumption for *Littorina littorea* under A) ambient temperature at three different pCO_2 levels, B) warming (ambient + 4 ˚C) temperature at two different pCO_2 levels, C) ambient and warming (ambient + 4 ˚C) temperatures at two different pCO_2 levels, and upon different lengths of exposure. Lowercase letters indicate significant differences between treatments $P < 0.05$ (N = 10 – 12 individuals per pCO_2 treatment, temperature regime, and time point). Values are expressed as means ± 1 S.E.

2.5 Discussion

I investigated whether long-term exposure to ocean warming (OW), and ocean acidification (OA) affected the metabolism of three intertidal marine gastropod species under seasonally-changing conditions. Based on the ecological effects observed under the same

exposure conditions (Godbold and Solan, 2013), I predicted that interactive effects between the stressors would only emerge in the long-term, and they would be highly influenced by seasonality in my exposure conditions (mainly temperature, tidal cycles, and photoperiods). Interestingly, I found that the interactive effects of warming and acidification on physiological responses followed those observed in Godbold and Solan (2013)'s study. I detected an interactive effect of OW and OA at summer conditions (6 months) of the exposure for two of the three species. In *O. lineatus*, there was a synergistic effect, i.e. combined exposure of OW and OA showed significantly higher metabolic demands that occurred under OW alone. This was in agreement with other studies on marine molluscs which applied shorter exposures under constant conditions and reported interactive synergetic effects of OW and OA (Matoo et al., 2013; Ivanina et al., 2013; Chatzinikolaou et al., 2017; Ong et al., 2017). Although these studies reported a decrease in the metabolism, *O. lineatus* increased MO_2. Interestingly, in contrast to *O. lineatus*, the observed interaction in *L. littorea* was antagonistic, where the combined exposure to OW and OA masked the increase in metabolic demand compared with OW alone. This was similar to other reported antagonistic effects of combined OW and OA in marine molluscs, such as in the flat oyster *Ostrea angas*. Here OA ameliorated the negative effects of elevated temperature on survival (Pereira et al., 2019). An antagonistic interaction effect has been previously reported in *L. littorea* under shorter exposures and more constant conditions, however there was a different pattern, when combined, with OW and OA decreasing the metabolic demand observed under OA alone (Melatunan et al., 2011). The observed differences may be a result of the longer length of exposure and the environmental variability applied here.

Secondly as predicted, when each driver is considered in isolation, responses to OW and OA where highly influenced by season, but they were also species-specific. In *N. lapillus*, under summer conditions, metabolic rates increased compared with winter conditions, irrespectively of whether snails were exposed to ambient or elevated temperatures. Under ambient temperature regime, elevated OA decreased metabolism under winter conditions (3 months), but this effect disappeared at the following two time points, the summer and second winter (6 and 12 month respectively) conditions, possibly demonstrating an acclimation response. A similar result has been reported for the abalone *Haliotis*

tuberculata where it was associated with ability for compensation of changes in extracellular pH under OA (Avignon et al., 2020). OW in isolation did not affect MO_2 in *N. lapillus*, as has been reported for other gastropod species (Melatunan et al., 2011; Hoefnagel & Verberk, 2017). Given the absence of any interactive effects between OW and OA, my results point to the ability of *N. lapillus* to cope metabolically with OW and OA both singly and in combination.

In *O. lineatus*, under ambient temperature, the effect of 1,000 ppm was dependent upon season. Summer conditions produced a significantly greater metabolic rate than winter conditions. Similar seasonal-dependent pattern was observed for 750 ppm under warming conditions, which also resulted in a greater MO_2 than the control. This sensitivity of the effects of OA under summer conditions further strengthens the case for an interactive effect of OW and OA. Moreover, OW in isolation increased MO_2 only under winter conditions. Increasing metabolism seemed to be the predominant response of *O. lineatus* under OW and OA singly and combined. Although increased MO_2 as a result of exposure to environmental stress has been reported in other gastropods (Gaty & Wilson, 1986; Vosloo & Vosloo, 2010; Lefevre et al., 2015; Leung et al. 2020), an increase in MO_2 under a combination of OW and OA is far less common in marine ectotherms, compared with no effect or even a reduction (reviewed in Lefevre, 2016). An increase in MO_2 has been suggested to be linked with the maintenance of internal pH in marine organisms (Melzner et al., 2009), or to increase calcification rates under OA (Calosi et al., 2017). However, increased metabolic rate could also be interpreted as maladaptive (Hoshijima et al., 2017), as these stressful conditions alter the ability to maintain cellular haemostasis and cell cycle processes (Calosi et al., 2017; Goncalves et al., 2017). My results provide support for the maladaptive nature of the increased metabolic rate, as the increased metabolic rates of *O. lineatus* were accompanied by severe reduction in survival in the long-term. This corroborates the previously reported sensitivity of *O. lineatus* to climatic stresses demonstrated as changes in its latitudinal range (Mieszkowska et al., 2007).

In *L. littorea*, under ambient temperature, OA did not have any effect under summer conditions, but was accompanied by a reduction in metabolism under winter conditions. This OA-related reduction in $MO_2$is in line with previous reported responses for *L. littorea*

(Bibby et al., 2007), and other marine calcifiers (Widdicombe and Spicer, 2008; Small et al. 2010; Christensen et al., 2011). Furthermore, metabolic depression is an energy-saving, stress tolerance strategy and a method to reduce oxidative damage (Marshall & Mcquaid, 2011; Teranishi and Stillman, 2007), and buffer negative effects of extreme conditions (Guppy & Withers, 1999; Storey and Storey, 2004). In contrast to the effect of OA, OW in isolation increased MO_2, however only under summer conditions, which may indicate that these snails are able to maintain aerobic capacity (Lefevre et al., 2015). If so, they may be able to increase energy budget as reported for marine molluscs under OW in isolation (Leung et al. 2020), as they showed high survival. *L. littorea* responses further highlight the importance of seasonality in physiological responses to environmental drivers, and that the physiological plasticity may be reversible (Brahim et al., 2019).

The large contrast between responses under summer and winter conditions highlights the importance of seasonal environmental context on gastropods responses to OW and OA as has been reported for other marine species (Godbold & Solan, 2013; Mitchell et al., 2023). Indeed, seasonal variations in physiological function under exposure to OW and OA have been reported for the bryozoan *Celleporaria nodulosa,* with negative impacts observed under summer not winter conditions (Durrant et al., 2013). My study points to the ability of *L. Littorea* and *N. lapillus* to physiologically cope to OW and OA, demonstrating an acclimation potential to ocean changing conditions (Seebacher et al., 2015; Watson, 2018). However, the long-term success of this acclimation is depending on the compensation for other fitness components (Wada & Sewall, 2014).

2.6 Conclusions

This study highlights the importance of understanding the long-term effects of exposure to environmental stress, in the context of seasonal changes in conditions and taking into account the species-specific responses. *N. lapillus* was able to cope with the isolated and combined exposure of OW and OA, with its metabolism only modulated by seasonal changes. While *L. littorea* showed the ability to modulate metabolism based on the seasonal environmental changes, pCO_2 concentration, and the temperature regime, with the lowest MO_2 was observed at pCO_2 1,000 ppm under ambient temperature in winter

conditions, while the highest MO_2 was observed at 380 ppm under warming in summer conditions, with antagonistic effects when combined with OA. My results show that while *O. lineatus* can cope with elevated pCO_2 under ambient temperature regime, it showed what appears to be a maladaptive higher MO_2 when combined with OW under summer conditions, displaying a synergistic interaction effect which may have negative impacts on its latitudinal distribution. This study does not support the notion of unifying effect of multi-stressors on physiological processes. Furthermore, it has emphasised that the seasonal responses and the species-specific variations can greatly complicate any general conclusions.

Chapter 3

Thermal acclimation affects fitness components differently in gastropods with different reproductive modes

3.1 Abstract

Evaluating the acclimation capacity of marine organisms to elevated temperatures is essential to predict populations' responses to ocean warming. Whilst many studies have documented the acclimation capacity of marine ectotherms, few attempt to quantify the fitness costs associated with this capacity, and how these costs differ between species with different reproductive modes. The aim of this study was to examine the trade-offs between survival and reproduction during thermal acclimation, in species with different reproductive modes. I acclimated two congeric gastropod species, the oviparous *Littorina littorea* and the ovoviviparous *Littorina saxatilis,* to one of three temperatures (15 °C, 18 °C and 20.5 °C) and measured adult physiological traits, from which I calculated scope for growth (SfG), survival, and reproductive output over a period of eight weeks. I observed a trade-off between reproduction and survival during thermal acclimation. The trade-off altered as a function of the magnitude of the temperature change, and was specific to reproductive mode of the species. While the oviparous *L. littorea* prioritized energy reallocation to survival, maintaining SfG at the cost of reduced reproductive output, the ovoviviparous *L. saxatilis* prioritised reproduction, reducing survival over time. I conclude that reproductive mode is an important factor in determining the costs of acclimation. Furthermore, whilst physiological plasticity can enhance the resilience of ectotherms to climate change, the cost of this plasticity is important in determining whether acclimation is adaptive long-term.

3.2 Introduction

The unprecedented rate of increase in ocean temperature (IPCC, 2023) is projected to have predominantly negative consequences for aquatic animals (Bindoff et al., 2019; Hoegh-Guldberg, et al., 2018). However, the potential for organisms to acclimate, *via* phenotypic

plasticity, may enable aquatic species to mitigate environmental change and enhance population persistence (Beaman et al., 2016; Fox et al., 2019; Seebacher et al., 2015). While physiological performance and survival may be enhanced during thermal acclimation, such adjustments may also lead to trade-offs between the different fitness components (e.g. survival, growth and reproduction) as a result of energy reallocation) (Jokela & Mutikainen, 1995; Donelson et al., 2010; Sokolova et al., 2012; Kühnhold et al., 2017, 2019). Indeed, exposure to elevated temperatures has been shown to have adverse effects on reproduction in many fish (Alix et al., 2020) and invertebrate species (Foo & Byrne, 2017; Gallo et al., 2020), effects that vary with the magnitude of stress (Donelson et al., 2010; Miller et al., 2015).

While numerous studies have identified increased thermal tolerance and performance upon thermal acclimation (Newell et al., 1971; Richard et al., 2012; Péden et al., 2016; Leung et al., 2021), the fitness costs associated with these change remain poorly known. It can be inferred from what is known that the impact of warming on reproduction is variable, ranging from a reduction in egg and hatchling sizes, as observed in the snails, *Crepidula atrasolea* and *C. ustulatulina* (Collin & Salazar, 2010), to the attenuation of gonadal function in the oyster, *Crassostrea virginica* (Nash et al., 2019), sperm deformations in the mussel, *Mytilus galloprovincialis* (Boni et al., 2016), and impairment of reproduction in the mussels *Perna canaliculus* (Petes et al., 2007). Therefore, for a more comprehensive assessment of the benefits and adaptive significance of acclimation, it is important to broaden our understanding of the potential trade-offs associated with the capacity of organisms to thermally acclimate.

Marine invertebrates tend to have complex life cycles, and present a wide range of reproductive modes (Reid, 1996). Marine gastropods in particular exhibit different reproductive and developmental modes that have a significant impact on their distribution and abundance (Fortunato, 2004;Foggo et al., 2007; Lee & Boulding, 2009; Hoffman et al., 2011). While direct developers can generate phenotypic variation, *via* local adaptation, planktonic dispensers tend to exhibit greater physiological flexibility (Yamada, 1987; Hollander, 2008; Sotka, 2012). Moreover, marine gastropods with different reproductive modes differentially allocate energy to reproduction (Hughes & Roberts, 1980; Perron,

1986; Gibson & Chia, 1991; Chaparro et al., 2012). However, whether phylogenetic divergence in reproductive strategies results in different energy allocation for physiological adjustments under thermal acclimation, and whether this subsequently influences the type of trade-off that may occur in fitness components, is largely unexplored.

Therefore, this study investigated the cost of acclimation to elevated temperatures in two marine gastropods congeners with different reproductive modes. Snails were exposed to three temperature treatments representing current year average, summer extreme, and projected end-of-century summer temperatures (T = 15 °C, 18°C and 20.5 °C, respectively) for eight weeks. During this period, I measured survival, reproductive output, and multiple energetic physiological traits in order to calculate a well-established measure of fitness, scope for growth (SfG) (Elliott & Davison, 1975; Widdows et al 1988; Filgueira et al., 2011). I predicted trade-offs between fitness components and acclimatory adjustments, where responses will be a function of thermal stress magnitude and reproductive mode of gastropods. Littorinids serve as excellent ecological models for intertidal environments with *Littorina. saxatilis* and *L. littorea* being the most studied species, due to their distinct morphological, biological, and life history characteristics (Rolán-Alvarez et al., 2015). *Littorina saxatilis* is characterized by direct development (ovoviviparous) with low dispersal, while *L. littorea* produces planktonic larvae (oviparous) with greater dispersal (Reid, 1996).Their capacity for thermal adaptation and physiological plasticity under thermal stress have been documented (Clarke et al., 2000a, 200 0b; Sokolova & Pörtner, 2001, 2003; Melatunan et al., 2011; Dwane et al., 2023). Therefore, these gastropods are excellent models for studying the potential trade-offs associated with thermal acclimation in closely related species with different modes of reproduction.

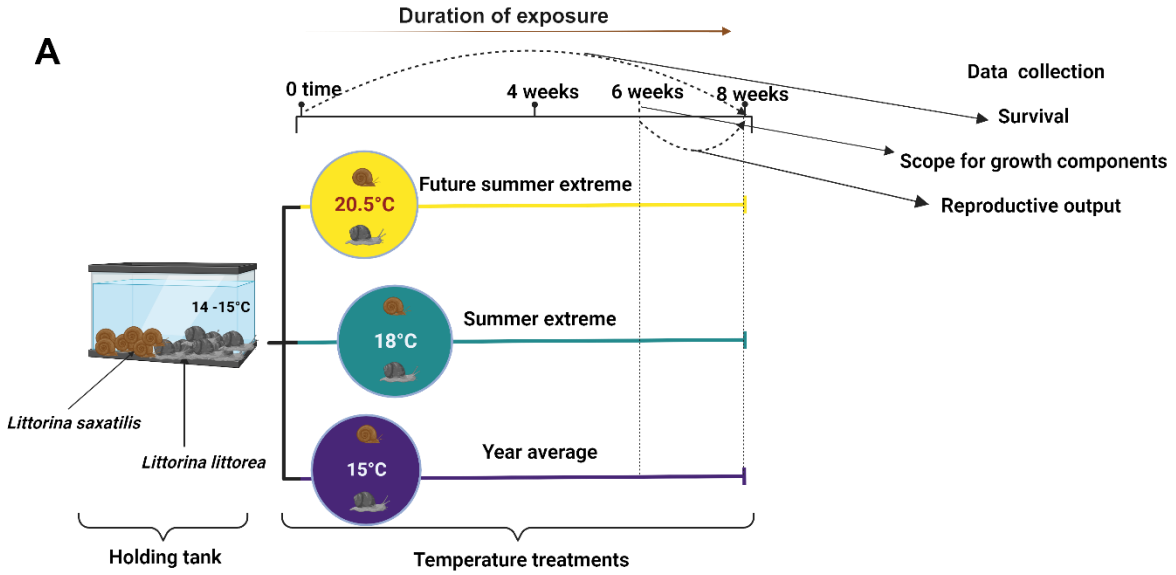
3.3 Materials and methods

3.3.1 Experimental design and animal husbandry

Adult *L. littorea* and *L. saxatilis* were collected from Mount Batten beach (50°21’30”N 4°07’50”W), Plymouth, UK, and transported to the wet laboratory at the University of Plymouth, UK. Here they were kept under ambient temperatures (T = 10 °C) for one week,

after which time the temperature was gradually increased (0.7 – 1 °C day^{-1}) until reaching the nominal treatment temperatures. The experiment included three temperature exposures, year average (T = 15 °C), summer extreme (T = 18 °C), and future summer extreme (+ 2.5 °C), based on end-of-the century predictions (IPCC, 2023)(T = 20.5 °C). Snails were exposed to each treatment for eight weeks (Fig. 3.1A).

Snails from each treatment were transferred to a recirculating holding unit comprising three replicate aquaria (Vol. = 60 L per aquarium) (N = 20 per replicate aquarium and species). Sea water in the aquaria overflowed and was gravity-fed into an 80 L sump fitted with biofilters and a protein skimmer (reef skimmer pro 500 DC, TMC, UK). A canister biofilter (Eheim Professionel 3, Eheim, Germany) pumped the water from the sump into a chiller (BOYU L075, China), and a UV filter (V2 Vecton 600, TMC, UK), for sterilisation, and then the recirculated sea water was supplied back to the treatment aquaria. Each unit was equipped with a heater (Eheim thermocontrol Aquarium Heater 300 w, Germany) synchronized with the chiller to maintain the desired temperature for each treatment (Fig. 3.1B). Deionized water top-ups were performed as necessary to maintain sea water salinity, while 10 – 15 % of the total water volume in each aquarium was siphoned to remove faeces and replaced with filtered and UV-sterilised sea water preadjusted to the corresponding temperatures twice a week. Physiochemical parameters were monitored twice weekly, and stability of abiotic parameters maintained stable *via* partial replacements of sea water (S = 33 – 35, nitrate < 30 ppm, ammonia < 0.25 ppm, pH = 7.97 ± 0.4, 12 h L:D cycle). Within each aquarium, snails were submerged at all times. The two species were kept separate by placing individuals from the same species in Perspex containers (Vol. = 10 L) (one for each aquarium and species), whose upper and lower sides were covered with a mesh (750 µm) to maintain good water exchange. Snails were fed *ad libitium* on fresh seaweed, *Ulva lactuca* and *Fucus serratus* (for *L. saxatilis* and *L. littorea* respectively) twice weekly.


A
Duration of exposure
0 time
4 weeks
6 weeks
8 weeks
Data collection
Survival
Scope for growth components
Reproductive output
20.5°C
Future summer extreme
14 -15°C
18°C
Summer extreme
Littorina saxatilis
Littorina littorea
15°C
Year average
Holding tank
Temperature treatments

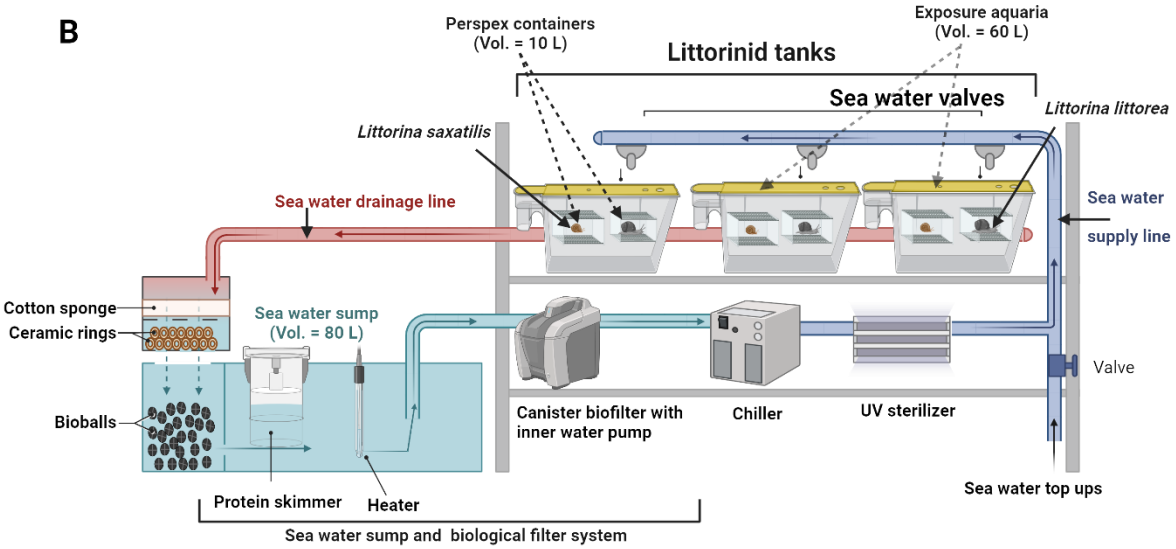

B
Perspex containers
(Vol. = 10 L)
Littorinid tanks
Exposure aquaria
(Vol. = 60 L)
Sea water valves
Littorina littorea
Littorina saxatilis
Sea water drainage line
Sea water
supply line
Cotton sponge
Ceramic rings
Sea water sump
(Vol. = 80 L)
Valve
Bioballs
Canister biofilter with
inner water pump
Chiller
UV sterilizer
Protein skimmer
Heater
Sea water top ups
Sea water sump and biological filter system

Figure 3.1. Experimental design. Littorinid species were exposed to three temperature treatments over an eight-week period (A). The temperature treatments included the year average (T = 15 °C), summer extreme (T = 18 °C), and future summer extreme (T = 20.5 °C). Data collection for survival spanned the entire exposure period, while measurements for components of scope for growth were taken at week six. Reproductive output data were collected during the last two weeks of exposure. (B) Experimental setup of a recirculating animal husbandry unit, with three units used in the experiment (one per treatment). Each unit consisted of three replicate aquaria (Vol. = 60 L, N = 20 individuals per aquarium per species which were housed in separate Perspex containers, each double-sided with perforated mesh), sump (Vol. = 80 L), equipped with a biological filter, protein skimmer, and heater. Additionally, a canister biofilter with an inner water pump, chiller, and UV sterilizer were part of the setup. Arrows indicate the flow direction of seawater in the experimental unit.

