

**The functional response of the Antarctic bivalve
Laternula elliptica to ocean warming and
acidification**

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Abstract

"It is a curious situation that the sea, from which life first arose, should now be threatened by the activities of one form of that life. But the sea, though changed in a sinister way, will continue to exist; the threat is rather to life itself."

– Rachel Carson

Marine life is currently under threat from large-scale, long-term changes to the marine environment. Anthropogenic emissions of greenhouse gases, particularly carbon dioxide (CO₂), are causing ongoing change to global marine systems, particularly through ocean warming and acidification. Greenhouse gases in the atmosphere are trapping radiation and heating the entire Earth surface, including the ocean. At the same time, oceanic uptake of CO₂ through absorption by surface waters is altering ocean chemistry, increasing acidity, reducing availability of carbonate ions (CO₃²⁻), and causing increasing dissolution of calcium carbonate (CaCO₃) structures.

Because atmospheric CO₂ diffuses more readily into cold water, the Southern Ocean (SO) will experience ocean acidification in a matter of decades. Warming in the SO is also occurring rapidly and represents a comparatively greater increase in temperature than elsewhere. SO marine fauna have evolved in constant, stable, cold conditions, and as a result are stenothermal and particularly at risk from ocean warming and acidification. The large infaunal bivalve *Laternula elliptica* is a prevalent keystone species found throughout the Antarctic benthos in high numbers, and contributes significantly to biodeposition and benthic-pelagic coupling.

This thesis examines how *L. elliptica* adults are affected over medium-term (5-mo) timescales by SO warming and acidification. Adult *L. elliptica* collected from Cape Evans in McMurdo Sound, Antarctica, were subjected to combinations of temperatures and pHs predicted for the SO by 2050 and 2100 (Temperatures: -1.4°C (control); -0.5°C; +0.5°C. pHs: pH 8.00 (control); pH 7.85; pH 7.65). *L. elliptica* were assessed at 5 wk and 5 mo to determine their cellular, metabolic, and whole-

organism responses to temperature increase and/or pH decrease. Survival parameters such as final survival percentage, survival curves, and time to 50% survival (LD₅₀) were compared among treatments. *L. elliptica* survival was severely reduced by warming of only 1-2°C above summer ambient temperatures. Physical and physiological condition indices were calculated to assess health, and show changes in shell and body tissue mass. Physical condition stayed similar amongst all treatments at both time points, while physiological condition decreased significantly at 5 mo with elevated temperature. Oxygen (O₂) consumption was measured as a proxy for standard metabolic rate to show whether animals had acclimated to conditions. O₂ consumption was significantly negatively correlated with physiological condition, and increased, becoming more variable, with both elevated temperature and lowered pH. This indicated that *L. elliptica* experienced increased metabolic demand in response to these conditions, and there was a general lack of acclimation to these conditions over time. Overall, pH had no significant effect on survival, metabolic rate, or condition. Heat shock protein 70 (HSP70) gene expression levels were measured to provide a preliminary indication of how the heat shock response of *L. elliptica* responds to both elevated temperature and reduced pH. Lowered pH appeared to stimulate an up-regulation of HSP70 gene expression at both time points, although this was smaller at 5 mo. *L. elliptica* did not seem to display a heat shock response at environmentally realistic levels of warming.

Overall, warming resulted in lowered survival and condition loss with no sign of acclimation after 5 mo. These responses occurred at smaller degrees of warming than are typically considered lethal for *L. elliptica*, indicating that successful longer-term maintenance is more thermally limited than short-term survival in this species. While physical (shell) condition was maintained in undersaturated conditions under both elevated temperature and reduced pH, this maintenance occurred alongside increased O₂ demand. Maintaining the aragonitic shell in combination with increased metabolic activity may have contributed to the decline in physiological (body mass) condition observed in *L. elliptica*. In combination, the results of this experiment indicate that warming of the SO may be more important than ocean acidification to the survival and functioning of adult *L. elliptica*.