3.3.2 Measurement of scope for growth components

3.3.2.1 Metabolic rate

After 6 weeks of exposure, rates of oxygen consumption (MO_2) were measured in 18 individuals from each treatment and species. Snails were individually housed in mesocosm tubes (Vol. = 400 mL) within the corresponding replicate aquaria and left to fast for 48 h before MO_2 was measured using a well-established closed respirometry, used successfully with both *Littorina species* in previous studies (Sokolova et al., 2000). Each respirometer (Vol. = 135 mL and 30 mL for *L. littorea* and *L. saxatilis* respectively) was fitted with an oxygen-sensitive dot (Presens, Germany) and supplied with a magnetic flea, to ensure adequate mixing, separated by a perforated mesh that worked as substrate for the snails to minimize activity. Twenty respirometers were supplied with aerated, filtered, and sterilised sea water. For each treatment, six snails from each tank were individually placed into respirometers and allowed to settle for 20 min, before chambers were sealed with gas tight lids under water. Two blanks were used per trial. All chambers were submerged in a flow-through water bath at the corresponding temperature and run simultaneously. The time was noted immediately upon closing the chamber, and measurements of decline in

pO_2 was measured at 10 min intervals using a fibre-optic cable and a Fibox 4 oxygen meter (Presens, Germany) for 1.5 – 2h. In each case, the decline in pO_2 was monitored until pO_2 levels reached 70 % air saturation (a.s.), to avoid hypoxic conditions. After measurement, snails were removed from the respirometers, blotted dry, dissected out of their shells, and their soft tissue was weighed to the nearest mg (Cubis Semi-Micro Balance, Sartorius, Germany). MO_2 was expressed as uL O_2 g^{-1} wet mass h^{-1} (salinity-temperature-pressure corrected). Values of MO_2 were transformed to the energy equivalent values (J g^{-1} h^{-1}) using the conversion factor: 1 mg O_2 = 14.4 J (Elliott and Davison, 1975).

3.3.2.2 Excretion rate

Following MO_2 measurements, 40-and 10-mL water samples were collected from each respirometry chamber (for *L. littorea* and *L. saxatilis* respectively) and used for measurements to estimate excretion rate (ER). Ammonia concentration was determined spectrophotometrically using a phenol–hypochlorite method (Soloranzo, 1969). ER was calculated using the equation:

$$(1)\ U = (C_{test} – C_{control}) \times (V/1000)/ t$$

Where U is the rate of ammonia excretion expressed as mg NH_4–N h^{-1}, C_{test} is the ammonia concentration (mg L^{-1}) in the sample, $C_{control}$ is the ammonia concentration (mg L^{-1}) in the control chamber, V is the volume (mL) of sea water in which the snail was incubated, and t is the incubation time (h). Values of ER were transformed to the energy equivalent values (J h^{-1}) using the conversion factor: 1 mg NH_4^{-1} = 25 J (Elliott & Davison, 1975), and then corrected by snail soft tissue mass (J g^{-1} h^{-1}).

3.3.2.3 Feeding rate

Feeding rate (FR) was measured for the same individuals used for MO_2 and ER measurements. Following respirometry, snails were returned to their individual mesocosm tubes. Each individual was allowed to feed for 48 h on fresh *Ulva lactuca* or *Fucus serratus*, for *L. saxatilis* and *L. littorea* respectively. Approximately a 1 g piece of seaweed was prepared for each snail, blotted dry, and pre-weighed (Cubis Semi-Micro Balance, Sartorius, Germany). Two mesocosm tubes, without snails, were used as blank to correct

for any mass changes of each macroalgae species during the feeding time. At the end of the feeding time, the remaining macroalgae were blotted dry, and re-weighted. FR was calculated as the difference in mass of the macroalgae before and after feeding, after being corrected by the blank, expressed as mg g^{-1} h^{-1}.

3.3.2.4 Absorption efficiency and absorption rate

Using a vacuum motor, each individual's faeces produced during the 48 h feeding period in the mesocosm tubes were collected on pre-weighed 45 mm glass fibre filters (Whatman GF/C). These filter papers were rinsed with deionised water, dried in an oven (T = 100 °C) for 24 h, weighed, ashed in a muffle furnace (T = 450 °C for 6 h) and reweighed. Three blank GF/C filters from each batch were treated in the same way and used for filter mass corrections. The ash-free dry mass (organic content) of faeces was determined by mass loss upon ignition at T = 450 °C in the muffle furnace for 6 h. The organic content of each macroalgae was measured using the aforementioned method in 10 X 1 g pieces for each species. The absorption efficiency (AE) was then calculated using the equation (Conover, 1966):

$$(2)\ AE = (F-E) / [(1-E) \times F]$$

where AE is the absorption efficiency; F is the ash-free dry mass to dry mass ratio of food, and E is the ash-free dry mass to dry mass ratio of faeces. The AE was expressed as a percentage.

The food absorption rate (AR) was calculated by multiplying FR by AE, where the AR was converted into energy equivalent using the energy content of the respective macroalgae (19.88 and 12.6 J mg^{-1} for *Ulva lactuca* and *Fucus serratus*, respectively) (A. Foggo, 2013, unpublished data).

3.3.2.5 Scope for growth

Scope for growth (SfG) was calculated using the equation:

$$(3)\ SfG = [AR - (MO_2 + ER)]$$

where AR is absorption rate; MO_2 is oxygen consumption rate; ER is excretion rate. SfG was expressed as J g^{-1} h^{-1}.

3.3.3 Reproductive output

During the last two weeks of the experiment, the mesh used for the Perspex containers were replaced with smaller mesh size (350 µm) to keep eggs and hatchlings inside. Eggs of *L. littorea* and hatchlings of *L. saxatilis* laid within the previous 24 h inside the Perspex containers were collected 3 – 4 times weekly. For *L. littorea*, eggs from individuals in each replicate tank were collected and concentrated in a 500 mL container, well mixed, counted in five subsamples of 5 mL and each examined under low power magnification (× 10–40). Using the average of the five counts, the total number of eggs produced was calculated. For *L. saxatilis*, hatchlings were carefully collected in a 50 mL Petri dish, and counted under low power magnification (× 10). The number of eggs and hatchlings was expressed as number per female per day. The number of females in the container from which eggs or hatchlings were collected was estimated using the male to female ratio identified in the subset of individuals used for respirometry (n = 18). Animals were sexed by examination under low power magnification (× 10 – 40).

At the end of the experimental period, eggs of *L. littorea* were collected from each replicate tank (n = 115 per treatment (year average and summer extreme)) and eggs of *L. saxatilis* were collected from the brood pouch of six females per treatment (n = 60 – 65 per treatment). Images of randomly selected eggs were captured for morphometric measurements using a Leica M205FA microscope (Leica Microsystems). Morphometric measurements of egg size for *L. littorea* and *L. saxatilis* were collected using the image analysis software ImageJ2 (Fiji) (Schindelin et al., 2012).

3.3.4 Survival

During the exposure period, deceased snails were removed and tallied for each replicate tank daily. The survival rate for each species was calculated three times a week for each treatment, represented as the average count of surviving snails during a specific week of

exposure, with the initial number of snails considered as the baseline. The cumulative survival rate was then expressed as an overall average, presented as a percentage.

3.3.5 Statistical analysis

Linear models (lm), using Type III sum of squares, were used to examine the effects of temperature treatments on each evaluated physiological trait in both littorinid species. Temperature treatment and species were used as fixed factors in five two-way ANOVA analysis for: SfG, MO_2, absorption, excretion, and survival rates as response variables. Temperature treatment was used as fixed factor in four one-way ANOVA analysis per species for reproductive output traits (number of eggs and hatchlings, and egg capsule size). Replicate tank was tested for random effect, but it had no effect, therefore it was discarded from the final analysis. The normality of residuals and homoscedasticity of variance were assessed through visual inspection of the Quantile-Quantile and scale-location plots. Pairwise differences between levels were identified by Tukey's HSD *post-hoc* tests. All data were analysed using the core Stats package in R v 4.3.1 (R Core Team, 2023). A P-value < 0.05 was considered statistically significant.

3.4 Results

3.4.1 Scope for growth and its components

Temperature significantly affected food absorption ($F_{2, 81} = 12.5$, $P < 0.001$) and excretion rates ($F_{2, 81} = 9.5$, $P < 0.001$), but did not affect MO_2 ($F_{2, 81} = 2.14$, $P = 0.12$). There was significant effect of species on food absorption ($F_{1, 81} = 30.2$, $P < 0.001$), MO_2 ($F_{2, 81} = 8.8$, $P = 0.003$), and excretion rate ($F_{2, 81} = 8.66$, $P = 0.004$). Moreover, there was no effect of the interaction between species and temperature on food absorption ($F_{2, 81} = 1.83$, $P = 0.167$) or excretion rate ($F_{2, 81} = 0.055$, $P = 0.94$), but there was a significant interaction on MO_2 ($F_{2, 81} = 5.098$, $P = 0.008$) (Fig. 3.2B – D). Snails at 20.5 °C had lower overall food absorption rate compared with those at either 15 °C ($P < 0.001$) or 18 °C treatments ($P = 0.003$). There was no significant difference between individuals kept at 15 and 18 °C ($P = 0.25$). At 20.5 °C, *L.*

littorea had lower food absorption rate than those at 15 °C (P = 0.042) and 18 °C (P = 0.021). By contrast *L. saxatilis* had significantly lower rates than those at 15 °C (P = 0.002) but similar to those at 18 °C (P = 0.68). *Littorina littorea* had an overall higher food absorption compared with *L. saxatilis* (P < 0.001), driven by higher rate at both 18 °C and 20.5 °C. Furthermore, *L. littorea* showed higher overall MO_2 compared with *L. saxatilis* (P = 0.003), driven by significant higher MO_2 of *L. littorea* snails at 20.5 °C compared to *L. saxatilis* in both the 18 °C (P = 0.042) and 20.5 °C treatments (P < 0.001). However, *L. littorea* snails under 18 °C showed MO_2 significantly lower than of those under 20.5 °C (P = 0.041), which was similar to *L. saxatilis* snails in all treatments (P > 0.05). Furthermore, at 20.5 °C treatment, snails showed overall higher excretion rate compared to those at 15 °C (P < 0.001), while those at 18 °C did not differ with those at 15 °C (P = 0.057) and those under 20.5 °C treatments (P = 0.11). *L. saxatilis* showed overall higher excretion rates compared to *L. littorea* (P = 0.004).

The plastic responses of those three physiological traits led to a significant effect of both temperature treatment ($F_{2, 81}$ = 15.98, P < 0.001) and species ($F_{1, 81}$ = 26.9, P < 0.001) on scope for growth, with no interaction between the two factors ($F_{2, 81}$ = 2.38, P = 0.098) (Fig. 3.2A). At 20.5 °C, snails demonstrated overall lower SfG compared to the 18 °C (P < 0.001) and 15 °C (P < 0.001) treatments. *Littorina saxatilis* showed overall lower SfG compared with *L. littorea* (P < 0.001). At 20.5 °C, while *L. littorea* exhibited significantly lower SfG than those at 15 °C (P = 0.004) and 18 °C (P = 0.001) treatments, *L. saxatilis* snails showed lower SfG than only 15 °C treatment (P = 0.002), while snails in the 18 °C treatment had no difference in SfG with either 15 °C or 20.5 °C treatments (P > 0.05). Moreover, the lower SfG of *L. littorea* snails under 20.5 °C treatment was not significantly different with that of all the three treatments of *L. saxatilis* (P > 0.05).

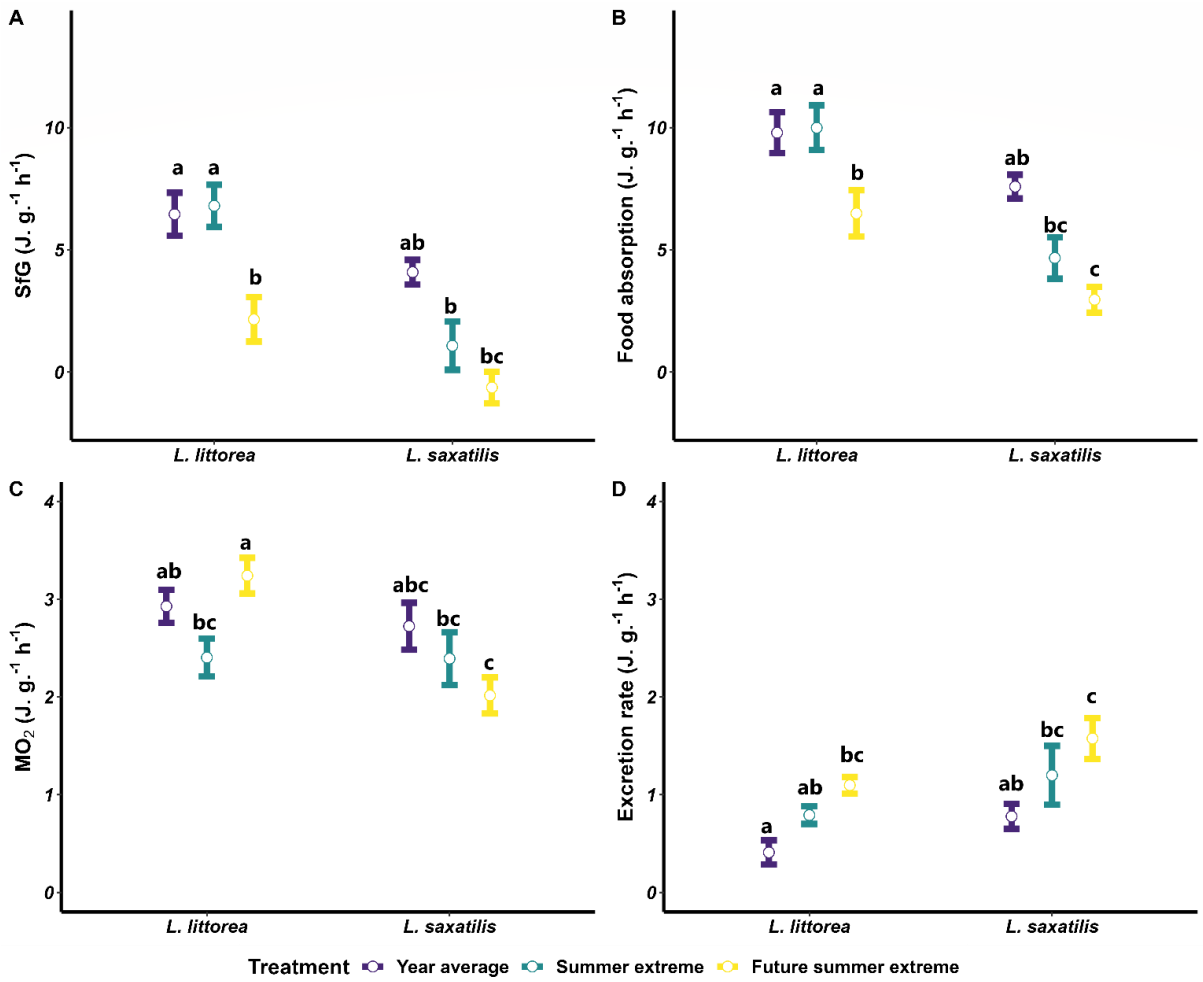


Figure 3.2. Physiological traits of *L. littorea* and *L. saxatilis* after 6 weeks of exposure to three temperature levels; year average (15 °C), extreme summer (18 °C), and future extreme summer (20.5 °C) temperatures. (A) scope for growth (SfG), (B) food absorption, (C) oxygen consumption (MO_2), (D) excretion rate. Lowercase letters show significant differences between treatments at $P < 0.05$ (n =14 – 17 individuals per treatment and per species for each measurement). Values are expressed as means ± 1 S.E.

3.4.2 Reproductive output

Temperature had a significant effect on the number of eggs released per female of *L. littorea* ($F_{2, 26} = 118.17$, $P < 0.001$) but it did not influence the number of hatchlings released per female of *L. saxatilis* ($F_{2, 26} = 1.88$, $P = 0.17$) (Fig. 3.3 A and B). *L. littorea* under 20.5 °C ceased egg release, while snails at 18 °C continued reproducing but at significant lower rate

compared to those at 15 °C treatment (P = 0.026). In contrast, *L. saxatilis* kept reproducing under all temperature treatments during the experimental period (P > 0.05). Egg size was affected by temperature in both *L. littorea* ($F_{1, 330}$ = 10.826, P = 0.001) and *L. saxatilis* ($F_{1, 184}$ = 9.47, P < 0.001) (Fig. 3.3 C and D, respectively). While *L. saxatilis* at 18 °C produced larger eggs compared with those at 15 °C (P = 0.004) and 20.5 °C (P < 0.001), L. *littorea* produced smaller eggs under 18 °C compared with those at 15 °C (P < 0.001).

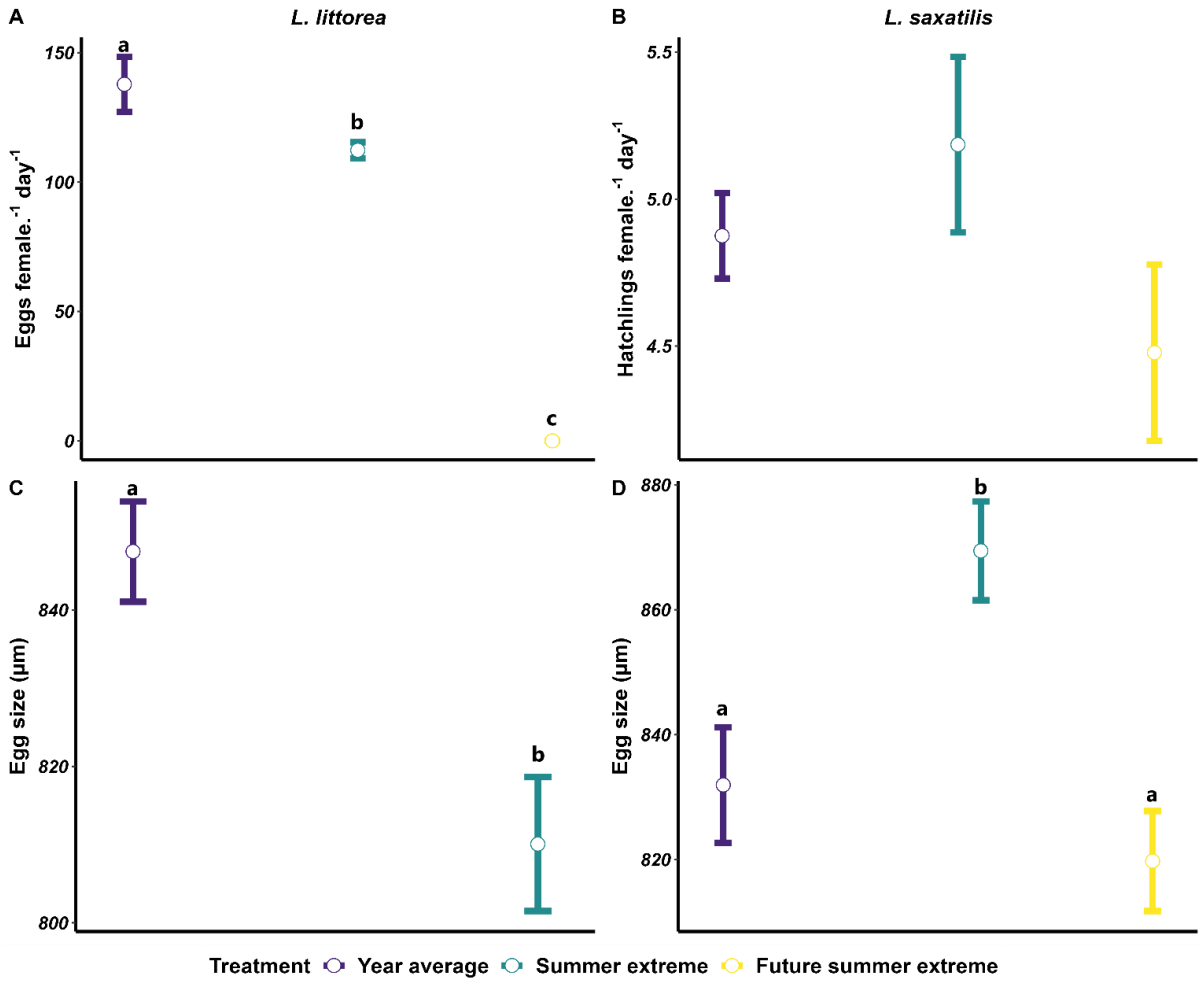


Figure 3.3. Reproductive output traits for *L. littorea* and *L. saxatilis* under exposure to three temperatures; year average (15 °C), extreme summer (18 °C), and future extreme summer (20.5 °C) temperatures. (A) fecundity, (C) egg size in *L. littorea*, while (B) fecundity, (D) egg size in *L. saxatilis* snails. Lowercase letters indicate significant differences between temperature treatments at $P < 0.05$ (n = 9 for A and B, n = 115 for C, and n = 60 – 67 for D per treatment). Values are expressed as means ± 1 S.E.

3.4.3 Survival rate

Survival was monitored over the whole exposure period (Fig. 3.4A). Overall survival rate was not affected by either temperature ($F_{2,36} = 0.61$, $P = 0.57$) or species ($F_{1,36} = 0.86$, $P = 0.38$), but by the interaction between both factors ($F_{2,36} = 29.39$, $P < 0.001$) (Fig. 3.4B). Under future summer extreme temperatures, *L. saxatilis* showed the lowest survival rate among all treatments ($P < 0.001$ in all cases), while under summer extreme treatment, it had lower survival rate than *L. littorea* ($P = 0.048$) but was similar to *L. saxatilis* under year average ($P = 0.079$). In contrast, *L. littorea* showed similar survival rate under all temperature treatments ($P > 0.05$).