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List of Abbreviations

Ω	Carbonate saturation state	H^+	Hydrogen ion
		HCO_3^-	Bicarbonate ion
Ω_{Ar}	Aragonite saturation state	H_2CO_3	Carbonic acid
		$HgCl_2$	Mercuric chloride
Ω_{Ca}	Calcite saturation state	HSP	Heat shock protein
AFDW	Ash-free dry weight	HSP70	70 kDa HSP
AIC	Akaike Information Criterion	LD₅₀	Time to 50% survival
		mo	Month(s)
ANOVA	Analysis of variance	mRNA	Messenger RNA
A_t	Total alkalinity	NIWA	National Institute of Water & Atmospheric Research
$CaCO_3$	Calcium carbonate		
cDNA	Complementary DNA		
CI	Condition index	O_2	Oxygen
$CI_{FW:SL}$	Ratio of dried flesh weight to shell length	OA	Ocean acidification
		pCO_2	Partial pressure of CO_2
$CI_{FW:SW}$	Ratio of dried flesh weight to dried shell weight	qPCR	Quantitative polymerase chain reaction
		RT-qPCR	Reverse transcription qPCR
$CI_{SW:SL}$	Ratio of dried shell weight to shell length	SE	Standard error
CO_2	Carbon dioxide	SL	Shell length
CO_3^{2-}	Carbonate ion	SO	Southern Ocean
C_t	Cycle threshold	SW	Dried shell weight
d	Day(s)	VUW	Victoria University of Wellington
DIC	Dissolved inorganic carbon		
		wk	Week(s)
FW	Dried flesh weight		

Chapter 1

Introduction

1.1 – Introduction

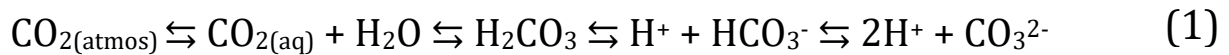
1.1.1 – Ocean warming and acidification

Anthropogenic greenhouse gases have been accumulating in the atmosphere since the Industrial Revolution. This is predominantly from anthropogenic carbon dioxide (CO₂) emissions from fossil fuel use and deforestation, though cement production also plays a role (Doney *et al.*, 2009; IPCC, 2013; Parker *et al.*, 2013). Increasing greenhouse gas concentrations in the atmosphere are contributing to global temperature increase and sea level rise (IPCC, 2013). Surface warming of both land and oceans has occurred since the Industrial Revolution, and is predicted to continue at a rapid pace unless rates of anthropogenic carbon emissions are reduced (Royal Society, 2005; IPCC, 2013). The oceans have absorbed a large proportion of the increased energy of the climate system in recent years, with ~70% absorbed in the upper ocean (0-700 m) and ~30% stored in the deeper ocean (> 700 m) (IPCC, 2013; Parker *et al.*, 2013). This has manifested in ocean warming of 0.11°C per decade over at least the past four decades, and likely longer, particularly in the upper 75 m (IPCC, 2013).

Atmospheric [CO₂] is not increasing at the same rate as anthropogenic outputs. Between 25 and 40% of atmospheric CO₂ emitted by humans is absorbed by the ocean and removed from the atmosphere (Feely *et al.*, 2004; Royal Society, 2005; Clark *et al.*, 2009). Consequently atmospheric [CO₂] levels are lower than they would be without oceanic uptake, and radiative forcing is somewhat reduced. Over time, if atmospheric [CO₂] continues to increase, the oceans will become saturated with CO₂, reducing their absorption potential. This will affect future rates of climate change (Royal Society, 2005; Guinotte & Fabry, 2008; Ingels *et al.*, 2012; IPCC, 2013). The CO₂ absorbed into the ocean modifies its chemistry, such as pH and the availability of ions. This process, known as ‘ocean acidification’ (OA), was previously not well understood and is regarded

as the secondary effect of CO₂ emissions. In fact, the effects of OA occur in addition to, and may exacerbate, the other effects of climate change, including ocean warming (Royal Society, 2005). OA and its implications have been the focus of dedicated research efforts over the last decade.

The natural oceanic carbonate buffering system is a multi-stage equilibrium reaction. When atmospheric CO₂ diffuses into the ocean across the air/sea interface, it reacts with water to form carbonic acid (H₂CO₃), which being a weak acid disassociates readily into water. This disassociation uses up free carbonate ions (CO₃²⁻), and produces hydrogen ions (H⁺) and bicarbonate ions (HCO₃⁻) (Royal Society, 2005; Doney *et al.*, 2009), as in the following equation (Doney *et al.*, 2009):



It is this increase in [H⁺] ions that reduces ocean pH and gives OA its name. Over longer timescales, the pH of the oceans is maintained by this buffering system. However, the rapid addition of CO₂ over short timescales from anthropogenic outputs is resulting in a [CO₂] increase more rapid than ocean buffering can neutralise (Caldeira & Wickett, 2003; Royal Society, 2005; Guinotte & Fabry, 2008; Hauck *et al.*, 2013). The rates of [H⁺] increase and [CO₃²⁻] decrease are relatively rapid, and this is changing ocean conditions at a correspondingly rapid rate. Oceanic pH has already decreased by 0.1 pH units in the 250 y since the Industrial Revolution. This corresponds to a 30% increase in [H⁺], representing a significant alteration in ocean chemistry (Caldeira & Wickett, 2003). Ocean pH is predicted to drop an additional 0.3-0.4 units by 2100, with an according 50% decrease in [CO₃²⁻] in the surface ocean (Orr *et al.*, 2005; Ries *et al.*, 2009; IPCC, 2013). This equates to a ~100-150% increase in [H⁺] since the Industrial Revolution (Orr *et al.*, 2005).