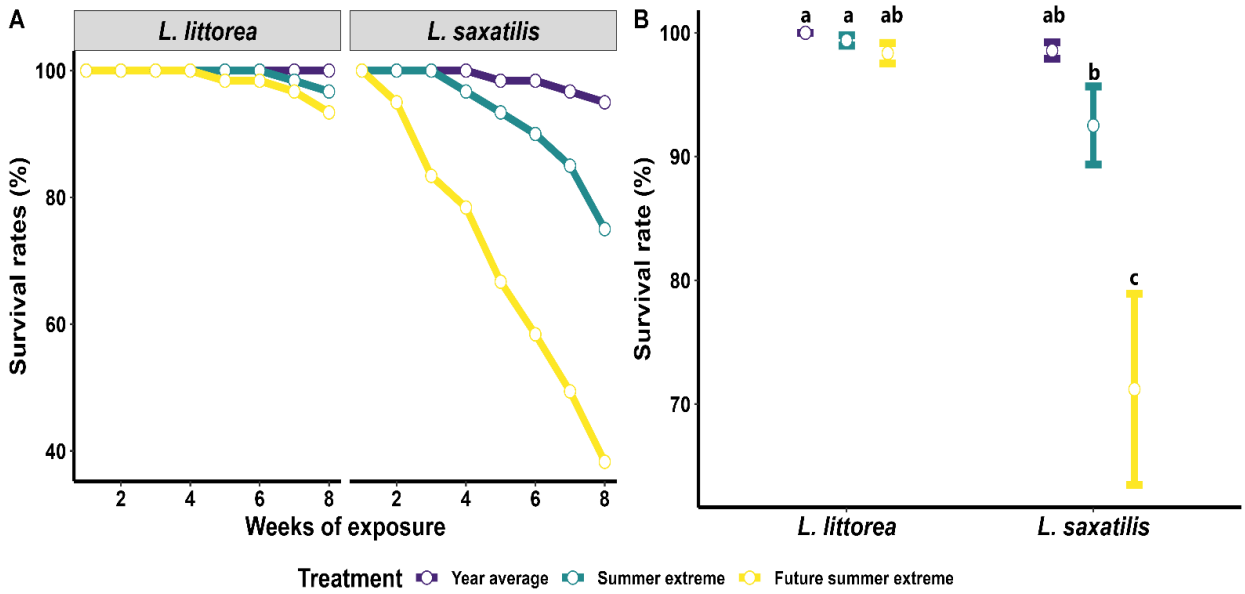


Figure 3.4. Survival rate of *L. littorea* and *L. saxatilis.* (A) weekly average survival over the period of exposure to three temperatures; year average (15 °C), extreme summer (18 °C), and future extreme summer (20.5 °C) temperatures, and (B) the overall average survival. Lowercase letters indicate significant difference between treatments at $\alpha < 0.05$ (n = 24 per treatment and species). Values are expressed as means ± 1 S.E.

3.5 Discussion

This study investigated the cost of thermal acclimation in two littorinid gastropods to determine whether the trade-offs associated with thermal acclimation differ between species with different reproductive modes. As predicted, thermal acclimation incurred trade-offs between fitness components which varied as a function of the magnitude of thermal stress and the reproductive mode of the gastropods. While *L. littorea* prioritized survival over reproduction, *L. saxatilis* continued to reproduce at elevated temperatures at the cost of long-term survival. The study demonstrates the importance of reproductive mode in shaping the cost of thermal acclimation. I emphasise the importance of considering multiple fitness traits for a more comprehensive understanding of the capacity for and consequences of thermal acclimation in the context of climate change.

Thermal acclimation can incur significant energetic costs (Kühnhold et al., 2017, 2019), as a consequence of the high energy demands associated with physiological adjustment mechanisms (Sokolova, 2021). Marine molluscs respond to thermal acclimation by balancing their energy budgets *via* adjustments in metabolic energy expenditure and/or energy intake (Newell & Branch, 1980; Brockington & Clarke, 2001). My findings reveal that snails exposed to the two elevated temperatures exhibited differential modifications in energy budget in comparison to those under control conditions. Specifically, at 18 °C, both species displayed similar food absorption, MO_2, excretion rates, and, consequently, similar SfG as they did at 15 °C. In contrast to my results, acclimation to elevated sea water temperatures has been shown to promote SfG in marine gastropods by increasing MO_2 and food absorption (Zhang et al., 2015; Leung et al., 2018, 2020). However, this higher energy budget has been associated with a trade-off in somatic growth (Leung et al., 2020). Both snail species were predicted to have similar acclimation capabilities at 18 °C, a temperature

well within the range they experience in their intertidal habitat, and significantly below their upper thermal limits. Indeed, coexisting populations of both species have similar heat coma temperatures, lethal thermal limits, and comparable capacities to increase thermal tolerances (Sandison, 1967; Sorte et al., 2011). In the present study, *L. saxatilis* exhibited lower food absorption, resulting in lower SfG and survival than *L. littorea*, suggesting high thermal sensitivity to elevated temperatures in this species. Despite this apparently high sensitivity, *L. saxatilis* acclimated to 18 °C demonstrated similar fecundity to those acclimated at 18 °C in terms of the number of hatchlings produced, with even larger egg capsules. This is consistent with a previous study demonstrating that, under various food rations, *L. saxatilis* maintains similar reproductive outputs, at the cost of somatic growth (Hughes, 1995). Moreover, elevated female fecundity in *L. saxatilis* populations has been reported as a possible compensatory mechanism in response to trematode infestation (Granovitch et al., 2009). In contrast, *L. littorea* exhibited reduced egg production and smaller egg capsules under 18 °C compared to 15 °C. Such reduction in reproductive output in *L. littorea* suggests that most of the energy budget was allocated to homeostatic maintenance. Reduction in reproduction during thermal acclimation has been documented for a range of invertebrate species (Collin & Salazar, 2010; Foo & Byrne, 2017; Gallo et al., 2020). This difference in reproductive strategies is intriguing, with *L. saxatilis* prioritizing energy reallocation to reproduction, while *L. littorea* limits reproduction. My study indicates trade-offs between fitness components, with their form dependent on the reproductive mode of gastropods.

A more significant cost of acclimation was evident with an increase in the magnitude of thermal stress. At 20.5 °C, both snail species displayed lower food absorption and higher excretion rates, but similar MO_2, resulting in lower SfG compared to those at 15 °C. A reduction in food absorption and, consequently, SfG as a result of elevated temperature has been previously demonstrated in marine molluscs (Wang et al., 2015). While metabolic depression has been shown to be an effective mechanism for conserving energy in gastropods under warming (Marshall & Mcquaid, 2011), both snail species in My study exhibited similar MO_2 at 18 °C and 15 °C. This result is supported by previous research that indicated MO_2 in *L. littorea* remained unaffected by acclimation to elevated temperatures (Melatunan et al., 2011). Furthermore, different mechanisms were responsible for the

maintenance of SfG in the two species. While *L. littorea* displayed significantly higher food absorption, *L. saxatilis* demonstrated lower MO_2. Additionally, ammonia excretion rates were not compensated by acclimation to 20.5 °C, which may indicate protein catabolism due to thermal stress, as previously reported in littorinids (Aldridge et al., 1995). The inability of both gastropod species to adequately regulate their metabolism and enhance or even maintain food absorption at 20.5 °C had a negative impact on their physiological state, consistent with reports in various invertebrate species under thermal stress (Leung & Connell, 2017; Harianto et al., 2018; Madeira et al., 2018 a; Hemraj et al., 2020). The reduction in SfG had a cascading effect on the available energy for other fitness components, leading to more obvious trade-offs between them.

Notably, *L. littorea* ceased reproduction, allowing them to maintain higher survival comparable to those at 15 °C. This could be considered an adaptive strategy to maintain high survival since surviving under sublethal thermal stress requires disproportionate energy allocation for maintenance mechanisms, as cellular and structural damage can occur (Pörtner, 2002; Sokolova, 2021). Similarly, reproduction in anemonefish, *Amphiprion melanopus*, ceased when exposed to the highest temperature compared to moderate and control temperatures (Miller et al., 2015). Moreover, stoppage to invest in reproduction under elevated temperatures has been reported to other marine gastropod (Mardones et al., 2021), and sea urchin (Delorme & Sewell, 2016). In contrast, *L. saxatilis* continued to reproduce, but exhibited significantly lower survival. The response of *L. saxatilis* may be linked to the absence of seasonality in reproduction as, despite some peak periods, adults breed throughout the year (Ellis, 1983; Ross & Berry, 1991), thus displaying less sensitivity to temperature. Consequently, the reduced ability of *L. saxatilis* to cease reproduction may render it more vulnerable to thermal stress (Arizmendi-Mejía et al., 2015). In contrast, *L. littorea* follows a spawning season (Fish, 1972, 1979; Grahame, 1975), which is influenced by the rate and onset of temperature increases within the natural temperature range (Chase & Thomas, 1995). Additionally, a seasonal shedding of the male penis at the end of the breeding season (summer) has been reported, along with the regression of the testes (Barroso et al., 2007), which may be a strategy to cope with high summer temperatures. One possible explanation for these differences could be variations in environmental-endocrine control of reproduction between gastropods with contrasting reproductive

modes (Wayne, 2001; Sternberg et al., 2010). Indeed, although the high mortality under acclimation to thermal stress in the brooder seahorse *Hippocampus erectus*, gonadal development and reproductive endocrine functionality were maintained (Qin et al., 2018). I conclude that reproduction is a plastic trait, with lower priority for energy reallocation under thermal stress in *L. littorea*, while it shows limited plasticity in *L. saxatilis*, maintaining priority in terms of energy allocation, even at the cost of survival.

3.6 Conclusions

My findings show that the intensity of thermal stress and the reproductive mode are crucial determinants of the cost of thermal acclimation, shaping the trade-offs between acclimation and fitness components. While *L. littorea* had high acclimation potential, the cessation of reproduction raises concerns about whether this acclimation is advantageous in the long-term. In contrast, even though *L. saxatilis* exhibits lower survival and limited acclimation capacity, the preservation of reproduction may ensure ongoing recruitment and prevent the population from extinction in the long-term, assuming offspring survival. As a result, while physiological plasticity may enhance the resilience of ectothermic animals to climate change, the cost of these adjustments will determine whether the acquired acclimation is adaptive in the long-term.

Chapter 4

Heat hardening improves thermal tolerance in abalone, without the trade-off of thermal acclimation to chronic warming

4.1 Abstract

Marine animals are challenged by chronically raised temperatures alongside an increased frequency of discrete, severe warming events. Exposure to repeated heat shocks could result in heat hardening, a phenomenon that enhances thermotolerance, and may be an important mechanism by which marine species will cope with future thermal challenges. However, we have relatively little understanding of the effects of heat hardening in comparison to chronic warming under elevated temperatures. Therefore, I aimed to compare the effects of heat hardening from repeated heat shock exposure and chronic warm on thermal tolerance in the European abalone, *Haliotis tuberculata.* Adult abalones were exposed to either control temperatures (T = 15 °C), chronic warming (T = 20 °C) or a regime of repeated acute heat shock cycles (T = 23 – 25 °C) for six months, and their thermal tolerance and performance (based upon cardiac activity) compared using a dynamic ramping assay. The energetic cost associated with each treatment was also estimated via measurements of condition index (CI). Abalone exposed to both temperature treatments had higher upper thermal limits than the control, but heat-hardened individuals had significantly higher CI values, indicating an enhancement in energetic status. Differences in the shape of the thermal performance curve (TPC) indicate different mechanisms are at play under different temperature exposure treatments. I conclude that heat hardening can boost thermal tolerance in this species, without the performance trade-offs associated with chronic warming. Heat hardening of abalone is suggested as an effective and viable strategy to enhance thermal tolerance and performance of commercially and ecologically important marine molluscs, both from a restoration and commercial perspective.

4.2 Introduction

The environmental stress associated with extreme weather events and climatic changes has far-reaching impacts on aquatic species, leading to performance impairment and disruption in marine ecosystems (Bindoff et al., 2019; Doney et al., 2020; Goncalves et al., 2017; Mayor et al., 2015; Oliveira et al., 2020). Indeed, the occurrence of severe warming events in coastal waters can lead to widespread mass mortality events, posing a threat to ecosystem functionality and food security (Masanja et al., 2023; Scanes & Byrne, 2023).
There is a growing consensus that phenotypic plasticity is a key factor in shaping and supporting the resilience of species and persistence of populations in the face of environmental change (Putnam, 2021; Leeuwis & Gamperl, 2022; Masanja et al., 2023). This plasticity allows organisms under environmental stress to quickly adjust their physiological phenotypes to maintain performance. The presence of adaptive physiological plasticity is a major factor influencing which species are able to persist in the context of rapid change (Somero, 2010; Seebacher et al., 2015; Watson, 2018). Consequently, understanding the capacity for physiological plasticity, and the mechanisms underpinning it, is not only essential for predicting resilience of different marine ectotherms, but also for informing conservation strategies (Donelson et al., 2023), stock enhancement programmes (Daly et al., 2021) and aquaculture productivity (Reid et al., 2019; Gavery & Roberts, 2017), increasing sustainability under a changing climate.

Thermal performance in marine ectotherms, as a plastic trait, can be influenced by acclimation to high temperatures (Ern et al., 2023; Vinagre et al., 2016; Jørgensen et al., 2021; Ørsted et al., 2022), and there is evidence that some species exhibit high acclimation potential, with enhanced thermal plasticity and increased tolerance limits (Newell et al., 1971; Stenseng et al., 2005; Leung et al., 2021; Madeira et al., 2017; Péden et al., 2016). Many studies evaluating acclimation potential, have used short-term exposure experiments, with significantly less attention given to long-term exposures. However, responses can vary with exposure time (Molina et al., 2023), with the change in thermal tolerance being a function of the duration of acclimation (Ern et al., 2023). Therefore, while short-term studies provide valuable insights into the plastic responses of marine ectotherms to acute environmental stress, they cannot be extrapolated to predict long-

term responses to climate change. It is therefore essential to consider longer exposure durations to more effectively assess the thermal acclimation capacity in these organisms.

Over recent years, it has been recognised that the nature of the thermal regime experienced by an organism in its natural setting needs to be carefully considered to accurately predict its capacity to acclimate to change and predict future performance (Morash et al., 2018). Many intertidal ectotherms do not experience static temperatures naturally, instead facing dynamic, fluctuating and unpredictable environmental conditions, with the potential for different effects on thermal tolerance and performance (Dong et al., 2021; Leeuwis & Gamperl, 2022; Dong et al., 2006; Kang et al., 2019; Kern et al., 2015; Nancollas & Todgham, 2022). A fluctuating thermal environment, where bouts of elevated temperatures are succeeded by periods of lower temperatures, may provide refuge and time for recovery, reducing the costs associated with chronic warming, and promoting acclimation (Auld et al., 2010; Chevin & Hoffmann, 2017). Additionally, there is the potential for heat hardening to occur, a phenomenon by which organisms acquire a short-term increase in heat tolerance following a sub-lethal extreme heat exposure (Bilyk et al., 2012). Hardening has been reported across various taxa including marine molluscs (Hackerott et al., 2021; Hilker et al., 2016; Moyen et al., 2019, 2020; Zhang et al.2021), and may be associated with activation of cellular stress responses, boosting oxidative stress and mitochondrial respiration capacities, and metabolic remodelling (Collins et al., 2023; Dunphy et al., 2018; Georgoulis et al., 2021, 2022). Despite its potential importance, the effects of heat hardening remain relatively understudied experimentally compared to the effects of chronic, static thermal exposures.

Therefore, this study aimed to compare the effects of repeated heat shocks, and chronic warm acclimation, on the thermal tolerance of the European abalone, *Haliotis tuberculata*. Abalone were either heat-hardened (repeated acute heat shocks, 23 – 25 °C), control (T = 15 °C) or acclimated to chronically raised temperatures (T = 20 °C) prior to determining thermal performance and tolerance (based upon cardiac activity) using a dynamic ramping assay. The costs associated with exposure to different thermal regimes was quantified using condition indices. I predicted that both long-term chronic warming and repeated heat shock exposure would increase thermal tolerance compared to the control treatment, and that this would incur an energy cost reflected in reduced condition index. As repeated heat

shock exposure allows for periods of recovery, I predicted that such costs will be lower in heat-hardened individuals. The European abalone, *H. tuberculata*, has a high value ecologically, and is an important aquaculture species that is threatened by warming, overfishing, and disease (Travers et al., 2008; Cenni et al., 2010; Van Wormhoudt et al., 2011; Cook, 2014; Kavousi et al., 2021). It shows high adaptive plasticity under aquaculture conditions (Lachambre, et al., 2017a, b), with positive implications for stock-enhancement (Chauvaud et al., 2021; Roussel,et al., 2019 a). Leveraging its well-understood husbandry techniques and spawning and larval development methods (Huchette et al., 2003, 2004; De Viçose et al., 2012; Roussel et al., 2019 b), abalone serves as an ideal model for investigating approaches influencing thermal plasticity within aquaculture and stock enhancement context. These findings, in turn, offer valuable insights applicable to other economically and ecologically important marine molluscs compromised with the adverse effects of elevated temperatures (Scanes & Byrne, 2023).

4.3 Materials and methods

4.3.1 Animal Husbandry

Adult abalones *H. tuberculata* (4 years old), were obtained from an abalone farm in France (48°36’50” N, 4°36’3” W; Plouguerneau, Brittany), where they were reared in land-based nursery tanks for 10 months before being transferred to sea-cages until collection. Abalones were transported to the University of Plymouth, UK, by road in an icebox, covered with a wet cloth and using ice packs to maintain low temperature. Upon arrival (within 12 h of collection), abalones (N = 160) were kept in holding tanks (2 X 1000 L, T = 14 – 15 °C, S = 33, 12 h:12 h L:D cycle) for a period of two weeks, where they were fed *ad libitum Palmaria palmata.*

Animals were reared in a recirculating unit comprising three replicate aquaria per treatment (Vol. = 60 L) (N = 15 – 17 per replicate aquarium). The sea water in the aquaria overflowed and was gravity-fed into a sump (Vol. = 85 L, one sump per treatment), fitted with a protein skimmer (reef skimmer pro 500 DC, TMC, UK), a canister biofilter (Eheim Professionel 3, Eheim, Germany), a chiller (BOYU L075, China), and a UV filter (V2 Vecton

600, TMC, UK) for sterilization (Fig. S.1). The aerated, filtered, UV-treated, and recirculated sea water was then supplied back to the treatment aquaria. All treatments were conducted in a temperature-controlled laboratory (15 °C). Each unit was equipped with a heater (Eheim thermocontrol Aquarium Heater 300 w, Germany) synchronized with the chiller to maintain the desired temperature for each treatment. Physicochemical parameters were monitored twice weekly, and stability of abiotic parameters maintained via partial water replacement at the corresponding temperature (salinity = 33 – 35, nitrate < 30 ppm, ammonia < 0.25 ppm, pH = 7.97 ± 0.06, 12 h L:D cycle). Deionized water top-ups were performed as necessary to maintain water salinity, while 10 – 15 % of the total water volume in each aquarium was siphoned to remove faeces and replaced with filtered and UV-sterilized sea water twice a week. Abalones were fed *ad libitum* with fresh *Palmaria palmata* twice a week.

4.3.2 Experimental design

Adult abalone were exposed to one of three thermal treatments over a period of 6 months: control (15 °C, N = 48), chronic warming (20 °C, N = 49), and acute thermal challenge treatment (15 °C plus two repeated heat shock cycles at 23 – 25 °C, termed repeated hear shocks, N = 48). The temperature for the chronically warmed treatment was set at the projected summer temperature for the end of the century (the current summer temperature of the collection site + 3 °C (Gac et al., 2020)), with temperature increased from 14 °C to 20 °C over the course of one week (~ 1 °C day^{-1}). The experimental design for the repeated heat shock exposure was adapted from Hutchison's "repeated critical thermal maximum (CTM)" method (Hutchison, 1961) which was modified from Georgoulis et al., (2022). The first heat shock event was carried out after three months of exposure to control temperatures under laboratory conditions. Preliminary trials of oxygen consumption measurements under resting conditions across a range of test temperatures (15, 18, 21, 24, and 27 °C) revealed that resting oxygen consumption was highest at approximately 24 °C. Consequently, abalone were subjected to repeated heat shocks of 23 °C (1 °C below the temperature at which oxygen consumption was highest) every 24 h over 5 consecutive days. The second heat shock event was applied after 6 months from the start of the experiment, following the same procedure with heat shock temperature raised

to 25 °C (1 °C above the temperature at which oxygen consumption was highest). To increase the sea water temperature in the exposure tanks, the flow of water was redirected to a heated tank containing sea water at 36 °C, where temperature was ramped from 15 °C to 23 °C or 25 °C within 1.5 h, via mixing of water bodies. Once the temperature reached 23 °C or 25 °C, the water flow from the tank was stopped, and the abalones were exposed to the target temperature for 3 h. After this period, the water flow was switched back to a tank containing sea water at 15 °C, allowing for a rapid decrease in water temperature in the exposure tanks to 15 °C within 1.5 h. Subsequently, the water flow was reconnected to the recirculating water system, and the abalones were left to recover at 15 °C for 18 h (Fig. 4.1).

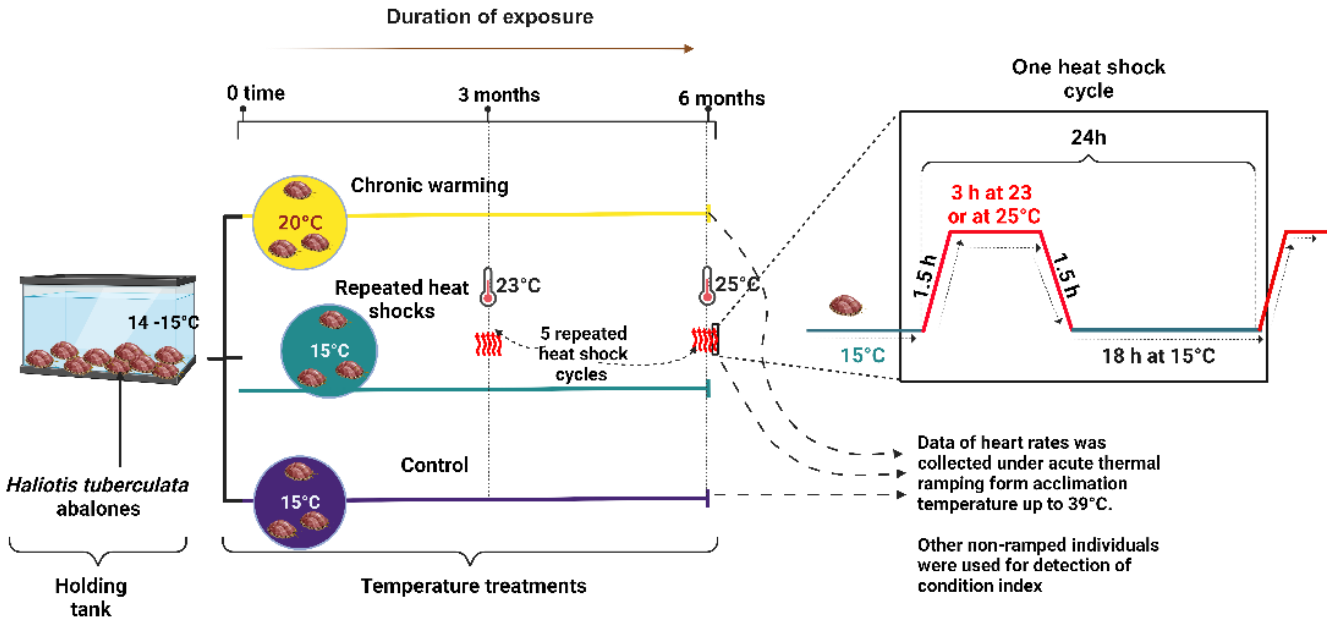


Figure 4.1. Experimental design for adult *Haliotis tuberculata* abalones exposed to three different temperature treatments; control (T = 15 °C, N = 48), repeated heat shocks (T = 15 °C but exposed to two warming events, after three and six months of exposure during each event animals exposed to five repeated 24 h heat shock cycles, N = 49), and chronically warmed (T = 20 °C, N = 48) over six months. Data for condition index and heart rate was collected by the end of the exposures after six months.

Following exposure to the different thermal regimes, condition of individuals was assessed and effects of heat hardening and chronic warming on thermal performance determined.

4.3.3 Condition index

The soft tissue and shell mass was determined (Cubis Semi-Micro Balance, Sartorius, Germany) (n = 15 per treatment) and a modified condition index was calculated by dividing the soft tissue weight by the shell weight according to Park et al., (2012).

4.3.4 Ramping protocol and cardiac performance

Following exposure to different thermal regimes, thermal performance and tolerance were assessed in animals exposed to each of the three temperature treatments using heart rate measurements under acute thermal ramping. Heart rate was measured in immersed abalones in response in two ramping trials for each treatment (n = 10 – 12 animals per treatment, divided across two trials). Abalones were settled individually in beakers (Vol. = 1 L) filled with aerated sea water for 30 min at the respective temperature to which organisms were acclimated (15 °C or 20 °C). Temperature control was achieved via immersion of beakers in a circulating water bath fitted with a thermostatic stirrer (Grant TX150 thermostatic stirrer, Grant Instruments, UK). Temperature was monitored continuously throughout the experiments (HH806U temperature logger, Omega). After the settlement period, sea water temperature was increased at a rate of ~ 0.1°C min^{-1} until all abalones reached cardiac flatline temperature (~36 – 39 °C). Heart rate measurements were performed using a modified non-invasive method first applied for abalone by Chen et al., (2016). An infrared sensor (CNY70, Vishay semiconductors, Germany) was attached to each shell above the heart to detect their heartbeat. Heartbeat signals were amplified, filtered, and recorded by the ElectricBlue heart frequency logger (PULSE V2, ElectricBlue, Portugal). The data were simultaneously displayed on a 3.5'' LCD screen and saved in the device's memory as plain CSV files. These files were then imported into Excel and R (v 3.5.2 (R Core Team, 2018)) for further analysis, following the manufacturer's manual (see data analysis section). Any inconsistent heartbeat traces resulting from sensor dislodgment during the trials were excluded from further analysis. After quality control, data for ten individuals per treatment were used. Heart rate data were used to establish thermal performance curves (TPCs), and the following thermal tolerance traits calculated based on

these TPCs: temperature at maximum heart rate (T_{peak}), maximum heart rate (R_{max}), thermal breadth or the temperature range over which heart rate was 80 % of its peak ($T_{breadth}$), flatline temperature or the temperature at which heart stops beating (FLT), and thermal safety margin, calculated here as the difference between T_{peak} and FLT, thus representing the difference between the animal's maximum performance for heart rate, and the temperature of maximum tolerance ($T_{s.margin}$). In addition, the Arrhenius breakpoint temperature (ABT), the temperature at which there was an abrupt decrease in heart rate, was calculated. The muscular foot critical thermal maximum (FM_{CTmax}), the temperature at which abalones detach from the vertical surface due to neuromuscular failure, was also measured. Following ramping, the soft tissue mass of each abalone was weighed (Cubis Semi-Micro Balance, Sartorius, Germany).

4.3.5 Data analysis

4.3.5.1 Thermal performance curves

The raw heart signals were converted into heart beats according to manufacturer protocols (Electric blue, https://electricblue.eu/pulse). Briefly, raw signal files were split into 10-minute intervals (equivalent to 1 °C ± 0.1 °C intervals) and then a custom-designed R shiny interface was used to count the heart beats. The counting process involved detecting voltage peaks in the raw signals, after adjusting the bandwidth (using the smooth function) and multiplier button tools of the shiny interface. Manual screening was performed to correct miscounted sections of the heartbeat signals, such as instances of doubly counted peaks (Burnett et al., 2013), specially before reaching the FLT. Subsequently, the heart rate in beats per minute (BPM) was calculated as an average of the recorded heart beats over the 10 min intervals.

To fit thermal performance curves (TPCs) to the heart rate data, a published pipeline was used (Padfield et al., 2021). This pipeline utilized the rTPC and nls.multstart packages in R version 4.3.0. It allowed for the fitting of mydata to 24 different mathematical TPC models, representing a wide diversity of nonlinear TPC models found in the literature, using nonlinear least squares (NLLS) regression. For each individual TPC, the pipeline identified

the best-fit model among the 24 TPC models based on their weights (AIC values), after they were visually validated. The "cal_params" function was then used to estimate the following TPC parameters: T_{peak}, R_{max}, $T_{breadth}$, $T_{s.margin}$, and FLT. These parameters were obtained from the function outputs as described by Padfield et al. (2021). High-resolution predictions (at 0.001 °C intervals) of the fitted model were used to calculate these derived TPC parameters. The fit of the non-linear models to the raw data were generally high (r^2 = 0.86 ± 0.074, mean ± sd).

4.3.5.2 ABT

To determine the ABT, the “break.line” function in the “segmented” R package was used. This function generated the best-fit regression lines on both sides of a potential break point, using the fitted heart rate values obtained from the best-fit TPC model of each individual. The ABT was calculated based on these fitted regression lines (Stillman and Somero, 1996).

4.3.5.3 Statistical analysis

To test for differences in thermal performance and tolerance traits between temperature treatments, separate analysis was conducted for each trait: FLT, T_{peak}, R_{max}, $T_{breadth}$, $T_{s.\ margin}$, ABT, FM_{CTmax}, and condition index. Linear mixed effect models were used to test the effects of treatment on each of the traits, with temperature treatment as a fixed factor, body mass as a covariate, and tank and trial as random factors. Models were fitted based on lowest AIC scores and there was no significant mass, tank, or trial effect for any of the traits. Accordingly, a type III SS linear model (lm) was used to examine the effects of temperature treatment on each trait. The normality of residuals and homoscedasticity of variance were assessed through visual inspection of the quantile-quantile and scale-location plots. Pairwise differences between levels were identified by Tukey’s HSD post-hoc tests. A p-value < 0.05 was considered statistically significant. All data were analysed using the core Stats package in R v. 3.5.2 (R Core Team, 2018).

4.4 Results

4.4.1 Condition index

The temperature treatment had a significant effect on the condition index of abalone ($F_{2,42}$ = 11.93, P < 0.001) (Fig. 4.2). The repeated heat shock treatment exhibited a higher condition index compared to both the control and chronically warmed treatments (P < 0.001), with no significant differences between the latter two (P = 0.205).

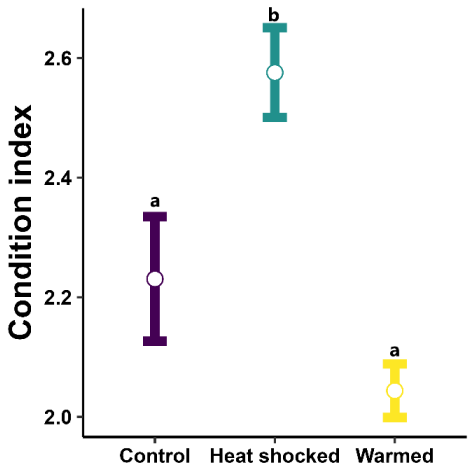


Figure 4.2. Condition index of *H. tuberculata* after exposure to three different thermal regimes; control (15 °C), repeated heat shocked, and chronically warmed abalones (20 °C). Lower case letters indicate significant post-hoc differences across treatments at P < 0.05 (n = 15 animals per treatment). Values are expressed as means ± 1 S.E.

4.4.2 Thermal performance and tolerance parameters

The different temperature treatments significantly influenced the shape of the TPC in abalone, with the overall shape of the TPC shifting towards higher temperatures and higher maximum performance for individuals in the chronically warmed treatment compared to the control. In contrast, for animals in the repeated heat shock treatment, the breadth of

the curve increased by increasing the upper thermal limit, while maximum performance was maintained, and equivalent to that of control individuals (Fig. 4.3).

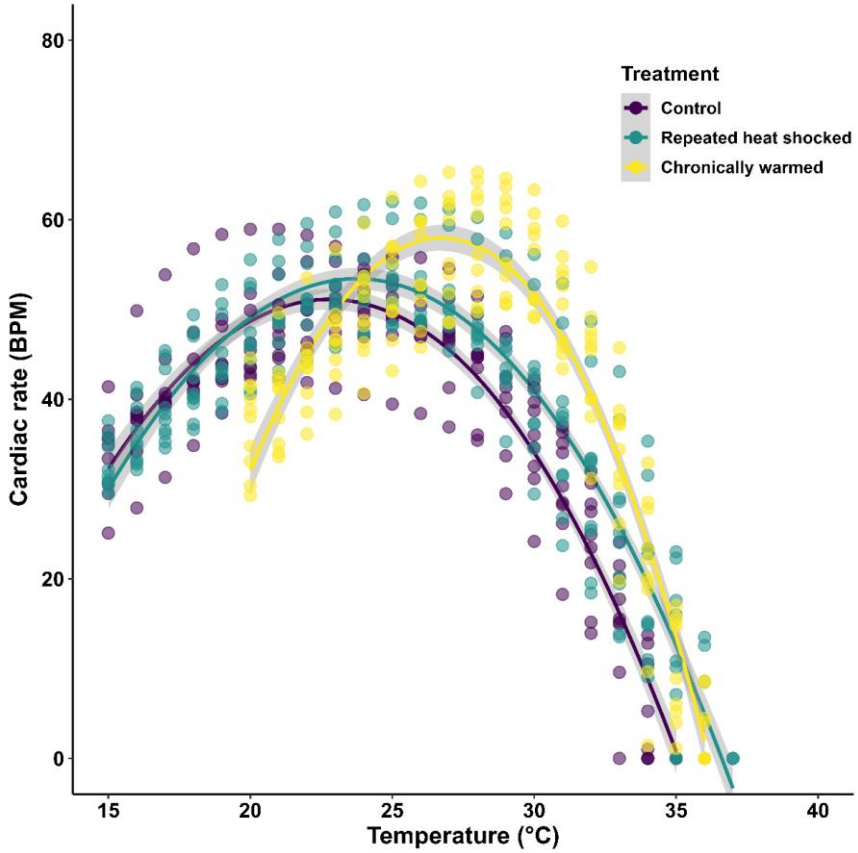


Figure 4.3. Thermal performance curves for cardiac activity (beats per minute, BPM) for adult *H. tuberculata* exposed to control (purple, n = 10), chronic warming (yellow, n = 10), and repeated heat shock exposures (teal, n = 10) over six months. Individual data points represent the heart beats of a ramped abalone at 1 °C intervals. Lines represent the smoothed conditional means for each treatment's overall TPC.

Whilst temperature treatment did not significantly affect maximum heart rate (R_{max}, $F_{2,27}$ = 3.357, P = 0.052) (Fig. 4.4A), a significant effect of treatment was observed on all other traits derived from the TPC: temperature at which maximum heart rate occurred (T_{peak}, $F_{2,27}$= 24.174, P < 0.001), flatline temperature (FLT, $F_{2,27}$ = 10.22, P <0.001), thermal safety margin ($T_{s.margin}$, $F_{2,27}$= 15.55, P < 0.001) and thermal breadth ($T_{breadth}$, $F_{2,27}$ = 6.83, P = 0.005). FLT was higher under the repeated heat shock (P < 0.001) and chronically warmed (P = 0.008) treatments compared to the control treatment, but the two temperature treatments did not differ significantly from each other (P = 0.53) (Fig. 4.4C). For all remaining traits, animals in the control and repeated heat shock treatments were similar to each other (P > 0.05), and differed from animals in the chronically warmed treatment, which had significantly lower mean values of $T_{breadth}$ (P = 0.008 and 0.010 for control and repeated heat shock, respectively) and $T_{s.margin}$ (P < 0.001 for both treatments) (Fig. 4.4D and E) and significantly higher values for T_{peak} (P < 0.001 for both treatments) (Fig. 4.4B). High T_{peak} and FLT values caused the shift of TPC towards higher temperatures in the chronically warmed animals, whilst the high FLT caused a shift in the upper TPC's limits in the repeated heat shocked animals, compared to control.

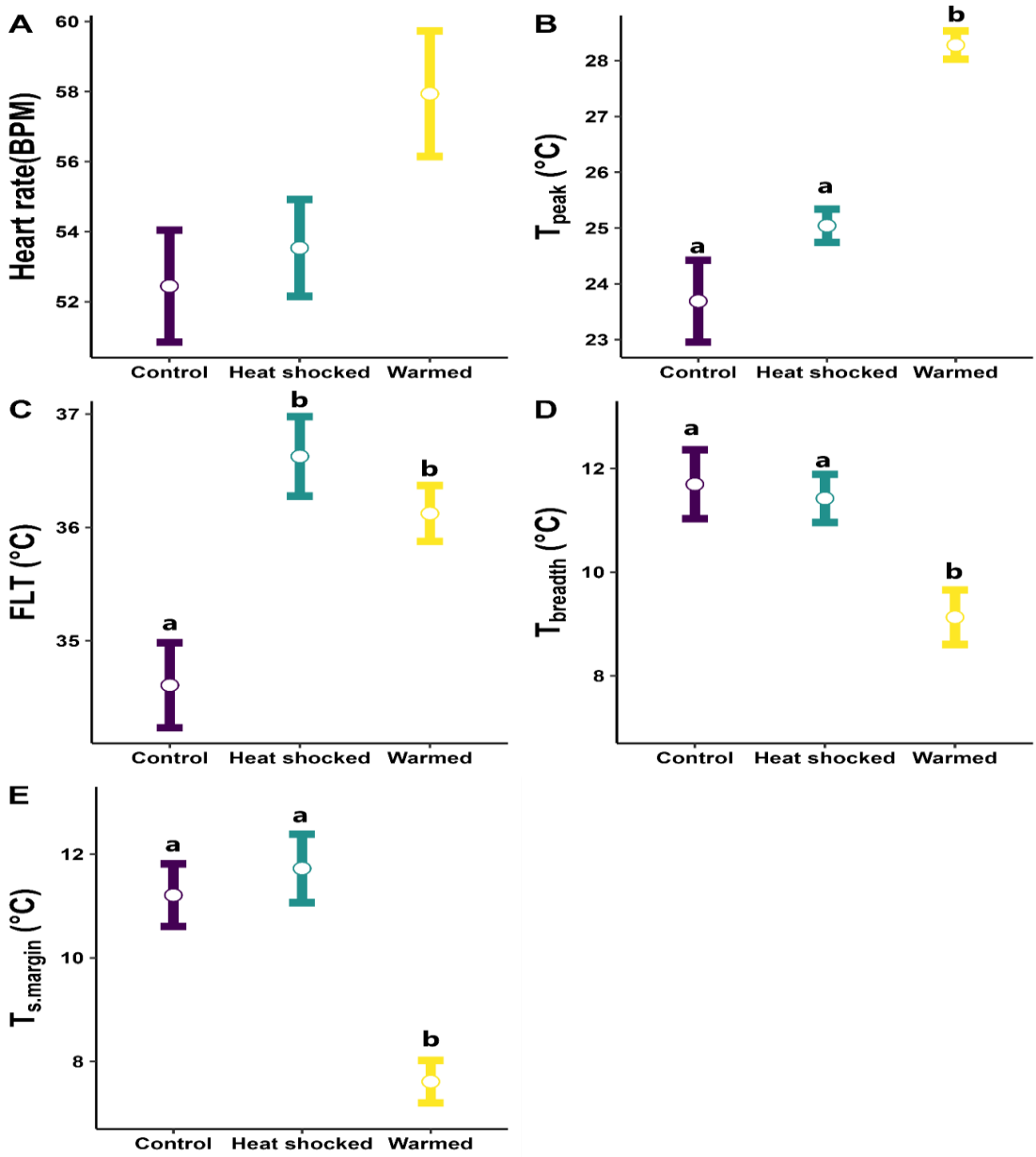


Figure 4.4. Thermal performance traits derived from TPCs in *H. tuberculata* after exposure to the three different treatments: control (15 °C), repeated heat shocked, and chronically warmed abalones (20 °C) for six months. Traits are as follows: (A) maximum heart rate (R_{max}), (B) temperature at maximum heart rate (T_{peak}), (C) temperature at which heart stops beating (flatline temperature) (FLT), (D) temperature range at which heart rate is 80 % of its peak ($T_{breadth}$), and (E) thermal safety margin, the difference between T_{peak} and FLT ($T_{s.margin}$). Lowercase letters indicate significant post-hoc differences between treatments at $P < 0.05$ (n = 10 individuals per treatment). Values are expressed as means ± 1 S.E.

Similarly to other TPC derived parameters, the Arrhenius break point (ABT) was significantly affected by temperature treatment ($F_{2,27}$ = 14.78, P < 0.001), with animals in the chronically warmed treatment having a significantly higher ABT than both the control (+ 2.83 °C, P < 0.001) and repeated heat shock (+ 2.57 °C, P < 0.001) treatments, which did not differ significantly from each other (Fig. 4.5A). The temperature at which animals lost the ability to remain attached to the substrate (FM_{CTmax}) was also significantly influenced by treatment ($F_{2,27}$ = 10.55, P < 0.001), with animals in both the repeated heat shock (P = 0.001) and chronically warmed (P < 0.000) treatments presenting higher FM_{CTmax} compared to animals kept under control conditions. Interestingly, in line with the FLT results, FM_{CTmax} did not differ significantly between the repeated heat shock and chronically warmed treatments (P = 0.066) (Fig. 4.5B).

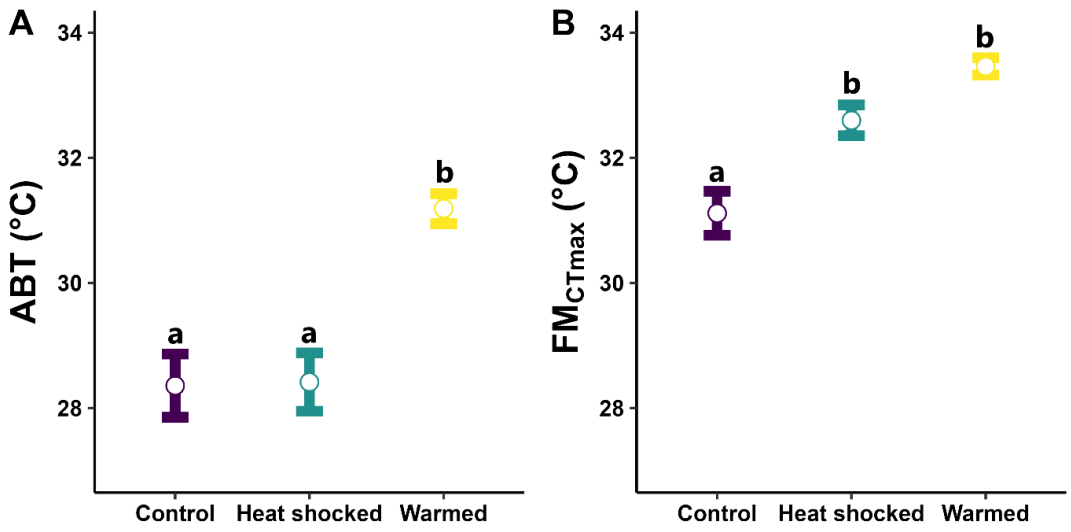


Figure 4.5. Thermal maximum temperature traits of *H. tuberculata* after exposure to the three different treatments; control (15 °C), repeated heat shocked, and chronically warmed abalones (20 °C) for six months. (A) Arrhenius breakpoint temperature (ABT), (B) Foot Muscle Critical Thermal maximum (FM_{Ctmax}). Lowercase letters indicate significant post-hoc differences across treatments at P < 0.05 (n = 10 animals per treatment). Values are expressed as means ± 1 S.E.