Ocean pH has not been more than 0.6 units below current levels in the last 300 M y, and current CO₂ levels have not occurred for the past 15 M y (Caldeira & Wickett, 2003; Wicks & Roberts, 2012; Hauck *et al.*, 2013). The conditions currently faced by marine organisms are changing at a pace that far outstrips previous climatic shifts. While there have been periods of warming and OA over the Earth's history, current and predicted

21st-century increases in atmospheric [CO₂], and associated ocean warming and acidification, are occurring at an unprecedented rate, particularly in the Western Antarctic Peninsula region (Meredith & King, 2005; Convey *et al.*, 2009; Crim *et al.*, 2011).

The combination of ocean warming and acidification presents a threat to the continued structure and function of marine ecosystems, particularly that of calcifying organisms. The construction of calcium carbonate (CaCO₃) structures is integral to the form and function of a wide range of marine taxa, which play an important role in primary production, food webs, habitat structure, and carbon cycling. Bivalves in particular, with carbonate shells, provide ecologically important sources of food, water purification, and habitat complexity over a wide range of latitudes and habitats (Wicks & Roberts, 2012; Gazeau *et al.*, 2013). Major calcifiers - calcareous algae, corals, coccolithophores, echinoderms, crustaceans, and molluscs - each display varying degrees of sensitivity to ocean acidification (Kroeker *et al.*, 2013).

Calcification relies on suitable [CO₃²⁻] and the maintenance of the pH of internal spaces. Both of these factors are altered by OA. In the process of forming CaCO₃ from aqueous HCO₃⁻, calcification releases one molecule of CO₂ for every molecule of CaCO₃ created (Royal Society, 2005). Consequently, calcification increases oceanic [CO₂], exacerbating OA, and this CO₂ could be released into the atmosphere if mixing brings it to the air/sea interface (Royal Society, 2005). This rate may increase as ocean [CO₂] increases over time (Royal Society, 2005). If calcifiers experience decreased survival or calcification as a result of ocean chemistry changes, the global carbon cycle will also be impacted, as dissolution and calcification of biological structures contribute to the carbon balance of the oceans (Royal Society, 2005; Wicks & Roberts, 2012).

Calcification in bivalves is a process under tight biological control whereby the ionic balance and pH of an internal chamber is maintained by passive and active transport of ions across cell membranes (Roleda *et al.*, 2012). Biomineralisation can only occur under specific conditions, and maintaining these becomes increasingly metabolically stressful and energetically costly as [CO₃²⁻] availability declines, or when internal pH drops (hypercapnia) (Fabry *et al.*, 2008; Doney *et al.*, 2009; Kroeker *et al.*, 2010; Wicks

& Roberts, 2012). Alternatively, respired CO_2 may be used by bivalves in biomineralisation after its conversion to HCO_3^- by carbonic anhydrase (Roleda *et al.*, 2012). Thus, it is the increased dissolution of shells in undersaturated conditions, not the external availability of CO_3^{2-} , that is the primary concern for bivalves under OA (Roleda *et al.*, 2012). Resistance to hypercapnia is dependent to a large degree on the acid-base regulatory capacity of the organism, often dictated by its mode of life (e.g., active vs. passive) (Wicks & Roberts, 2012). Bivalves in particular are affected by hypercapnia across a range of life stages (Barnes & Peck, 2008; Gazeau *et al.*, 2013; Matoo *et al.*, 2013; Parker *et al.*, 2013). They have a poor capacity to regulate their ionic and acid-base status, and are less able to compensate for changes in extracellular pH and ion balance than more active or higher-level organisms (Pörtner *et al.*, 2004; Pörtner, 2008; Jakubowska & Normant, 2015).

Calcium carbonate structures can take several forms depending on the polymorphs of CaCO_3 used in their construction. In order of increasing solubility these are: low magnesium calcite, aragonite, and magnesium-rich (high-magnesium) calcite (Barnes & Peck, 2008; Gazeau *et al.*, 2013; Hauck *et al.*, 2013). Bivalve shells can be solely aragonitic or calcitic, or may have layers of aragonite and calcite (Royal Society, 2005; Barnes & Peck, 2008; Gazeau *et al.*, 2013; Hauck *et al.*, 2013). Calcification occurs more easily above the calcium carbonate saturation horizon, the depth where the carbonate saturation state (Ω) > 1, and once formed, CaCO_3 does not dissolve above this horizon. Different saturation horizons exist, with different depths for aragonite and calcite. As aragonite is more soluble, its saturation horizon depth (Ω_{Ar}) is consistently shallower than that of calcite (Ω_{Ca}) (Royal Society, 2005; Wicks & Roberts, 2012).