4.5 Discussion

The aim of this study was to explore the potential of repeated heat shock exposures to induce heat hardening in the European abalone, *H. tuberculata,* and assess whether the effect of heat hardening on thermal tolerance is comparable to that of acclimation to chronic warming. Both chronic warming and repeated heat shock exposures induced plasticity in thermal tolerance traits of *H. tuberculata,* when exposed to acute thermal ramping. Exposure to repeated heat shocks resulted in heat hardening in abalone via significant physiological improvement. Improved thermal tolerance in heat-hardened animals was comparable to that observed in animals acclimated to chronic warming, but occurred in the absence of any compromise being noted in other performance measures, such as thermal safety margin and thermal breadth, unlike for the chronic warming treatment. Moreover, repeated heat shock exposures resulted in higher condition index compared to other treatments. These findings highlight the potential benefits of using repeated heat shocks as an effective method to enhance the thermal tolerance and overall performance of molluscs under changing environmental conditions in restoration and commercial settings.

4.5.1 Different effects of heat hardening and chronic acclimation elicit different effects on thermal performance

Intertidal animals can increase their upper thermal limit via acclimation to elevated or fluctuating temperatures (Collins et al., 2023; Dong et al., 2021). The three traits for thermal tolerance used here (ABT, $FM_{CTmax,}$ FLT) indicated that abalone exposed to chronically elevated temperatures for six months presented higher thermal tolerance than animals kept under control conditions. My results showed similar patterns of changes in ABT and FLT for chronically warmed abalone to those noted previously in other acclimated marine molluscs, where greater shifts were observed in ABT compared to FLT following thermal acclimation (Drake et al., 2017; Moyen et al., 2022; Stenseng et al., 2005), confirming that ABT is more strongly influenced by thermal acclimation than FLT (Dong et al., 2021). The plasticity of ABT under thermal acclimation has been previously reported in abalone species, with the level of change depending upon exposure time and magnitude

(Alter et al., 2017; Yu et al., 2023). For heat-hardened abalone, although the ABT did not change significantly from control, thermal tolerance (FM_{CTmax} and FLT) was enhanced similarly to chronically warmed individuals. However, integrating all the observed traits together reveals that the mechanisms at play differ between the two treatments.

The mechanisms mediating the observed differences between the effects of chronic warming and heat hardening are unclear. Chronic warming involved prolonged exposure to elevated temperature, allowing for gradual acclimation and the potential for long-term physiological adjustments, as reported in abalones (Xiao et al., 2021; Yu et al., 2023). My results clearly demonstrate that chronically warmed abalones experienced lower temperature sensitivity of heart rate, indicated as a shift in their TPCs towards a higher thermal range with significant higher T_{peak}, indicating a "perfect compensation" of the TPC (Seebacher & Little, 2021). However, this was associated with narrower thermal range of $T_{s.margin}$ and $T_{breadth}$, which is commonly observed for elevated acclimation temperatures (Ern et al., 2023). In contrast, repeated heat shocks involve acute thermal challenges with resting periods at lower temperatures, thus the contrasting timeframes and magnitude of temperature change experienced may be responsible for triggering different responses. Indeed, there were performance similarities between heat-hardened and control abalones at lower ramping temperatures (evidenced by similar T_{peak} and ABT), however, notable divergence was observed at 23 – 25 °C, the temperature to which abalones were repeatedly exposed during 3 h warming events. Thus, heat-hardened abalone had a higher FLT = compared to the control group, but was comparable to that = of the chronically warmed animals. Such a response is likely as a result of the activation of thermal tolerance mechanisms being "trained" during hardening events, and reactivated under higher ranges of temperature during ramping (Collins et al., 2023; Dunphy et al., 2018; Georgoulis et al., 2021, 2022). Indeed, heat hardening in marine molluscs has been shown to be associated with heightened antioxidant capacities (Georgoulis et al., 2022; Zhang et al., 2023) and the induction of pathways that maintain neural functionality under thermal stress (Dunphy et al., 2018). Moreover, the differences in the thermal range from the ABT to the FLT between heat hardening and chronic warming treatments may indicate that these separate exposures resulted in distinct physiological adjustments that differentially influenced

homeostatic maintenance, and thus the disruption of homeostasis under thermal ramping (Ørsted et al., 2022).

The ABTs for cardiac activity did not coincide with the temperature at which foot muscle function was lost. This mismatch is likely a result of the different anaerobic capacities of heart and foot muscles. Foot muscles in gastropods tend to have higher anaerobic capacities and rely primarily on anaerobic metabolism for energy generation (Baldwin et al., 2007; Venter et al., 2018), thus physiologically compromised abalone may not be able to meet the aerobic demands to support cardiac activity, but may retain anaerobic capacity to sustain foot adhesion over a greater range of temperatures. Similar disparity between critical thermal maxima for cardiac and foot activity have been observed in other gastropods (Wang et al, 2019). The higher FM_{CTmax} observed in both chronically warmed and heat-hardened abalones compared to the control, therefore implies that anaerobic metabolism may play a significant role in their thermal tolerance. Indeed, some marine gastropods and bivalves can utilise anaerobic metabolism under thermal ramping, increasing tolerance limits (Artigaud et al., 2015; Han et al., 2013; Chen et al., 2021). Interestingly, heat-hardened abalones maintained foot muscle function for longer after reaching their ABT, potentially indicating greater capacity for anaerobic metabolism compared to chronically warmed animals (e.g Han et al., 2017), a response which could be linked to different energy storage in organisms under different thermal regimes, as discussed below.

4.5.2 Unique effects of hardening and acclimation on organism condition

When facing stress, energy is redirected towards various biochemical pathways to maintain homeostasis (Sokolova, 2013; 2021). However, a reallocation of energy resources may come at the expense of other vital ecological functions (Clarke, 1987; Donelson et al., 2010). As a result, trade-offs appear, organisms may prioritize survival and adaptation mechanisms, temporarily compromising their ability to allocate energy to growth (Leung et al., 2020). Interestingly, chronically warmed abalones were able to manage their energy resources to support the physiological adjustments observed during long-term warming,

as evidenced by the maintenance of their condition index, similar to that of the control group. Surprisingly, the repeated heat shock exposures treatment was not only less energetically demanding, but led to a greater condition index. These findings are in agreement with previous results in marine invertebrates, where exposure to fluctuating temperatures enhanced the capacity to regulate protein expression in the abalone, *H. discus hannai* (Kang et al., 2019), glycogen content in the Californian mussel, *Mytilus californianus* (Nancollas & Todgham, 2022), and growth in the sea cucumber, *Apostichopus japonicus* (Dong et al 2006) compared to static exposures. The improved condition index in heat-hardened abalones may indicate higher initial energy stores, which likely enabled the prolonged maintenance of performance under the higher temperature range from ABT towards FLT. This result confirms that elevated initial energy stores may play an important role for enhancing thermal tolerance.

4.6 Conclusions

My results underscore the potential of heat hardening to enhance upper thermal tolerance limits in *H. tuberculata* abalones comparably to a chronic warming acclimation, without incurring potential trade-offs on performance, and increasing condition index. This increase in tolerance is underpinned by a complex interplay of physiological adjustments, influenced by the mode of thermal exposure. Heat hardening, as a stress conditioning approach, shows potential for improving thermal performance, and thus offers substantial potential for bolstering thermal resilience of ecologically important marine molluscs in the face of changing oceanic conditions.

Chapter 5

Parental heat hardening enhances offspring thermal performance in the abalone *Haliotis tuberculata*

5.1 Abstract

Offspring phenotype can be shaped by the parental environment, potentially triggering transgenerational plasticity (TGP). Yet, TGP elicits a variety of responses, and so our understanding of the ideal parental conditions for inducing TGP remains limited. Notably, while heat hardening has been observed to rapidly enhance thermal tolerance in adult molluscs and corals, it is not known whether this beneficial effect extends to the performance of their offspring and how it compares to parental chronic warming. Here, I compared the effects of exposing parents of the European abalone, *Haliotis tuberculata,* to repeated heat shocks and a chronic warming regime on aspects of the thermal performance of their offspring. I exposed adult abalones, during the reproductive phase, to either no warming, chronic warming, or a regime of repeated acute heat shock cycles for six months. Offspring were cultured under control (T= 15 °C) or warm (T = 20 °C) conditions until 81 days post fertilisation (dpf), during which time key morphological and physiological parameters were determined in three developmental stages. Parental exposure to chronic warming increased maternal provisioning but had negative effects on hatching success, larval development, and performance. It also led to significant larval mortality prior to reaching the post larvae stage compared to other parental treatments, irrespective of developmental conditions. In contrast, heat-hardened parents produced eggs of lower average size but displayed similar hatching success as control parents. Compared with control parent offspring, heat-hardened parent offspring showed similar survival and physiological performance at 15 °C. At 20 °C, early veliger had similar performance but improved survival, post larvae displayed enhanced survival and performance, and 81 dpf old juveniles presented greater performance. These findings point to a stress conditioning mechanism for harnessing thermal TGP in marine molluscs, *via* parental heat hardening. This method of heat hardening offers promising avenues for augmenting thermal adaptation for conservation and commercial management of important molluscs.

5.2 Introduction

Parental effects refer to the phenomenon where the environment experienced by parents influences the phenotype of their offspring (Burgess & Marshall, 2014; Crean et al., 2013; Diaz et al., 2018). This form of phenotypic plasticity involves the transmission of non-genetic and/or epigenetic developmental factors, across generations, without any changes in the offspring's DNA sequence (Mousseau & Fox, 1998; Uller, 2008). Indeed, parental effects can lead to trans-generational plasticity (TGP), whereby the environment experienced by the parent influences their progeny's ability to maintain or improve performance in that environment. TGP has been proposed as an important mechanism in enabling populations to persist in the face of climate change stressors (Donelson et al., 2012; Munday et al., 2017).

While many studies have highlighted the beneficial effects of exposing parents to environmental stressors on the performance of their offspring in marine species (Ryu et al., 2018; Parker et al., 2012; Spencer et al., 2020; Wang et al., 2023), others have reported neutral or even maladaptive effects (see Byrne et al., 2020; Donelson et al., 2018). The underlying cause of this variability is not clear but could be associated with trade-offs between parental and offspring fitness in different environments (Burgess & Marshall, 2014; Waite & Sorte, 2022). Therefore, crucial questions remain regarding the optimal conditions that facilitate beneficial TGP.

In the context of climate change, many studies investigating the effects of parental environment on offspring performance focus on subjecting adult parents to short-term stress exposures (see Byrne et al., 2020). Such an approach may not favour parental plasticity due to the extremely high costs of homeostatic maintenance under high levels of stress (Auld et al., 2010; Beaman et al., 2016), which in turn reduces the capacity to trigger offspring plasticity. Conversely, when compared to short-term parental exposures, longer periods of exposure to stress during the reproductive phase have been observed to lead to more pronounced positive parental effects (Dupont et al., 2012; Suckling et al., 2015). Indeed, parental conditioning to ocean acidification and ocean warming, of, the sea urchin *Psammechinus miliaris* and the endangered sea cucumber *Apostichopus japonicus*

produced different responses in the offspring's performance, with greater performance and an enhanced tolerance exhibited when the parental exposure period was increased (Suckling et al., 2014; Wang et al., 2015). Therefore, extended time periods may be required to induce parental acclimation to chronic warming, to increase the investment in offspring fitness that facilitates TGP.

On the other hand, repeated exposure to acute heat challenges over a prolonged time period, can lead to heat hardening, a phenomenon by which organisms acquire a short-term increase in heat tolerance following a sub-lethal extreme heat exposure (Bilyk et al., 2012). Inducing stress hardening in corals has shown that artificial preconditioning treatments, involving exposure to fluctuating temperatures, can effectively enhance the thermal stress tolerances (Hawkins & Warner, 2017; Majerova et al., 2021). Moreover, in marine molluscs, mainly bivalves, heat hardening enhances adult's survival and thermal tolerance during subsequent thermal stress (Georgoulis et al., 2021, 2022; Moyen et al., 2020; Zhang et al., 2021; Zhang et al., 2023), demonstrating the benefits of this rapid adaptive plasticity. These enhancements in thermal tolerance, achieved *via* adjustments to multiple physiological systems, occurred after exposure to only a single or a few warming events. Therefore, such a heat hardening approach is anticipated to increase parental fitness and alleviate the thermal stress costs incurred by parents experiencing chronic warming (see Chapter 4), which in turn increases the likelihood of triggering the offspring's plasticity. However, even though this approach holds substantial promise, the potential impacts of heat hardening parents during the reproductive phase and the resulting impact on offspring remain largely unexplored. How this approach compares to the effects of chronic warming exposure is one of the key outstanding questions concerning TGP and heat hardening on thermal performance of offspring.

Therefore, my aim was to compare the effects of parental exposure to repeated heat shock events or chronic warming on the thermal performance of European abalone, *Haliotis tuberculata*, offspring. I exposed adult abalone to one of three temperature regimes for a period of 6 months: control (15 °C), chronic warming (summer temperature + 3 °C (20 °C)) or heat hardening (also kept at 15 °C but subjected to repeated heat shock events on two separate occasions (23 – 25 °C)). Following exposure, individuals were induced to spawn,

with egg size and albumin diameter measured. Fertilized eggs obtained from each parental treatment were developed under either control (T = 15 °C) or chronic warm (T = 20 °C) conditions, with hatching success subsequently measured. Hatched larvae were left to develop until 81 days post-fertilization (dpf), with physiological measurements conducted at three developmental stages: early veliger, post larvae, and juvenile stages. I measured morphometrics and larval deformations, as well as metabolic, survival, and settlement rates. I predicted that both the chronic warming and repeated heat shock exposures will influence the thermal performance of *H. tuberculata* offspring compared to the control treatment. However, as the thermal exposures are different and thus differential acclimation responses are expected to be triggered in parents, the effects on offspring's performance are expected to differ between the two treatments. The European abalone, *H. tuberculata,* is a highly valued aquaculture species (Cook, 2014), of great ecological importance (Mgaya & Mercer, 1994). However, similarly to other commercially important marine molluscs in coastal systems, larval survival is compromised by rising environmental temperatures (Kavousi et al., 2021). Therefore, it is critical to understand how this species may cope with increasing temperatures, in the context of exploring approaches that can artificially bolster TGP within intensive production settings. This understanding is required for ensuring the sustainable cultivation of economically and ecologically significant species under future predicted coastal and oceanic conditions.

5.3 Materials and methods

5.3.1 Animal Husbandry and parental broodstock conditioning design

Adult abalone *H. tuberculata* (4 years old), were obtained from an abalone farm in France (48°36'50" N, 4°36'3" W Plouguerneau, Brittany). Here they were reared in land-based nursery tanks for 10 months before transfer to sea-cages until they were 4 years of age. Abalone were transported by road to the University of Plymouth, UK, in an icebox, covered with a wet cloth and using ice packs to maintain low temperatures, arriving within 12 hours of collection. Upon arrival, abalones (N = 160) were allowed to recover and acclimate to site of collection's ambient temperatures (T = 14 – 15 °C) in sea water holding tanks (2 X 1000 L, S = 33, 12h:12 h L:D cycles) for two weeks. Subsequently, abalone parents of each

treatment were transferred to a number of recirculating holding units. The design of the units and the husbandry of abalones were described previously in Chapter 4 (Fig. S.1).

At the beginning of the experiment adult abalone were exposed to one of three thermal treatments over a period of 6 months: control (T = 15 °C, control, N = 48), chronic warming (T = 20 °C, chronically warmed, N = 49), and acute thermal challenges treatment (T = 15 °C, plus two heat shock periods at T = 23 – 25 °C, repeated heat shocks, N = 48). The experimental design is shown in figure 5.1. The chronically warmed treatment temperature was set at the projected summer temperature for the end of the century (collection site's mean summer temperatures (Gac et al., 2020) + 3°C), with temperature increased from 14 °C to 20 °C over the course of one week (~ 1 °C day^{-1}). The experimental design for the repeated heat shock exposures was identical to that described in Chapter 4, except that the timing of the two warming events was modified. Briefly, the first sublethal heat shock event was carried out at the start of the experiment. Abalone were subjected to repeated heat shocks at 23 °C, every 24 h, over 5 consecutive days. The second heat shock event was applied following the same procedure, with temperature increased to 25 °C (1 °C above maximum performance temperature) after 3.5 months from the start of the experiment. Between the 2 warming events, and following the 2nd one, repeated heat-hardened abalones were kept under 15 °C until the termination of the experiment.

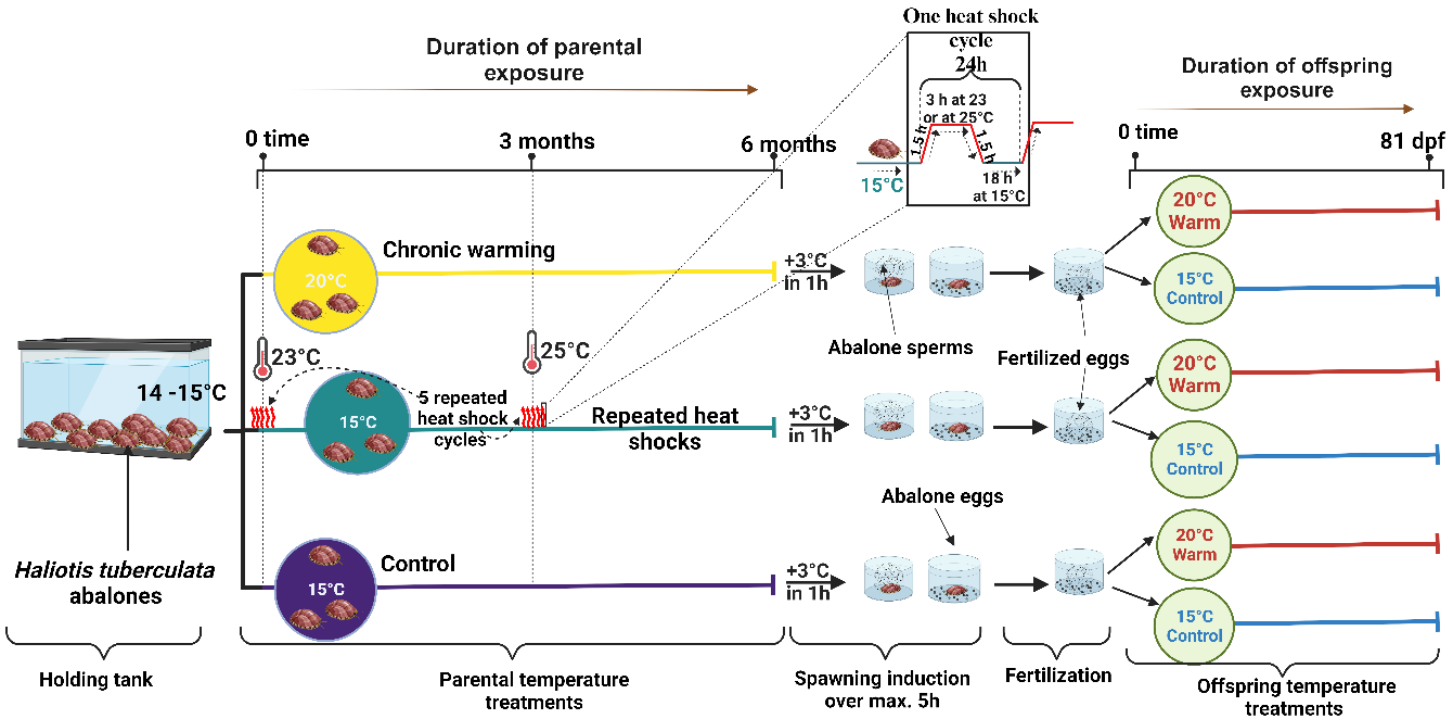


Figure 5.1. Experimental design for parental and offspring exposures of *Haliotis tuberculata* abalones. Parents were subjected to three temperature treatments over a six-month period: control (T = 15 °C, N = 48), repeated heat shocks (T = 15 °C but exposed to two warming events - at 0 h and after three months of exposure, during each event animals were exposed to five repeated 24 h heat shock cycles at 23 °C and 25°C, respectively, N = 49), and chronically warmed (T = 20 °C, N = 48). After six months, spawning was induced by raising the temperature by 3 °C within 1 h, and maintaining it for 5 h. Fertilization occurred within 1 h of gamete release, and fertilized eggs were transferred to larval exposure treatments. In the larval exposure conditions, embryos from each parental treatment were exposed to two different temperatures: control (T = 15 °C) and warm (T = 20 °C) over 81 days post-fertilization (dpf). Data for larval development and physiological performance were collected at three different developmental stages: early veliger, post-larvae, and 81 dpf juveniles.

5.3.2 Spawning induction and larval production

After the 6-month exposure period, ripe, haphazardly selected abalones were individually placed in buckets (Vol. = 4 L) filled with filtered (1 µm), UV sterilized sea water. Spawning was stimulated by gradually heating individuals by 3 °C from the acclimation temperature over 1 h, abalones were allowed to spawn for a maximum of 5 h. After egg release, an equal number of eggs from three females were pooled per treatment, divided into three batches, and each batch fertilized with sperm obtained from a single male from the corresponding parental treatment, to avoid spermatic competition (Harney et al., 2018). Fertilization occurred within 1 h of gamete release. Each egg batch (120,000 eggs) was collected in a container (Vol. = 10 L), where fertilization occurred at an optimal sperm concentration of approximately 100,000 spermatozoa mL^{-1} for 15 – 20 min (Huchette et al., 2004). Following that period, eggs were collected in a 75 µm bag filter and gently rinsed with sea water to avoid polyspermy, and fertilization success checked by assessing the proportion of eggs showing 1st or 2nd polar bodies. At 1 h post-fertilization (hpf), the three fertilized egg batches were mixed, equally divided into six pools of 60,000 embryos and moved to hatching tanks under the corresponding developmental temperature (15 or 20 °C, three pools for each).

5.3.3 Offspring experimental design

To investigate parental effects on offspring thermal performance, I subjected the fertilized eggs from each parental treatment to two different developmental temperatures (control-15 °C, and warm 20 °C) over a period of 81 dpf. Three developmental stages were sampled over this period: 21 day-degree early veliger, 100 day-degree post larvae, and 81 dpf juveniles. Fertilised eggs developed in flow-through systems through larval development until they reached post larvae stage, after which settled post larvae were transferred to static tanks for the rest of the experiment. Each developmental temperature treatment was carried out in three replicate flow-through systems, each consisting of a header tank, hatching tank and larval tank. The header tank (Vol. = 85 L) was supplied with natural 1-µm filtered, UV-sterilized, and well-aerated, sea water. The hatching tank (Vol. = 25 L) received reduced-flow, running sea water from the header tank to prevent flushing out the eggs. An overflow evacuation pipe mounted to the hatching tank allowed automatic transfer of larvae to the larval tank once hatchlings were capable of swimming. The larval tank (Vol. = 32 L) was supplied with sea water from the header tank to rear hatched larvae that had left the hatching tank. A 50-µm net was placed at the outflow to prevent larvae from escaping which was replaced regularly (Fig. S.2).

At 58 day-degree, approximately 2,500 larvae from each larval tank were carefully transferred to a static tank (Vol. = 20 L) under the corresponding temperature conditions, allowed to settle on two settlement plates, and left to grow and develop until they reached 81 dpf. To maintain optimal water quality, water was replaced three times a week using natural 1-µm filtered, UV-sterilized, and well-aerated sea water. Larvae fed *ad libitium* on the encrusting green algae *Ulvela lens,* grown on the settlement plates, and on small pieces of seaweed, *Ulva* sp. and *Palmaria palmata*.

5.3.4 Egg size, albumin ratio and hatching success rate measurements

Oocyte samples were collected and imaged using a Leica M205FA microscope (Leica Microsystems) (n = 250 – 300 per treatment), egg sizes and albumen diameters were

measured using ImageJ2 (Fiji) (Schindelin et al., 2012). The albumin to egg size ratio was calculated and expressed as a percentage. After hatching, the unhatched embryos that had settled at the bottom of the hatching tank were collected and concentrated in 4 L bucket, from which 3 X 5 mL replicates were sampled per larval replicate. The number of unhatched embryos in each tank was counted and subtracted from the total to estimate the rate of hatching success of each tank which was expressed as a percentage.

5.3.5 Morphometric, larval deformation and survival rate measurements

Preliminary trials indicated that embryos in the warm treatment exhibited a developmental rate, from fertilization to post larvae, approximately X 1.5 faster than those in the control treatment. Consequently, sampling times were adjusted during early development accordingly to ensure the capture of the same larval stages under different treatments. Morphometric measurements were performed on the three sampled stages: 21 day-degree early veliger, 100 day-degree post larvae, and 81 dpf juveniles. In addition, survival was measured for the first two stages, and larval deformations only assessed on early veliger. For each developmental treatment, free-swimming early veliger larvae were sampled by collecting sea water (Vol. = 1 L) from each larval tank and concentrating larvae in a 15 mL tube. Survival was determined in 5 X 1 mL replicates (n = 15 replicate), with the remainder preserved in 70 % ethanol for subsequent examination for deformations and morphometric analysis. Post larvae in each larval tank were concentrated in a 2 L container, 5 X 5 mL samples were taken for survival determination (n = 15 replicates), while 100 mL were aliquoted and preserved in 70 % ethanol for subsequent morphometric analysis. Under a binocular microscope (X 10), the number of live individuals was counted. Survival of early veliger was expressed as a percentage of the total hatched larvae, while post larvae, survival was expressed as a percentage of the number of early veliger larvae.

To evaluate larval deformation in *H. tuberculata*, I employed a phenotype categorization method adapted from Auzoux-Bordenave et al. (2022). The categorization included two classes: normal phenotype, characterized by normal tissue and shell development, and altered phenotype, characterized by abnormal tissues and partial or no shell formation. Images of lateral-sided early veliger larvae were captured for each sampled replicate

using a Leica M205FA microscope (X 4 – 10, Leica Microsystems), deformed larvae were counted, and larval deformation was expressed as a percentage of the total imaged larvae in each sample (n = 180 – 200 larvae per developmental treatment). Similarly, images were captured for larval length of non-deformed early veliger larvae, and shell length of post larvae (n = 180 – 200 larvae per stage per developmental treatment), which were measured using ImageJ2 (Fiji) software.

For juvenile abalone, as with the larval stages, shell length was measured in haphazardly-selected individuals, subsequent to being manually, gently detached from the settlement plate (n = 27 – 35 juveniles per treatment). Since measuring individual mass was not practicable, batches of individuals (n = 3 – 5) were blotted with tissue paper to remove excess water and weighed together to obtain a precise mass (n = 15 – 20 batches per treatment) (Cubis Semi-Micro Balance, Sartorius, Germany). The average mass per individual was then estimated from the batch mass. These data were used to calculate the daily growth rate in terms of both shell length and mass.

5.3.6 Larval settlement

The rate of larval settlement was assessed using free-swimming veliger larvae, after the complete formation of the epipodal tentacles on foot muscle (De Viçose et al., 2007), at 58 day-degree. For each larval tank, 2 X 4 L containers were prepared with 1-µm filtered, UV-sterilized, well-aerated sea water at the corresponding temperature (n = 6). In each settlement container, two 10 cm × 15 cm semi-vertical (75 °) polycarbonate plates, covered with a thin layer of encrusting microalga *U. lens*, were installed. Settlement plates used in this study were produced in the laboratory before commencing the larval development experiments, where *U. lens* was induced to release its sporophytes using the method of Hannon et al. (2014).

Approximately 1,000 larvae were transferred using a pipette into each settlement container (n = 6). After 24 h, the sea water was filtered through a 50-µm filter to collect any larvae that had not settled. Settlement was then calculated by subtracting the number

of unsettled larvae from the total and expressing it as a percentage, using the average of the two containers for each developmental tank.

5.3.7 Metabolic rate and larval developmental cost

To assess metabolic adjustments under the thermal developmental treatments, I measured the rate of oxygen uptake (MO_2) of abalone offspring at their corresponding developmental temperature. MO_2 rates were estimated using closed respirometry. Each glass respirometer, Vol. = 1.8 mL, was fitted with an oxygen-sensitive dot (PreSens, Germany), a magnetic flea was used to ensure proper mixing which was powered by an underwater magnetic stirrer (Thermo Scientific Multi Position Stirrer, USA). Approximately 70 larvae mL^{-1} or 3 – 5 juveniles were placed inside each respirometer. Two respirometers were run without abalone to act as a blank for each larval or juvenile tank (n = 6 per developmental treatment). Each respirometer was filled with air-saturated, filtered, and sterilized sea water, and gas-tight lids were securely sealed underwater to prevent the formation of air bubbles. The decline in partial pressure of oxygen (pO_2) over time (between 85 and 75 % air saturation) was monitored using a multi-channel oxygen meter (OXY-4 SMA (G3), PreSens, Germany), and the oxygen measurements were collated using the PreSens measurement studio software (PreSens, Germany). For early veliger and post larvae, MO_2 was expressed as nmol O_2 h^{-1} $larva^{-1}$. MO_2 data were then used to estimate the cost of development for those two larval stages according to the developmental cost theory (Marshall et al., 2020; Pettersen et al., 2019), by multiplying the MO_2 value at specific stage by the time (h) of development to that stage since hatching.

While for juveniles, after MO_2 measurement, juveniles used in respirometry measurements were removed from the respirometer, blotted dry, and weighed to nearest mg (Cubis Semi-Micro Balance, Sartorius, Germany). In this case MO_2 as expressed as nmol O_2 h^{-1} wet mass mg^{-1}.

5.3.8 Data analysis

Linear models (lm) using Type III sum of squares were used to examine the effects of parental and developmental treatments on each evaluated physiological trait. Parental and developmental treatments were used as fixed factors in two-way ANOVA. Larval tank was initially tested for random effect, but was subsequently discarded as it was shown to have no significant effect. The normality of residuals and homoscedasticity of variance were assessed through visual inspection of the Quantile-Quantile and scale-location plots. Pairwise differences between levels of each factor were identified by Tukey's HSD *post-hoc* tests. For egg size and albumin diameter, a similar approach was followed, where parental treatment was used as a fixed factor in a one-way ANOVA. Female mass was used as a covariate, but as it was shown to have no significant effect, it was subsequently discarded from the final analysis. All data were analysed using the core Stats package in R v 4.3.1 (R Core Team, 2023). A p-value < 0.05 was considered statistically significant.

5.4 Results

The chronic warming of parents resulted in increased maternal provisioning compared to the other two parental treatments, but it adversely affected larval development, survival, and performance, as well as MO_2 and the cost of development under both developmental conditions. In contrast, when parents were exposed to repeated heat shocks, broadly speaking, offspring performance was similar to those obtained from the parental control group when raised under control conditions. Interestingly, when larvae were raised under war temperature, parental heat hardening improved overall larval development and performance. Furthermore, this enhanced thermal performance persisted and was observed in the juveniles after 81 dpf, as detailed in the following sections.

5.4.1 Egg size and albumin ratio

Parental treatment significantly influenced egg size ($F_{2, 816} = 49.5$, $P < 0.001$) and albumin size ($F_{2, 816} = 98.8$, $P < 0.001$). Heat-hardened parents produced significantly smaller eggs than both parents in the control ($P < 0.001$) and chronic warming ($P < 0.001$) groups. There

was no significant effect of parental warming (P = 0.62) (Fig. 5.2A). However, eggs from chronically warmed parents had a greater proportion of albumin compared to both hardened (P < 0.001) and Control (P < 0.001) parents, respectively (Fig. 5.2B).

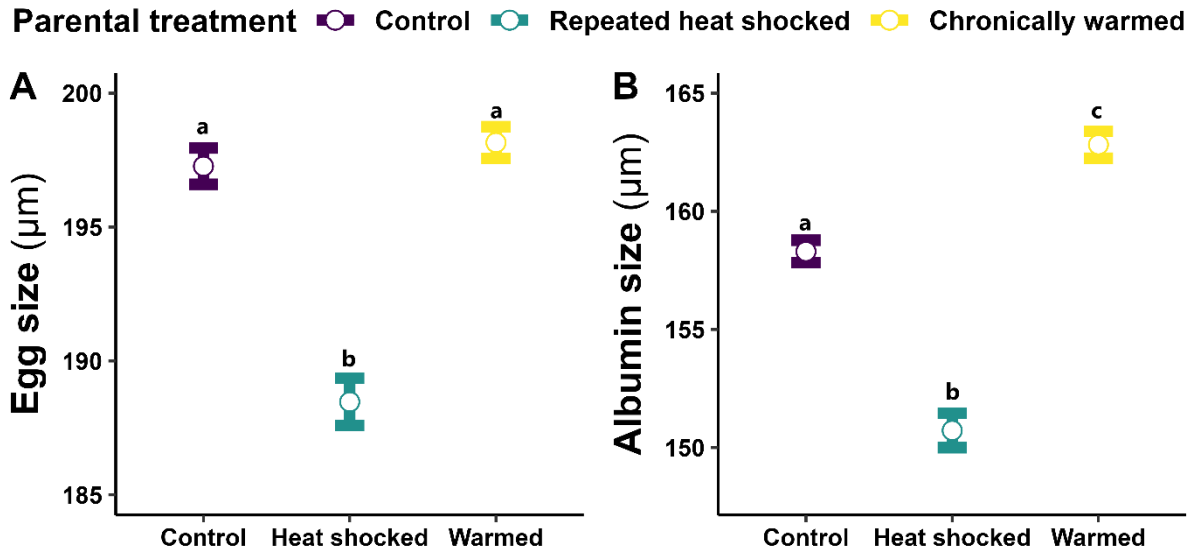


Figure 5.2. Egg size measurements from adult *Haliotis tuberculata* abalones after broodstock conditioning under three thermal treatments. (A) Egg size and (B) Albumin to egg size ratio. Lowercase letters indicate significant post-hoc differences across treatments at P < 0.05 (n = 250 – 300 per treatment). Values are expressed as means ± 1 S.E.

5.4.2 Larval development and survival

Parental treatment had a significant effect on hatching success ($F_{2, 48}$ = 27.06, P < 0.001), larval deformities ($F_{2, 81}$ = 240.23, P < 0.001), and survival rates of early veliger larvae ($F_{2, 81}$ = 162.41, P < 0.001). However larval settlement ($F_{2, 20}$ = 0.057, P = 0.81) and post larvae survival ($F_{1, 52}$ = 0.12, P = 0.72) were not affected (Fig. 5.3A – E). Offspring from chronically warmed parents had lower hatching success and early veliger survival, in addition to higher larval abnormalities, compared to offspring from control and heat-hardened parents (P < 0.001 in all cases). While there was no effect of developmental temperature, or any interaction between parental and developmental treatment, on hatching success ($F_{1, 48}$= 0.3, P = 0.57 and $F_{2, 48}$ = 0.06, P = 0.93, respectively), an effect of developmental

temperature, and an interaction were observed for larval deformities ($F_{1, 81} = 62.5$, $P < 0.001$ and $F_{2, 81} = 39.1$, $P < 0.001$, respectively) and survival rate of early veliger ($F_{1, 81} = 37.41$, $P < 0.001$ and $F_{2, 81} = 9.7$, $P < 0.001$, respectively) and post larvae ($F_{1, 52} = 63.1$, $P < 0.001$ and $F_{1, 52} = 15.6$, $P < 0.001$, respectively) (Fig. 5.3A – D). Moreover, larval settlement was not affected by the developmental temperature ($F_{1, 20} = 0.69$, $P = 0.41$) with no interaction observed between parental and larval treatments ($F_{1, 20} = 1.48$, $P = 0.23$). However, *post hoc* analysis detected a significant difference between developmental temperatures ($P = 0.026$) which seems to be driven by a non-significant increase in settlement rates of larvae from heat-hardened parents under warm conditions (Fig. 5.3E). Notably, under control conditions, the reduced larval deformity observed in offspring from both heat-hardened and control parents was correlated with similar larval survival and settlement rates. Interestingly, when developed under warm conditions, both early veliger and post larvae originating from heat-hardened parents displayed higher survival rates in comparison to those from control parents ($P = 0.003$ and < 0.001, respectively). Furthermore, offspring obtained from heat-hardened parents had similar performance in terms of larval deformities, early veliger, and post larvae survival under both developmental temperatures ($P > 0.05$ in all cases), showing no sensitivity to warmer conditions. While for both control and chronically warmed parents, offspring had lower performance when developed under warm conditions compared with development under control conditions ($P < 0.001$ in all cases).

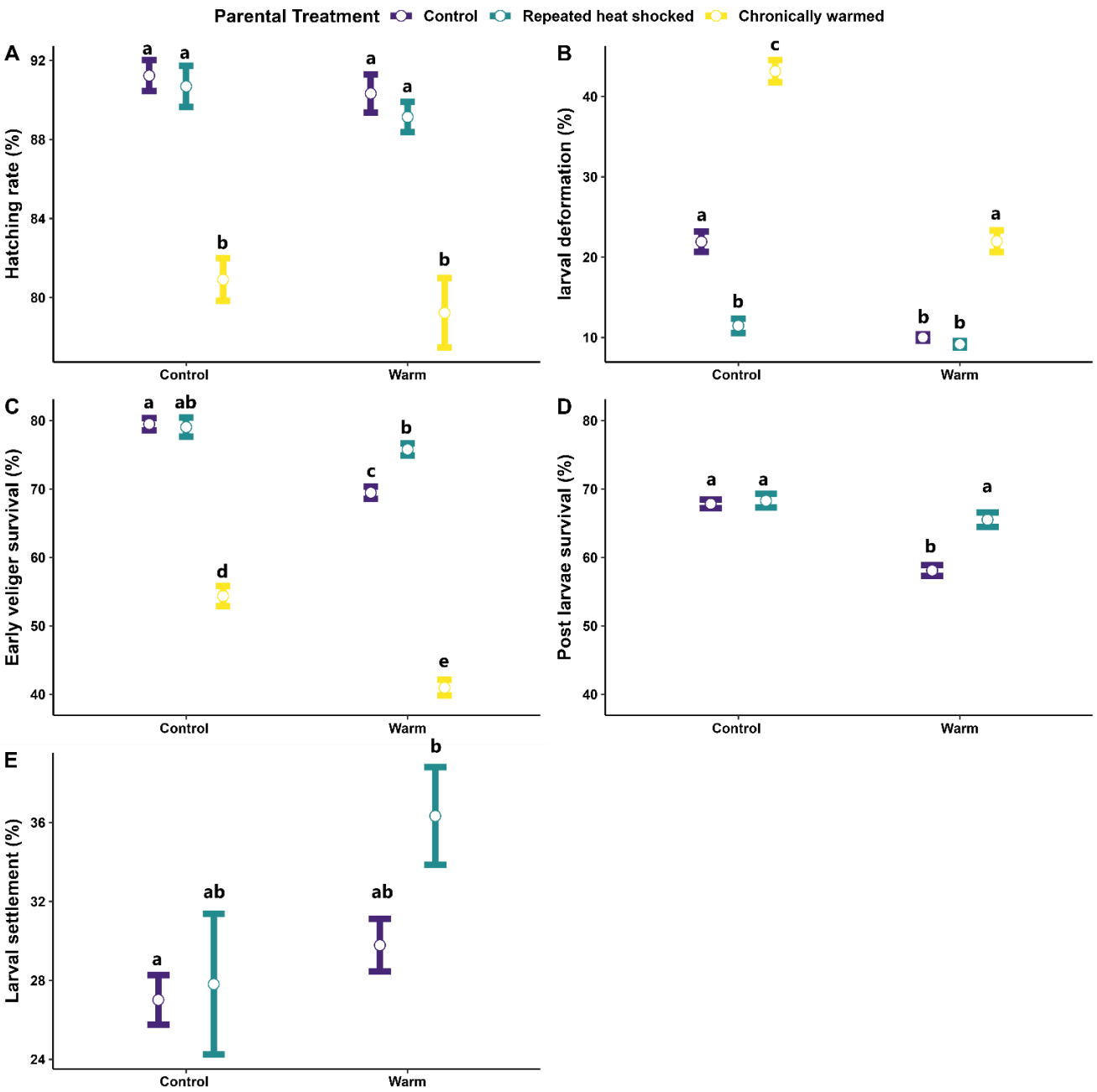


Figure 5.3. Effects of parental and developmental temperatures on development, survival, and settlement rates of *Haliotis tuberculata* offspring. (A) Hatching success, (B) Larval deformation rates, (C) Survival of early veliger stage, (D) Survival of post larvae stage, and (E) Larval settlement rates. Lowercase letters indicate significant post-hoc differences across treatments at $P < 0.05$ (N = 9 per parental treatment for (A), and N = 13 – 15 for (B) and (C), and (D), whilst N = 6 per developmental treatment for (E). Values are expressed as means ± 1 S.E.