The aragonite saturation state is a useful proxy for the ease of biomineralisation in aragonitic calcifiers (Miller *et al.*, 2009). Once aragonite is undersaturated ($\Omega_{\text{Ar}} < 1$), its scarcity means it is more energetically costly to remove it from seawater, transport across cellular membranes, and utilise (Barnes & Peck, 2008; Wicks & Roberts, 2012). Alternatively, it is the enhanced dissolution of shells that is the most costly to compensate (Roleda *et al.*, 2012). Aragonite undersaturation does not automatically lead to an inability to calcify, but rather increases the cellular costs of doing so, and the associated stress for the organism (Miller *et al.*, 2009; Gazeau *et al.*, 2013).

Subsequently, the energetic costs of calcification and the depth restrictions this places on calcifying taxa in the future depends on the form of CaCO_3 they secrete. Decreased pH may lead to reduced calcification rates in a range of species (Clark *et al.*, 2009). Shifts in species composition could occur, with a loss of species unable to calcify under reduced pH conditions and an increase in dominance of species able to calcify, or which do not calcify at all (Clark *et al.*, 2009). Generally, weaker skeletons, decreased growth rates, and increased metabolic demand are expected (Clark *et al.*, 2009).

When exposed to reduced pH, many species exhibit physiological trade-offs in responses, reducing calcification and reproductive output (Kroeker *et al.*, 2010, 2013; Wicks & Roberts, 2012). Subtle effects of depressed reproduction have potentially serious long-term consequences (Wicks & Roberts, 2012; Guy *et al.*, 2014), though if adults can acclimate to conditions then their larvae may have some adaptive advantage (Parker *et al.*, 2011; Suckling *et al.*, 2015). Ocean warming increases trade-offs in energy use in order to maintain basic metabolic functions such as calcification and basal metabolic rate, and if environmental temperatures rise outside a species' thermal tolerance window, reproductive output, growth, and behaviour can all be negatively affected (Clarke, 1987; Pörtner, 2008; Alcaraz *et al.*, 2014).

1.1.2 – Southern Ocean warming and acidification

Warming is of particular concern in the Southern Ocean (SO). The SO comprises all ocean south of 60°S, beyond the Polar Front (Barnes & Peck, 2008; Lenton *et al.*, 2009). It covers 360,000 km² and represents ~8% of the global oceans, as well as ~11% of global continental shelf area (Peck, 2005b; Linse *et al.*, 2006). SO water temperatures have been stable and cold for millennia (Barnes & Conlan, 2007; Barnes & Peck, 2008) and areas of the SO, particularly the Western Antarctic Peninsula, are experiencing the fastest warming in the Southern Hemisphere, with nearly +3.0°C of atmospheric warming and a +1°C rise in summer seawater surface temperatures since 1951 (Meredith & King, 2005; Montes-Hugo *et al.*, 2009; IPCC, 2013). An additional 2°C of warming is predicted for the SO by the end of the century (IPCC, 2013). Peck *et al.* (2004) and Meredith & King (2005) all suggest that a 1-2°C increase in ocean

temperature in summer would place the majority of benthic Antarctic species at risk of major population loss, considering their known sensitivity to warming through reduced aerobic capacity as temperatures increase.

Elevated temperatures increase metabolic rates (Christensen *et al.*, 2011; Doney *et al.*, 2012; Morley *et al.*, 2012a; Alcaraz *et al.*, 2014), though the different processes contributing to this will respond at different rates (Alcaraz *et al.*, 2014). This is particularly noticeable in invertebrate Antarctic marine ectotherms, as they lack the ability to adjust their internal mechanisms compared to endothermic species (Pörtner *et al.*, 2007). As temperatures increase, metabolic demand increases until aerobic demand outstrips respiration rate, at which point there is a switch to anaerobic respiration (Pörtner & Farrell, 2008; Pörtner, 2010). This mismatch between available energy and energy demand as temperature increases affects the total energy available to the animal for processes such as growth and reproduction (Doney *et al.*, 2012; Alcaraz *et al.*, 2014), and they may ultimately die.

Shallow marine Antarctic environments are regularly disturbed by iceberg scour and anchor ice, yet are very productive (Pearse *et al.*, 1991). OA and warming pose a comparatively greater threat in the SO, where even small decreases in pH or increases in temperature represent extreme environmental changes. Rates of temperature increase and pH decrease predicted for this century are unprecedented for SO fauna on geological timescales, representing a major physiological challenge their evolutionary history has not prepared them for (Royal Society, 2005; Barnes & Peck, 2008; Convey *et al.*, 2009; Barnes *et al.*, 2009).

The SO takes up a large amount of atmospheric CO₂ due to its cold waters absorbing more CO₂, and the high wind speeds that maximise CO₂ absorption into the surface ocean (Sabine *et al.*, 2004; Orr *et al.*, 2005; IPCC, 2013). The SO exerts considerable influence on the global ocean system, as a large amount of its now carbon-rich water sinks and is transported northward by thermohaline circulation (Griffiths, 2010; Ingels *et al.*, 2012). Therefore the SO is an area where CO₂ introduction into the deep sea, usually a slow process, is relatively rapid (Royal Society, 2005), and results in particularly low carbonate saturation states. The aragonite saturation horizon in SO

surface waters is already shallower than in temperate or tropical waters, and is predicted to shoal earlier than at lower latitudes (Barnes & Peck, 2008). Aragonite undersaturation in the surface waters of the SO will be first reached in winter, as greater cooling results in higher aqueous [CO₂] and there is greater upwelling of carbon-rich deep water (Feely *et al.*, 2004; Orr *et al.*, 2005). This could occur as early as 2035 (McNeill & Matear, 2008; Mattsdotter Björk *et al.*, 2014).

The Ross Sea is part of the East Antarctic sub-region, and is a distinct species richness hotspot (Linse *et al.*, 2006). It is the most productive system in the SO, with high sedimentation rates, and intensely productive plankton blooms each spring and summer (Heilmayer *et al.*, 2003; Thrush *et al.*, 2006; Smith Jr, 2007; Smith Jr *et al.*, 2014). McMurdo Sound, at the margin of the Ross Sea ice shelf, is only free of sea ice for part of the year, and is the most southern open water habitat (Morley *et al.*, 2012b). It has a particularly low, stable seawater temperature range throughout the year, especially in winter (Peck, 2005a; Peck *et al.*, 2014; Kapsenberg *et al.*, 2015). Southward species' range shifts in McMurdo Sound in response to climate change and OA are not possible, as it is the most southerly shelf area; therefore any escape from warming is limited to depth shifts into deeper waters, and if species are unable to cope with or adapt to future ocean conditions, there is a real threat of local extinction (Barnes *et al.*, 2009).

1.1.2.1 – Susceptibility of Southern Ocean fauna

The susceptibility of SO marine fauna to ocean warming and acidification, particularly of calcifying invertebrates, is due to the physical and chemical characteristics of the water, and the evolutionary history of these specialised taxa that limits their ability to cope with rapid changes. SO temperatures have been stable, cold, and constant for millennia (Peck *et al.*, 2014). This has resulted in fauna that is highly adapted to cold environments, with slow movement, growth, reproduction and protein synthesis, as well as gigantism, long overall lifespan, long development times for gametes and larvae, and low metabolic rates all common (Pearse *et al.*, 1991; Peck, 2005b; Aronson *et al.*, 2007, 2009; Barnes & Peck, 2007; Barnes *et al.*, 2009; Convey *et al.*, 2009; Gonzalez-

Bernat *et al.*, 2013). Several My of constant cool climate have limited the thermal range of Antarctic marine biota and restricted their upper temperature limit. Functional limits can be even more stenothermal. Some SO species are unable to perform critical behaviours (e.g., predator avoidance behaviours) even at temperatures lower than their lethal upper temperature limits (Peck *et al.*, 2004; Barnes *et al.*, 2009; Clark & Peck, 2009a,b).

Consequently, many Antarctic marine species are unable to acclimate to elevated temperatures, even over several months (Peck *et al.*, 2014). Metabolic repression is an adaptation to allow SO marine fauna to survive the limited energy and intense seasonality of primary production and light availability in their environment (Brockington, 2001; Peck, 2005b; Thrush *et al.*, 2006; Morley *et al.*, 2007). This metabolic repression may now become a hindrance (Peck, 2005a,b). As a consequence of their low metabolic rates and slow way of life, SO organisms may struggle to regulate internal ion concentrations, necessary to preserve function under OA (Gazeau *et al.*, 2013; Gonzalez-Bernat *et al.*, 2013; Parker *et al.*, 2013).

1.1.3 – Measuring an organism's response to ocean warming and acidification

Multiple effects to OA and warming are expected, with stresses at the cellular level, such as increased energy demand or protein production, influencing metabolic processes such as respiration which, in turn, affect the whole-organism response (function and survival) (Clarke, 1991; Llabres *et al.*, 2013; Morley *et al.*, 2009). Ultimately, the survival and performance of an animal are constrained by the efficiency of its cellular processes, such as acid-base regulation, and how well these adjust to changes in pH and temperature (Pörtner & Farrell, 2008; Pörtner, 2002; Pörtner *et al.*, 2007; Pörtner, 2010; Pan *et al.*, 2015).