5.4.3 Larval performance

Parental treatment significantly influenced larval length ($F_{2, 1141} = 153.97$, $P < 0.001$), MO_2 ($F_{2, 29} = 19.54$, $P < 0.001$), and consequently cost of development ($F_{2, 29} = 24.56$, $P < 0.001$) of the early veliger larvae. Parental chronic warming resulted in lowest early veliger larval lengths, which were associated with higher MO_2 and cost of development compared with both heat-hardened ($P < 0.001$) and control ($P < 0.001$) parents. In contrast, parental heat hardening resulted in similar early veliger MO_2 and cost of development to parental control ($P > 0.05$), nonetheless larvae were shorter ($P < 0.001$) (Fig. 5.4A, C, and E). For post larvae, parental treatment did not affect length ($F_{1, 835} = 1.54$, $P = 0.21$), MO_2 ($F_{1, 20} = 1.69$, $P = 0.20$) or cost of development ($F_{1, 20} = 2.88$, $P = 0.10$) (Fig. 5.4B, D, and F). The developmental treatment and its interaction with parental treatment had an effect on the body length of both early veliger ($F_{1, 1141} = 19.07$, $P < 0.001$ and $F_{2, 1141} = 27.47$, $P < 0.001$, respectively) and post larvae ($F_{1, 835} = 22.03$, $P < 0.001$ and $F_{1, 835} = 12.78$, $P < 0.001$, respectively). Furthermore, an effect of developmental treatment was observed on MO_2 of both early veliger ($F_{1, 29} = 8.92$, $P = 0.005$) and post larvae ($F_{1, 20} = 13.52$, $P = 0.001$), with no effect on cost of development for either stage ($F_{1, 29} = 0.39$, $P = 0.53$ and $F_{1, 20} = 3.02$, $P = 0.09$, respectively). When developed under warm conditions, early veliger larvae from heat-hardened and control parents were shorter ($P = < 0.001$ and < 0.001, respectively), had higher MO_2 values ($P = 0.025$ and 0.047, respectively) but a similar cost of development ($P = 0.99$ and 0.98, respectively), compared to larvae developed under control conditions. Conversely, chronically warmed parents had larger early veliger ($P = 0.008$), but these individuals had a similar MO_2 ($P = 0.055$), and cost of development ($P = 0.37$) compared to those developed under control conditions. For both early veliger and post larvae, there was no interaction between parental and developmental treatment on MO_2 ($F_{2, 29} = 0.027$, $P = 0.97$ and $F_{1, 20} = 3.58$, $P = 0.07$, respectively) or the cost of development ($F_{2, 29} = 2.16$, $P = 0.13$ and $F_{1, 20} = 2.02$, $P = 0.17$, respectively). However, interestingly, when developed under warm conditions, the post larvae from heat-hardened parents were larger ($P < 0.001$) than those from control parents, with a lower MO_2 ($P = 0.003$) and lower cost of development ($P = 0.006$). Both of which were comparable those measured in individuals developed under control conditions ($P = 0.98$, 0.75, and 0.99, respectively). Thus, heat hardening of the parents ameliorated the effect of developing under warm conditions on larval MO_2 and

cost of development, which underpins the enhanced development, survival, and growth measured in this group.

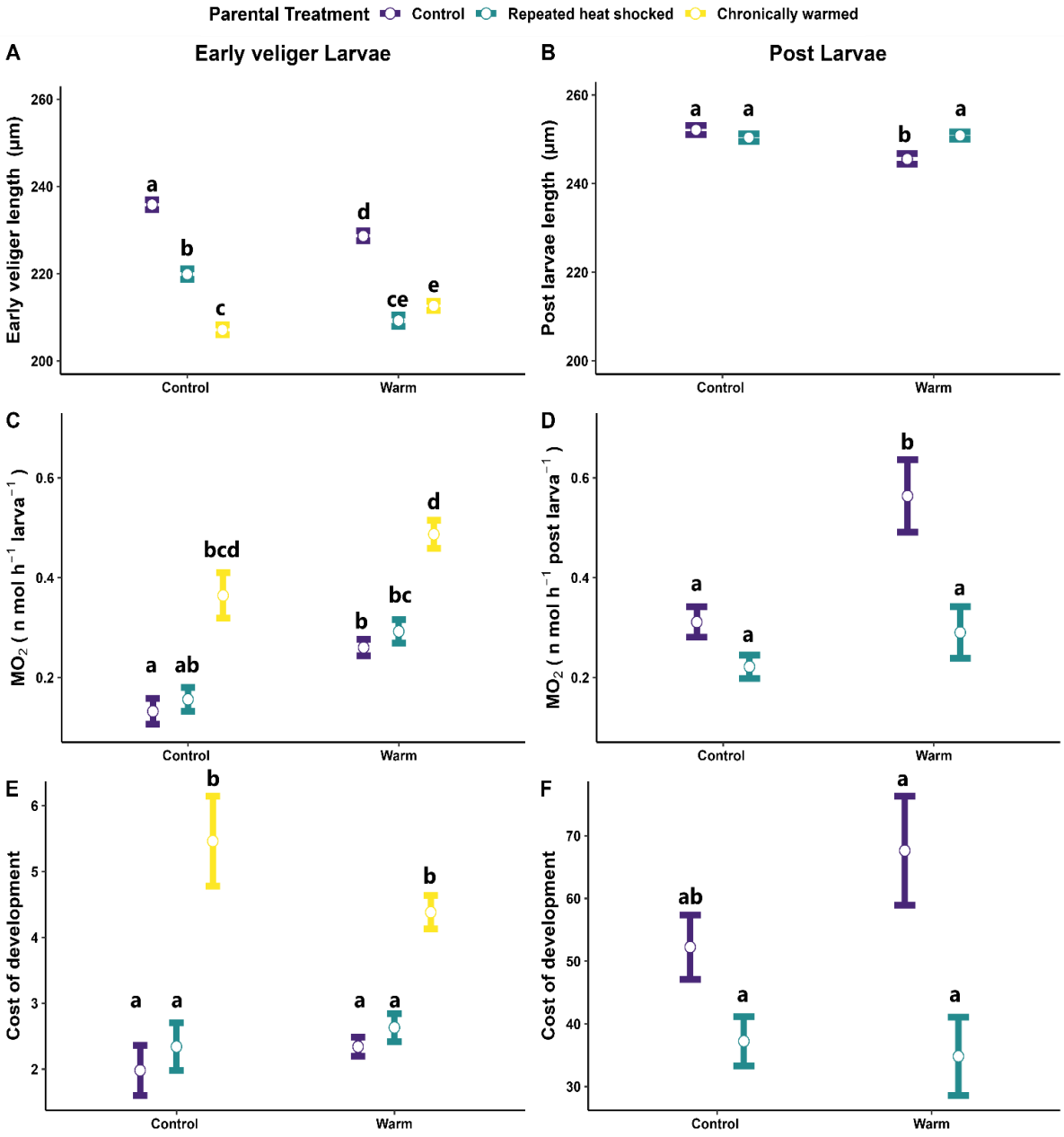


Figure 5.4. Effects of parental and developmental temperatures on larval performance traits of *Haliotis tuberculata* abalone. Early veliger (A) and post larval (B) lengths, metabolic rates of early veliger (C) and post larvae (D), and developmental costs of early veliger (E) and post larvae (F). Lowercase letters indicate significant post-hoc differences across treatments at P <0.05 (n = 180 – 200 per developmental treatment for (A) and (B), while n = 6 per developmental treatment for (C – F)). Values are expressed as means ± 1 S.E.

5.4.4 Juvenile performance

The growth rate of shell and body mass of *H. tuberculata* juveniles were only affected by an interaction between parental treatment and developmental temperature ($F_{1, 106} = 4.81$, P = 0.03 and $F_{1, 73} = 10.23$, P = 0.002, respectively), while MO_2 of juveniles was affected only by developmental treatment ($F_{1, 20} = 6.25$, P = 0.019) (Fig. 5.5A – C), 81 dpf. For all three traits, juveniles from control and heat-hardened parents were similar when developed under control temperatures (P > 0.05). However, when developed under warm temperatures, juveniles form heat-hardened parents had significantly higher shell (P < 0.001) and body mass (P < 0.001) growth rates, and higher MO_2 (P = 0.049), further confirming the beneficial effects of parental heat hardening that persisted from larvae to juveniles.

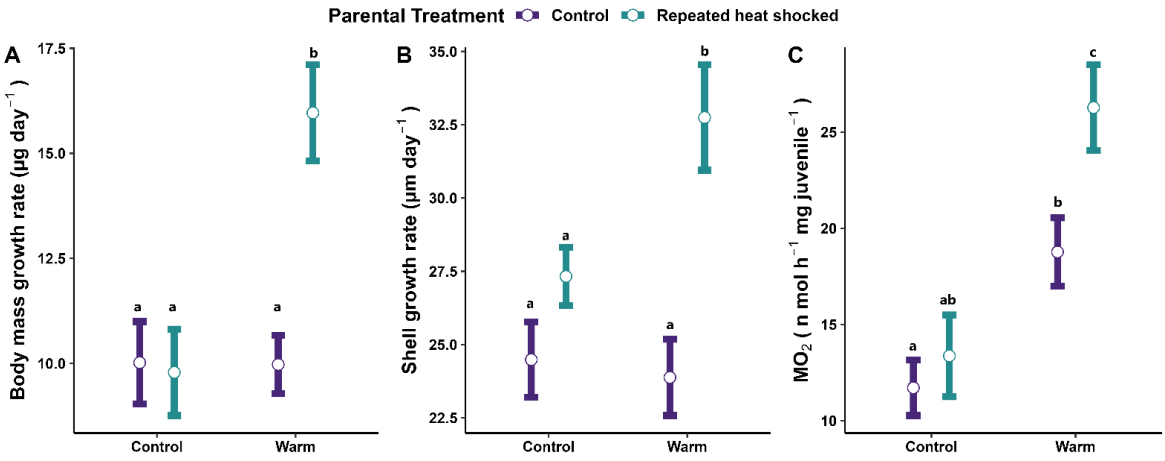


Figure 5.5. Effects of parental and developmental temperature on growth and metabolic rates of *H. tuberculata* juveniles. (A) Shell growth rates, (B) Body mass growth rates, and (C) Metabolic rates. Lowercase letters indicate significant post-hoc differences across treatments at $P < 0.05$ ($n = 27 – 35$ for (A), $n = 15 – 20$ for(B), while $n = 6$ for (C), per developmental treatment). Values are expressed as means ± 1 S.E.

5.5 Discussion

The aim of this study was to compare the effect of parental exposure to repeated heat shock with the effects of parental chronic warming, to investigate whether different thermal exposure regimes trigger different parental effects. My prediction that offspring performance would be differentially influenced by the thermal regime experienced by the parent was supported. Chronic warming had negative impacts on hatching success and larval performance and was associated with lower larval survival and deformations, irrespectively of offspring developmental temperature. In contrast, offspring from heat-hardened parents had similar performance to those of control parents when developed under control conditions and, importantly, this group had greater performance when developed under warm conditions. These beneficial effects of heat hardening were associated with metabolic adjustments that reduced the cost of development during larval stages and improved performance for juveniles, indicating adaptive parental effects.

The differences in egg size and albumin content observed here between eggs from parents exposed to different thermal regimes are indicative of differences in maternal provisioning in abalone (Huchette et al., 2004). Chronically warmed parents produced eggs with slightly greater albumin content (2 %) than control parents, despite those eggs being of similar size. This is consistent with observations in other species such as sea urchins (Chamorro et al., 2023). However, in contrast with previous studies, changes to albumin content were not associated with increased hatching success (Leicht & Seppälä, 2019). In contrast, heat-hardened parents produced smaller eggs compared to control parents, though maintained similar albumin content, indicating lower maternal investment as parents in the control group. Interestingly, their embryos exhibited comparable hatching rates to the control.

The negative effect of parental chronic warming on larval hatching success was exhibited by greater occurrence of larval deformation and mortality under both developmental treatments, which reduced the numbers of individuals achieving the post larvae stage. In contrast, early veliger larvae from heat-hardened parents demonstrated improved performance under both developmental treatments. Under control conditions, they had lower larval deformations, but similar survival rates, while under the warm treatment, they exhibited higher survival rates with similar deformations as the control group. Moreover, post larvae from heat-hardened parents showed higher survival rates under warm conditions compared to those from control parents. This contrasts with previous research showing that when the embryos of abalone develop under high temperatures they experienced high mortality (Kavousi et al., 2021; Pedroso, 2017). However, in my study the fact that chronically warmed parents resulted in negative parental effects on offspring performance suggests the duration and stability of heat exposure is critical for determining transgenerational effects. Similarly, Waite & Sorte, (2022) reported negative parental effects for the mussel *Mytilus californianus* collected from a natural thermal gradient, where warmed parents produced less thermally tolerant offspring, with these authors attributing their results to the trade-off between parental and offspring fitness. Conversely, my results show significant positive effects of parental heat hardening exposure on offspring physiological plasticity and overall performance. Differences in parental effects may be attributed to the different costs of parental thermal stress between the two thermal exposures, as hypothesised, which influenced the fitness trade-offs between parents and offspring.

Mechanistically, it is critical to understand what causes these differences in larval performance. Offspring size is influenced by the temperature-dependent nature of both MO_2 and developmental rate, factors which collectively shape the developmental costs incurred by individuals prior to becoming independent feeders (Marshall et al., 2020; Pettersen et al., 2019). Therefore, to enhance overall offspring fitness, it is crucial to maximize the resources available during the nutritionally dependent phase, by minimizing developmental costs (Pettersen et al., 2019). As a result, any parental and/or developmental conditions that alter the sensitivity of MO_2 and/or developmental rates to temperature will directly impact the overall fitness of the offspring. In this sense, at the

early veliger stage, larvae obtained from chronically warmed parents exhibited the highest MO_2, consequently, they incurred the highest developmental costs under both developmental treatments, which were higher than those incurred by larvae originating from the other two parental treatments. This led to larvae from the chronically warmed parental group having the lowest overall performance. A similar result was reported for *Octopus maya*, where thermally stressed females produced thermally stressed offspring, displaying smaller sizes, elevated MO_2, and increased mortality compared to offspring from non-stressed females (Domínguez-Castanedo et al., 2023). It was proposed that thermally stressed females activate epigenetic alterations that modify the energetic metabolism of embryos, linked to a breakdown in the antioxidant defence system and high oxidative damage. Moreover, higher mortality of gastropods embryos and larvae was associated with higher oxidative stress and lipid peroxidation when developed under elevated temperature (Deschaseaux et al., 2010; 2011). Therefore, as chronically warmed parents demonstrated higher maternal provisioning compared to the control group in this study, it is possible that the effects of metabolic maladaptation may be transferred from these parents to their offspring, resulting in higher developmental costs and thus diminished performance during early larval stages.

In contrast, in the case of heat-hardened parents, the early veliger larvae under both control and warm conditions exhibited a similar MO_2 and cost of development compared to those obtained from control parents. However, these larvae were smaller. This discrepancy could be attributed to the fact heat-hardened mothers produced smaller eggs. Interestingly, post larvae from heat-hardened parents managed to compensate for this reduced larval size under control conditions, with post larval size resembling that of control parents. Whilst when developed under warm conditions post larvae of heat-hardened parents achieved a larger size than the control group, with these three groups showing a similar MO_2 and cost of development. In contrast, the post larvae from control parents, when developed under warm conditions, exhibited a reduced size along with higher MO_2, increasing the developmental costs compared with larvae from heat-hardened parents. These results suggest the positive effects resulting from the parental heat hardening treatment as effective metabolic adjustments decreasing developmental costs, subsequently enhancing the overall offspring fitness. Supporting this assumption, previous

studies focusing on the impact of heat hardening on adult molluscs indicated metabolism remodelling, improvements in metabolic capacity, and bolstered antioxidant mechanisms (Georgoulis et al., 2021, 2022; Zhang et al., 2021; Zhang et al., 2023).

After 81 dpf, the effect of parental heat hardening was still obvious in abalone juveniles developed under warm conditions. When individuals are nutritionally independent, a higher MO_2 offers a greater potential for growth, provided sufficient energy availability, ultimately contributing to increased overall performance. In accordance, my results showed that juveniles of heat-hardened parents enhanced growth rates, accompanied by elevated MO_2. Similarly, in marine sticklebacks, thermally stressed parents produced larger offspring with higher MO_2 when raised under high temperatures. This was attributed to the possible inheritance of maternal epigenetic modifications that enabled thermal adaptation, as outlined by Shama et al., (2014). In contrast, juveniles from control parents, although showing compensation for warming conditions evidenced by comparable growth rates to their counterparts under control conditions, they exhibited lower MO_2 and consequently, slower growth rates compared to those from heat-hardened parents. This result could potentially be attributed to a selection process in warmed offspring from control parents which favoured slower growing (Jarrold et al., 2019), with lower MO_2 individuals (Pettersen et al., in press). These observations reveal that without a proper protocol to artificially acquire thermal plasticity, such as parental heat hardening, ocean warming may favour slower-growing individuals with a negative impact on molluscan aquaculture, where higher growth rate is a priority trait (Venter et al., 2018).

Whilst investigation of the direct effects of heat hardening on TGP are rare, compelling evidence supports the efficacy of induced parental thermal stress as a mechanism to enable offspring to withstand elevated temperatures across various taxa (Chamorro et al., 2023; Fellous et al., 2021; Ryu et al., 2018; Wang et al., 2023). Wang et al. (2023) observed that in the case of Pacific oysters, *Crassostrea gigas*, environmentally fluctuating intertidal parental conditions led to the induction of thermal TGP in two successive generations. In contrast, subtidal oysters exhibited distinct thermal responses in terms of fitness-related traits, gene expression profiles, and DNA methylation patterns. While our knowledge of best practice for inducing positive parental effects is still growing, my results demonstrate

a positive example of thermal TGP stemming from parental exposure to heat hardening, adding to this knowledge. This exposure leads to improved performance in both larval and juvenile stages under elevated temperatures, which resulted in advantageous growth rates.

5.6 Conclusions

My study demonstrates the importance of investigating different parental exposure treatments to determine the optimum conditions that induce TGP in marine ectotherms. Notably, my research unveiled a substantial enhancement in offspring performance under elevated temperatures via parental heat hardening, leading to improved growth and survival. These findings emphasize the potential advantages of employing this method as an efficient and feasible approach to bolster the thermal tolerance and overall performance of commercially and ecologically significant molluscs under the future environmental conditions. Indeed, this approach can be used for harnessing TGP to produce more thermally tolerant fast-growing seeds that will support conservation strategies and enhance production in commercial settings under changing conditions, ensuring the sustainability of aquatic ecosystems.

Chapter 6

General discussion and conclusions

6.1 Thesis summary

The central aim of my thesis was to examine the physiological plasticity of some key marine molluscs in response to ocean warming (OW) and/or ocean acidification (OA) across various life history stages. The central focus was on assessing trade-offs associated with this plasticity and investigating exposure approaches and response modifiers that could enhance my understanding of their capacity for acclimation within and across generations. In Chapter 2, I concentrated on long-term, ecologically relevant exposure to OA and OW, aiming to evaluate differences in metabolic plasticity, among three intertidal gastropods, and how this plasticity is affected by seasonal variations in exposure conditions. In Chapter 3, I shifted the focus to assessing the fitness costs associated with thermal acclimation, comparing scope for growth (SfG), survival, and reproductive outputs between two congener gastropods with different reproductive modes. In Chapter 4, I employed two distinct thermal exposure regimes to evaluate the effect of exposure nature on the condition index and thermal tolerance of adults of a commercially important gastropod. In Chapter 5, I explored the parental effects of the same thermal exposure regimes used in Chapter 4, examining their impact on offspring performance. This concluding chapter aims to highlight the key findings of my thesis and provide recommendations for future research directions, ending with thesis conclusions.

6.2 Metabolic plasticity under climate change

Temperature is a primary driver for physiological and life-history traits in ectotherms, influencing growth, reproduction, developmental rate, and recruitment. This makes it a key factor in determining the distribution and abundance of species in marine ecosystems (Cossins and Bowler 1987; Somero, 2005). Indeed, oxygen uptake rates and energy metabolism in ectotherms are highly influenced by temperature (Prosser 1991; Angilletta 2009). In the context of climate change, metabolic plasticity is fundamental for survival and

adaptation to changing environmental conditions (Calosi et al., 2017; Leung et al., 2020; Leung & Connell, 2017), and thus assessing this plasticity becomes increasingly important to understand species susceptibility to climatic stressors. To characterize metabolic plasticity, I utilized an ecologically relevant exposure approach in Chapter 2, which applied different CO_2 levels while temperature, photoperiod, and tidal cycle were adjusted monthly to follow a seasonal cycle of local conditions under the two temperatures regimes (ambient and ambient + 4 °C). I identified differences in metabolic rate (MO_2) between the three studied species. These differences were influenced by the type of stressor, length of exposure, and seasonality in exposure conditions. In isolation, ocean warming (OW) exhibited no effect on *N. lapillus*, irrespective of season, but increased MO_2 in *L. littorea* and *O. lineatus* under summer conditions. Meanwhile, ocean acidification (OA) led to lower MO_2 in *N. lapillus* and *L. littorea* under winter conditions, but had no effect on MO_2 under summer conditions. Intertidal ectotherms showed similar changes in MO_2 under OW and/or OA (Widdicombe and Spicer, 2008; Small et al. 2010; Vosloo & Vosloo, 2010; Melatunan et al., 2011; Kordas et al., 2022). Significantly, my study demonstrated that the interactive effects of OW and OA were only observed under summer conditions after 6 months of exposure. Interestingly, *N. lapillus* showed no interaction and *L. littorea* showed an antagonistic effect indicating good adaptive responses. In contrast, *O. lineatus* showed synergistic effects. My study highlights the significance of taking into account potential response modifiers, in this case the interaction between multiple stressors, an extended duration of exposure, and the consideration of seasonality in the exposure context. While length of exposure and seasonal changes in exposure conditions have been reported to influence the interactive ecological responses to OW and OA in marine ectotherms (Godbold & Solan, 2013), this study is the first to emphasize the effect of seasonal dynamics of a set of multiple exposure parameters on physiological responses to multiple climatic stressors. Based on these results, I argue that such temporal dynamics should be considered in experimental designs, as interactive effects between stressors may only unfold under specific conditions. Furthermore, my findings do not support a unifying metabolic response to multiple stressors, and instead suggest that species-specific responses can greatly complicate any general conclusions. This confirms previously reported conclusions when reviewing metabolic responses to OW and OA in marine ectotherms (Lefevre, 2016). Results of my study call for caution when making conclusions

about species resilience or susceptibility to climatic stressors in the wild, scaling it up to ecosystem levels, based on short-term low-ecologically relevant exposures. However, as experimental setups like that used in my study may be logistically difficult and expensive to establish in many laboratories, efforts to apply such a comprehensive approach could be directed to the most endangered, vulnerable species to help with their conservation and sustainability plans.