1.1.3.1 – Survival, condition, and metabolism

Death is the definitive measure of whether environmental conditions are tolerable. Extended exposure to sufficiently stressful conditions beyond an organism's tolerance limits can result in death, sometimes with a decline in health beforehand (Peck *et al.*, 2002; Beukema *et al.*, 2009). This occurs in molluscs as a result of both OA and warming. For example, the survival rates of large juvenile paua (*Haliotis iris*) were negatively affected by reduced pH (7.6) (Cunningham *et al.*, 2015), and the bivalve *Macoma balthica* experienced increased mortality with warming (Beukema *et al.*, 2009). Consequently, measuring survival is a useful metric for determining how tolerable a stressor is over the long term. However, there are other measures of stress that may become apparent while the animal is still alive.

A decline in the general health of an organism is a clear indication that environmental circumstances are stressful and that it is unable to maintain condition. Health in bivalves is measured by various condition indices (Crosby & Gale, 1990; Roper *et al.*, 1991). Physical (shell) condition is a measure of relative weight change of the shell, while physiological (flesh) condition shows whether flesh mass has changed relative to shell length or internal shell volume (Crosby & Gale, 1990; Roper *et al.*, 1991; Cunningham *et al.*, 2015). OA is predicted to increase shell dissolution (Green *et al.*, 2004; Orr *et al.*, 2005; McClintock *et al.*, 2009) and bivalve shells may be thinner and more brittle under reduced pH (Fitzer *et al.*, 2014). This would result in reduced physical condition. Body mass loss has been noted in bivalves as a result of warmer-than-average temperatures (Beukema *et al.*, 2009). Increased energy expenditure under OA and warming will likely result in an associated decline in physiological condition.

Respiration rate is the final product of a number of processes operating at the molecular, cellular, and whole-organism level. Consequently, it is a good holistic measure of energy demand. Energy demand increases with OA and warming as marine calcifiers must maintain their acid-base balance to calcify under reduced pH, and elevated temperature results in an elevated aerobic demand (Pörtner, 2010; Jakubowska & Normant, 2015). Oxygen consumption is a standard measure of basal

metabolic rate (e.g., Brockington [2001]; Peck *et al.* [2002]; Heilmayer *et al.* [2004]; Morley *et al.* [2007, 2009]; Wood *et al.* [2008]; Zittier *et al.* [2015]). Metabolic rate increases as a result of elevated aerobic demand, which increases with temperature increase (Heilmayer *et al.*, 2004; Pörtner, 2010; Doney *et al.*, 2012), as do all physiological processes (Clarke, 1991). In Antarctic bivalves, temperature elevation increases respiration rates; in response to +2.0°C in *Yoldia eightsi* (Abele *et al.*, 2001) and to +3°C and to +6-9°C in *L. elliptica* (Peck *et al.*, 2002).

Metabolic rate has increased in response to OA in nearly all mollusc species tested to date (Parker *et al.*, 2013), though see Cunningham *et al.* (2015) and Jakubowska & Normant (2015) (both found no change). Alternatively, metabolic rate can be depressed if hypercapnia continues uncompensated, and this can be lethal if continued long-term (Christensen *et al.*, 2011; Gazeau *et al.*, 2013; Parker *et al.*, 2013). Oxygen uptake can also be repressed under both OA and warming, as in the brittle star *Ophionereis schayeri* (Christensen *et al.*, 2011). Consequently, measuring respiration rates to determine metabolic demand in response to both OA and warming in Antarctic bivalves is an interesting and valuable metric.

1.1.3.2 – HSP70 and the heat shock response

When exposed to environmental conditions outside their tolerance envelope, the homeostasis of an organism can be disturbed (Clark *et al.*, 2008a,b; Fabbri *et al.*, 2008). If this disruption continues, species can experience a loss in performance, reduced population size, and reduced species ranges (Pörtner, 2008, 2010; Pörtner *et al.*, 2007). Understanding a species' heat shock response helps identify its lower and upper tolerance limits to specific environmental variables. These are the boundaries for a species' range, and indicate how this range may change with environmental stress, such as the changes predicted to occur as a result of ocean warming and acidification (Pörtner *et al.*, 2007; Clark & Peck, 2009b). Measuring the expression levels of the inducible 70 kDa heat shock protein (HSP70) is a good way to achieve this.

The induction of HSP70 is a highly-conserved measure of both cellular and physiological stress in an organism that indicates the necessity of the additional energy expenditure of producing this protein (Clark & Peck, 2009b; Tomanek, 2010). While absent in a number of Antarctic species, heat-stimulated HSP70 up-regulation is found in two Antarctic mollusc species; the infaunal bivalve *Laternula elliptica* and the gastropod *Nacella concinna* both show a strong heat shock response at +10 and +15°C, respectively (Park *et al.*, 2007; Clark *et al.*, 2008a; Clark & Peck, 2009b). *L. elliptica* also induces HSP70 gene expression in response to both raised and lowered pH (Cummings *et al.*, 2011).

1.1.4 – *Laternula elliptica*

Laternula elliptica is a large infaunal bivalve that has a shell considerably longer than most other Antarctic molluscs. It is one of the largest bivalves in the SO ecosystem (the other being the scallop *Adamussium colbecki*), with a maximum shell length of 120 mm (Berkman *et al.*, 1991; Ahn, 1993, 1994; Urban & Mercuri, 1998; Clark *et al.*, 2010). *L. elliptica* dates from the Cretaceous and produces a thin aragonitic shell of a primitive, simple structure (Berkman *et al.*, 1991; Sato-Okoshi *et al.*, 2010). The relatively thick periostracum helps protect the shell, but the shell itself is thin, forming the minimum protection needed, possibly to reduce production costs in the cold Antarctic environment (Sato-Okoshi *et al.*, 2010). It exhibits slow rates of shell growth and shell repair (Brey & Mackensen, 1977; Sleight *et al.*, 2015). *L. elliptica* also displays the low metabolic rate, extended lifespan (up to 36 y), extended gametogenesis, and long larval development time typical of Antarctic species (Ahn & Shim, 1998; Urban & Mercuri, 1998; Peck *et al.*, 2004; Peck, 2005a; Morley *et al.*, 2007; Kang *et al.*, 2009).

L. elliptica is a common species with a circumpolar distribution, and is found in high densities (~70-100 ind m⁻²) in soft sediments, representing high biomass (~4800 g wet weight m⁻²) (Ahn, 1993, 1994; Ahn *et al.*, 2001). *L. elliptica* buries itself upright in sediment and uses its extendable siphon to reach the benthic/demersal interface to breathe and feed. Therefore its internal structures will be exposed to any change in water conditions, because despite its body being buried, it still takes in water from the

pelagic environment above it. The response of *L. elliptica* to future SO warming and acidification is of concern because it is a key species in the SO benthos, playing a dominant role in biodeposition and benthic-pelagic coupling (Ahn, 1993; Clark *et al.*, 2010). *L. elliptica* already relies on metabolic repression to survive resource-limited winters with little to no food (Ahn *et al.*, 2001, 2003; Brockington, 2001; Morley *et al.*, 2007), so any increased metabolic demand resulting from rising temperatures and declining pH (as discussed above) may negatively impact this species.

As a highly stenothermal species, *L. elliptica* is strongly negatively affected by temperature increase. The lethal limit for this species is +7.5-12.5°C (Peck *et al.*, 2004; Peck, 2005a,b; Morley *et al.*, 2009). Not only does *L. elliptica* die if taken ~10°C above its normal temperature range, but it is unable to rebury itself at 2-5°C (Peck *et al.*, 2004; Morley *et al.*, 2012a,b). Burial is an important behaviour that allows it to avoid iceberg scour (Ahn, 1994; Peck *et al.*, 2004; Morley *et al.*, 2012a,b). Only 50% of *L. elliptica* can bury at +2-3°C, and there is 100% failure to bury at +5°C (Peck *et al.*, 2004). *L. elliptica* is also unable to acclimate to increased temperatures of +3.0°C over 3 mo timescales (Morley *et al.*, 2012a). Therefore even small temperature changes, such as the +2°C warming predicted for the Ross Sea by 2100 (IPCC, 2013), would leave the species unable to perform vital behaviours and may greatly increase the risk of mortality.

L. elliptica also appears vulnerable to reductions in pH. The only current study on the effect of pH change on adult *L. elliptica* revealed that basal metabolism (measured via oxygen consumption rate) at 4 mo and HSP70 gene expression levels at 3 wk increased with both elevated (pH 8.32) and reduced (pH 7.78) pH relative to ambient conditions (pH 7.99) (Cummings *et al.*, 2011). In addition, chitin synthase, an enzyme involved in biomineralisation in bivalves, was up-regulated after 4 mo at pH 7.78 ($\Omega_{Ar} = 0.71$) (Cummings *et al.*, 2011). These responses are expected to be energetically expensive to maintain over the long term, especially in water undersaturated with aragonite (Cummings *et al.*, 2011), and this has the potential to reduce reproductive potential and subsequent population size in this species (Guy *et al.*, 2014).