6.3 The cost of physiological acclimation is dependent on reproduction mode

Physiological plasticity is vital for animals under environmental stress, allowing them to respond immediately to maintain performance and enhance survival. Understanding the cost of such plasticity is crucial to evaluate its long-term impact on ecological success. This is because these physiological adjustments can lead to trade-offs between different fitness components. For instance, survival, growth, and reproduction may be affected due to energy reallocation (Donelson et al., 2010; Kühnhold et al., 2017, 2019; Sokolova et al., 2012; Petes et al., 2007). Therefore, a comprehensive understanding of these trade-offs becomes essential to fully grasp the capacity and implications of acclimation to climatic stressors. In Chapter 3, I characterized the cost of physiological plasticity in terms of fitness components under thermal acclimation in two intertidal gastropods with distinct reproductive modes. My study demonstrated that a trade-off exists between physiological adjustments, reproduction, and survival during thermal acclimation, which was dependent on the magnitude of the temperature change and the reproductive mode of the species. The oviparous *L. littorea* favoured reallocating energy towards survival, even if it resulted in reduced reproductive output. Conversely, the ovoviviparous *L. saxatilis* prioritized reproduction, even at the expense of long-term survival.

Numerous studies speculate that climate change will affect the fitness of marine organisms, potentially leading to detrimental effects on populations and ecosystems. However, these predictions often rely on short-term, indirect measures of physiological performance as proxies for fitness. In contrast, my study stands out as one of the few that directly measured multiple fitness components associated with physiological adjustments under thermal stress. Moreover, it is the first to investigate the effects of

reproductive mode on the trade-offs among these fitness components. The findings from my study reveal significant variability in the costs of physiological plasticity, influenced not only by the magnitude of thermal stress but also by the inherent biological characteristics of the species. Notably, different reproductive modes resulted in distinct strategies for coping with elevated temperatures. This underlies the importance of considering species-specific attributes in understanding the implications of climate-induced physiological adjustments on overall fitness. While *L. littorea* exhibits high acclimation potential, the cessation of reproduction raises concerns about the long-term advantages of this acclimation. Some previous work on marine ectotherms reported the potential for acclimation of reproduction under climatic stressors when longer durations were applied (Suckling et al., 2015) or when the rate of warming was gradually increased through generations (Donelson et al., 2016). Further work is required to understand whether *L. littorea* can compensate for reproduction over a longer exposure time period. On the other hand, *L. saxatilis*, despite its lower survival and limited acclimation capacity, may ensure ongoing recruitment and prevent long-term population extinction through the preservation of reproduction, assuming offspring survival. Here, the potential for developmental plasticity may play a crucial role in population persistence (Burggren, 2018). The observed diversity in fitness consequences among species with distinct reproductive modes under thermal stress needs to be taken into account when predicting the effects on long-term ecological change, as it is likely to affect our ability to identify winners and losers.

6.4 Parental thermal exposure influences thermal performance of parents and offspring

The cost of physiological plasticity in terms of fitness components such as reproduction can have consequences that extend to offspring, potentially lowering their fitness. While parental effects can enhance offspring plasticity (Parker et al., 2012; Spencer et al., 2020; Wang et al., 2023), the trade-off between parent and offspring fitness could result in negative parental effects in marine ectotherms (Burgess & Marshall, 2014; Waite & Sorte, 2022). Using short-term parental exposure to climatic stressors, which does not provide enough time for parental acclimation, could result in negative parental effects, compared with longer exposure durations (Suckling et al., 2014, 2015; Wang et al., 2015). To explore

the full potential of parental effects on plasticity within and across generations, I used two long-term thermal regimes which vary in their nature, a repeated heat shock exposure and a long-term chronic warming. These regimes were applied over a period of 6 months, in an attempt to reduce or prevent the trade-offs between parent and offspring fitness, by allowing enough time to facilitate the enhancement of adult fitness and positive parental effects in the commercial gastropod, *Haliotis tuberculata.* In Chapter 4, I assessed adult thermal tolerance traits and condition index, and, in Chapter 5, I evaluated the thermal performance of their offspring during development under control and warm conditions. This approach provided a more comprehensive understanding of the responses of marine molluscs to thermal stress within and across generations.

In *H. tuberculata* adults, the repeated heat shock exposure resulted in a higher condition index compared to both the control and chronic warming treatments. Furthermore, both chronic warming and repeated heat shock exposures led to an increase in upper thermal limits compared to control, with repeated heat shock exposure resulting in heat hardening in abalone. The upper thermal limits were similar in heat-hardened animals and those acclimated to chronic warming. However, other performance measures, such as thermal safety margin and thermal breadth, were different in animals exposed to the two different thermal regimes. Heat hardening seemed to be more advantageous in terms of adult fitness and thermal performance, as indicated by increasing both condition index and upper thermal limits. This result emphasizes the need to consider the nature of the thermal regime experienced by an organism in its natural environment for accurate predictions of its capacity to acclimate to change and anticipate future performance. This recognition has gained prominence in recent years, as highlighted by Morash et al. (2018). In my study, the application of repeated heat shocks followed by a period of lower temperatures, allowing for recovery, represents an attempt to mimic the way abalones experience thermal stress in their natural habitats. This approach acknowledges that many intertidal ectotherms do not encounter static temperatures; instead, they contend with dynamic, fluctuating, and unpredictable environmental conditions (Dong et al., 2021; Leeuwis & Gamperl, 2022; Collins et al., 2023). In addition to uncovering the advantageous effects of heat hardening on thermal tolerance, my study also demonstrated positive impacts on the overall fitness. This finding holds particular significance for commercial species, highlighting the potential

of heat hardening as a stress conditioning approach to enhance performance of such organisms. I then explored whether these differential physiological adjustments in adult fitness, as a result of the differences in thermal regime, could lead to varying parental effects.

In Chapter 5, chronic warming of parents resulted in slightly larger eggs with increased albumin content, while heat-hardened parents produced smaller eggs with similar albumin content compared with parents at control. Unexpectedly, embryos from chronically warmed parents exhibited lower hatching rates, while those from heat-hardened parents exhibited comparable hatching rates to the control group. Furthermore, chronically warmed parents produced offspring with lower performance and maladaptive developmental costs, which resulted in high mortality before reaching post larvae stage irrespective to developmental temperature compared to other parental treatments. In contrast, early veliger larvae from heat-hardened parents showed improved performance under both developmental conditions with adaptive developmental costs. Post larvae and juveniles from heat-hardened parents demonstrated higher performance under warm developmental conditions compared to those from control parents. My study underscores the significance of the nature of parental thermal exposure as a critical factor in determining the influence of parental effects on offspring performance. Integrated with other studies, such as Waite & Sorte (2022), my study further emphasizes that the status of trade-offs between parental and offspring fitness should be rigorously tested to properly evaluate the TGP responses in marine ectotherms. Despite chronically warmed adults managing to maintain a condition index similar to the control treatment, while simultaneously increasing thermal tolerance, it appears that this enhancement of parental fitness exerted a negative effect on offspring fitness. In contrast, the better physiological state of heat-hardened parents resulted in better performance of their offspring. These results further confirm the importance of considering ecological realism in laboratory exposures when exploring environmentally induced plasticity. Moreover, this study underscores the substantial benefits of heat hardening, as a stress conditioning approach, in significantly improving adult thermal tolerances and offspring performance under elevated temperatures, resulting in enhanced growth and survival. I therefore propose heat hardening as a method to boost the thermal tolerance and overall performance of

marine molluscs within and across generations of key mollusc species. When applied to broodstock conditioning, heat hardening becomes a valuable, relatively inexpensive tool in restoration and sustainable food production in a rapidly changing climate.

6.5 Future research directions

The findings from my thesis have provided valuable insights into the physiological acclimation capacity of marine molluscs, highlighting the potential for adaptive within-generation responses and parental transgenerational effects. However, our understanding of the enduring nature of such plasticity across generations remains limited. To address this gap in knowledge, future research should focus on better understanding the persistence of these responses. Exploring the underlying molecular and epigenetic mechanisms that facilitate TGP will contribute to a more comprehensive understanding of the adaptive potential of marine molluscs in the face of environmental change.

6.5.1 Inheritance of parental effects

The role of parental effects in adaptation to environmental change, particularly whether they involve permanent changes, has been a subject of debate (Hoppeler, 2015). Epigenetic inheritance, which is suggested to occur predominantly through DNA methylation, histone modification, and non-coding RNAs, is increasingly recognized as a key mechanism for phenotypic plasticity inheritance across various taxa, following environmental stress exposure (Clarke & Vieux, 2015; Eirin-Lopez & Putnam, 2019; Vandegehuchte & Janssen, 2014). In marine molluscs, DNA methylation is the most studied epigenetic carrier of environmental memory within a single generation (Gavery & Roberts, 2010; Song et al., 2017; Sun et al., 2014; Wang et al., 2023). However, they exhibit lower global cytosine methylation variation (5 to 15 % of CpG) compared with fish species (80 % of CpG being methylated) (Metzger and Schulte, 2016; Fallet et al., 2020). Adrian-Kalchhauser et al. (2020) proposed that epigenetic marks may not necessarily be inherited by the same mechanism or fixed on a specific gene locus. Instead, they may occur through inherited gene regulation machinery with normal wax and wash mechanisms, a phenomenon that may be used to argue against epigenetic inheritance potentiality

(Burggren, 2015). This suggests that physiologists could adopt broader scope methods, such as RNA sequencing, to study TGP responses in non-model species (Adrian-Kalchhauser et al., 2020). This approach will be helpful, especially in non-model species, with low tendency to exhibit variation in genome methylation. This could potentially contribute to the diversification of animal models utilized in research, as alternative models might be more apt for addressing our biological inquiries (Burggren, 2021; Krogh, 1929). This approach, when integrated with measurements of whole-organism and cellular responses as well as epigenetic markers, could help to understand the observed parental effects and better evaluate the potential for its inheritance as permanent effects in marine molluscs. Indeed, the relationship between epigenetic markers and transcriptomic responses to climatic stressors is still unclear in marine molluscs (Fallet et al., 2020), especially in the context of TGP. Therefore, the interplay between epigenetic markers and the transcriptomic profiles that may underline TGP responses of marine molluscs is an open question, necessitating further research. Understanding this approach will be a first step in establishing alternative or complementary approaches to genetic selection programs, such as epigenetic selection approach, to enhance aquaculture sustainability and secure future production, especially in the context of climate change (Eirin-Lopez and Putnam, 2019).

6.5.2 Epigenetic selection in aquaculture

Brood-stock conditioning is an important approach to explore environmentally induced epigenetic modifications and their potential transmission from parents to offspring. Recent interest in this area has grown, particularly in aquatic animals like fish and shellfish, focusing on transgenerational epigenetic inheritance and its potential role on environmentally induced changes to fitness (Munday, 2014; Norouzitallab et al., 2014; Roy et al., 2019, 2020; Wang et al., 2023). Despite the nascent state of epigenetic inheritance studies in marine animals, some research has linked epigenetic modifications to physiologically and commercially significant traits. Notably, studies involving environmental manipulation for brood-stock conditioning in Manila clams and Sydney rock oysters, exposing them to ocean acidification, resulted in offspring with enhanced growth and fitness (Zhao et al., 2018; Parker et al., 2012). These findings, alongside the results presented in this thesis (Chapter 5), suggest that environmentally hardening brood-stock

holds the potential to improve the performance of subsequent generations under environmental stressors. Given the ability of animals to exhibit environmentally induced phenotypic plasticity, where genomic information alone cannot account for all phenotypic variation, the role of epigenetic variation associated with phenotypic plasticity becomes important in selecting individuals based on desired traits (Burggren, 2014; Gavery and Roberts, 2017; Roy et al., 2019). Therefore, incorporating epigenetic markers into selection programs for brood-stock in aquaculture seems plausible. A study in Pacific oysters *Crassostrea gigas*, investigated the correlation between genetic and epigenetic variation in different populations of mass selection line, revealing some correlation but also indicating distinct epigenetic profiles in individuals with similar genetic backgrounds, suggesting that epigenetic variation may be partly dependent on the genetic context in *C. gigas* (Jiang et al., 2013). These results imply a potential role for epigenetic variation in selective breeding under captivity. Notably, in Nile tilapia *Oreochromis niloticus*, an epigenetic regulation after just one generation of domestication was reported (Konstantinidis et al., 2020), with sex-specific epigenetic regulation of growth observed during early stages of domestication (Podgorniak et al., 2019). Additionally, a genome-wide methylation profile study revealed significant epigenetic variation in hatchery-reared Pacific salmon compared to their wild counterparts, suggesting that epigenetic modifications induced by hatchery conditions may be used to trace the fitness of hatchery-reared salmon when released in the wild (Le Luyer et al., 2017).

In summary, there is potential for environmentally hardening brood-stock in marine ectotherms, for improving the performance of subsequent generations under environmental stressors. Moreover, the incorporation of epigenetic markers in selection programs could offer a promising avenue, with potential role of epigenetic variation in selective breeding under captivity in commercial settings. This will not only help in securing food production from aquaculture species but also could be beneficial in conservation efforts under predicted climate change scenarios, putting phenotypic plasticity into practice (Donelson et al., 2023).

6.6 Thesis conclusions

In the introduction section, I highlighted the significance of phenotypic plasticity as an immediate response strategy to environmental change, providing animals with the potential to maintain performance within and across generations amidst climatic stressors. Addressing the limitations of short-term studies with low ecological relevance, my thesis focused on often-overlooked factors such as context variations, the nature of exposure, and the fitness costs of plasticity. Firstly, the findings revealed that there were differential species-specific effects of OA and OW on metabolic rates in isolation and when combined, influenced by the seasonal dynamics of exposure conditions, with interactive effects observed only over long-term exposures under summer conditions. While some gastropods demonstrated adaptive metabolic plasticity and acclimation, others were severely impacted. The study emphasized the importance of an ecologically relevant approach with long-term exposure durations for better predictions of animal responses to climatic stressors in the wild. Secondly, although some gastropods exhibited the ability to physiologically acclimate, this capacity was species-specific and constrained by trade-offs due to differential energy supply for various biological processes under stress. Notably, a trade-off was observed between fitness components (SfG, survival, and reproductive output) during thermal acclimation, where the pattern of this trade-off was dependent, not only on the magnitude of stress, but also on the reproductive mode of the tested gastropods. Finally, this thesis highlighted the significance of considering the potential trade-offs between parent and offspring fitness as a potential source of discrepancies in the outcomes of parental effects, highlighting the role of exposure nature. Additionally, it underscored the beneficial effects of parental heat hardening on enhancing thermal tolerances in adults and improving thermal performance in offspring under thermal stress. I hope that this thesis contributes to a better understanding of the capacity for physiological acclimation within and across generations in marine molluscs, highlighting the effect of response modifiers, providing insights for broader inquiries into the capacities and potential inheritance of physiological acclimation in marine animals. This understanding is crucial for predicting the impacts of climate change on biodiversity and ecosystem function, guiding the development of effective management, conservation, and sustainability strategies.

Appendix

Supplementary figures

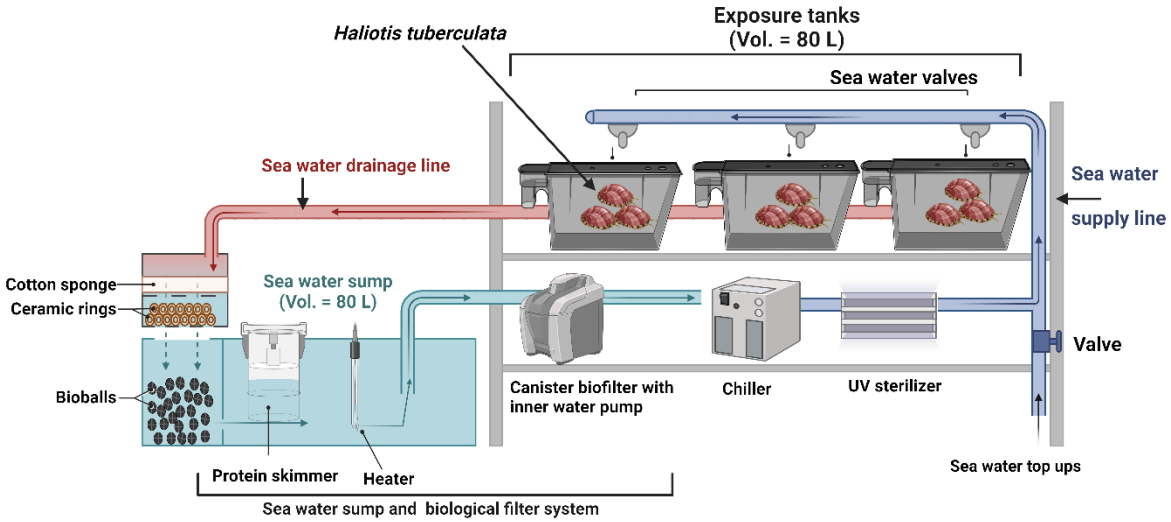


Figure S.1. Experimental setup of a recirculating *Haliotis tuberculata* husbandry unit, with three units used in the experiment (one per treatment). Each unit consisted of three replicate aquaria (Vol. = 60 L, n = 20 individuals per aquarium per species which were housed in separate Perspex containers, each double-sided with perforated mesh), sump (Vol. = 80 L), equipped with a biological filter, protein skimmer, and heater. Additionally, a canister biofilter with an inner water pump, chiller, and UV sterilizer were part of the setup. Arrows indicate the flow direction of seawater in the experimental unit.

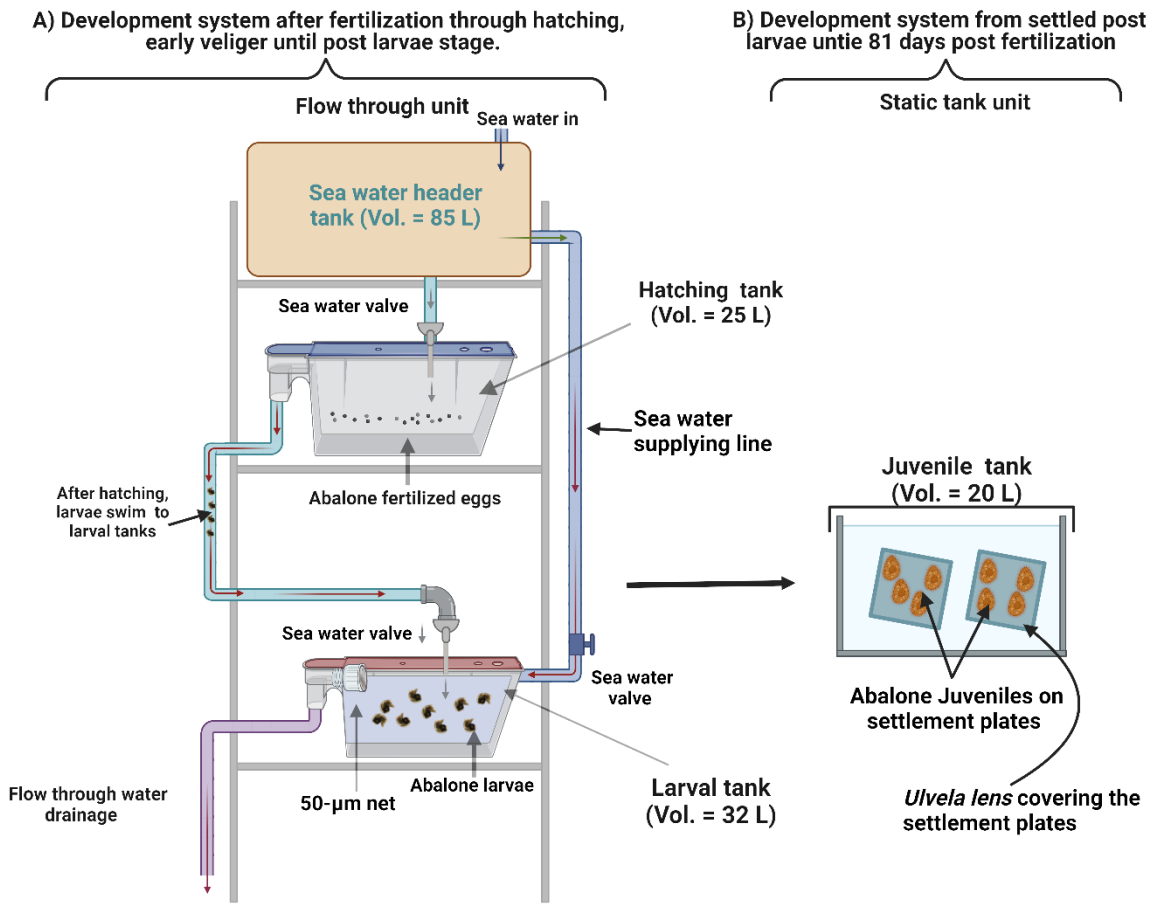


Figure S.2. Experimental setup for *Haliotis tuberculata* larval rearing, with each unit used for one larval replicate (n = 2 (larval treatment) x 3 (parental treatment) = 6). (A) The unit comprises a header tank (Vol. = 85 L), a hatching tank (Vol. = 25 L), an overflow evacuation pipe (facilitating automatic larval transfer to the larval tank), and a larval tank (Vol. = 32 L) equipped with a 50-µm net at the outflow to prevent larval escape. Fertilized eggs are transferred to this unit and allowed to develop under the respective temperature conditions until the post-larvae stage. (B) At 58 day-degrees, some of the still swimming larvae are permitted to settle on settlement plates in a static tank (Vol. = 20 L), where they continue developing under the same temperature until 81 days post-fertilization.

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