Most knowledge of species' reactions to expected environmental changes is currently based on short-term, single-stressor experiments (e.g., studies by Barnes & Conlan

[2007] and Barnes & Peck [2008]) (Kroeker *et al.*, 2013). Multi-stressor responses are important in OA research (e.g., Parker *et al.* [2013]; Byrne *et al.* [2014]; Kapsenberg & Hoffman [2014]; Suckling *et al.* [2015]; Zittier *et al.* [2015]). *L. elliptica* is a common and regionally dominant bivalve in the SO benthos whose response to future predicted ocean conditions is of concern given its susceptibility. The response of common species with high biomass is of interest as any changes in behaviour, survival, or biomass of that species will influence a large number of other species and processes in the system. *L. elliptica* is therefore an ideal study species to examine the effects of future predictions of comparatively rapid ocean pH and temperature change in the SO.

This thesis will focus on adult *L. elliptica* and fill several knowledge gaps in ocean acidification and warming research, namely the need for: multi-stressor studies; studies for periods longer than 1-3 mo; and studies on crucial, common species in an ecosystem. By focusing on processes at both the cellular level (HSP70 production) and the whole-organism level (O₂ consumption, health, condition, and survival), this thesis elucidates the functional response of *L. elliptica* to SO warming and acidification, and will examine its ability to acclimate to these conditions over time.

Specifically, the hypotheses of this thesis are that:

1. Both an increase in temperature and a decrease in pH will induce a negative stress response in *L. elliptica* across all processes (increased HSP70 gene expression, increased O₂ consumption, reduced physical and physiological condition, and reduced survival).
2. This negative response will be exacerbated when warming is combined with acidification.
3. *L. elliptica* will be unable to acclimate over time, and over the course of the study the magnitude of this negative response will stay constant or increase, but not decrease.

1.2 – Thesis aims and structure

Chapter 2 describes the results of multiple medium-term (5 mo) experiments on adult *L. elliptica* exposed to levels of pH and temperature predicted for 2050 and 2100. The experiments show how *L. elliptica* survival, O₂ consumption, and overall health (assessed using physical and physiological condition indices) respond to elevated temperature, lowered pH, and combinations of these stressors. The change in functional status of *L. elliptica* between 5 wk and 5 mo was used to ascertain whether they can acclimate to conditions over time, and if there were indications of individual phenotypic plasticity. This work is presented in manuscript form in preparation for submission, and there may be some repetition of information also provided in Chapters 1 and 4.

Chapter 3 presents preliminary work on HSP70 expression of *L. elliptica* using tissue samples collected during the experiments in Chapter 2, and is presented in short note format. The time-consuming nature of these analyses limited the sample size to n = 1; additional analysis was not possible within the timeframe of this thesis. This preliminary experiment refines RNA extraction and quantitative polymerase chain reaction (qPCR) techniques for *L. elliptica*, and shows trends of how the heat shock response of *L. elliptica* responds to both elevated temperature and lowered pH.

An overall synthesis and recommendations for future work are discussed in Chapter 4.

Chapter 2

The response of *Laternula elliptica* to ocean warming and acidification

2.1 – Introduction

In the 250 y since the Industrial Revolution, anthropogenic emissions of greenhouse gases, particularly carbon dioxide (CO_2), have increased, and are accumulating in the atmosphere at an alarming rate. As a result, global average temperatures have risen by 0.85°C in the last 130 years (IPCC, 2013). CO_2 dissolves into the ocean at a greater rate under high atmospheric loadings, and this causes changes in ocean chemistry, unbalancing ion concentrations. As a result, carbonate ions (CO_3^{2-}) decrease and hydrogen ions (H^+) and bicarbonate ions (HCO_3^-) increase. The increase in $[\text{H}^+]$ ultimately reduces the pH (on the total hydrogen scale, hereafter referred to as ‘pH’) of the oceans, a process termed ‘ocean acidification’ (OA) (Orr *et al.*, 2005; Royal Society, 2005; IPCC, 2013).

As $[\text{CO}_3^{2-}]$ decreases, the saturation states of calcite and aragonite (Ω_{Ca} and Ω_{Ar} , respectively) decrease and calcification becomes more difficult (Miller *et al.*, 2009; Barnes & Peck, 2008; Gazeau *et al.*, 2013; Bednaršek *et al.*, 2014). Dissolution due to undersaturation (Ω_{Ar} or $\Omega_{\text{Ca}} < 1$) is also considered to be challenging for calcifiers (Green *et al.*, 2004; Roleda *et al.*, 2012). This is especially problematic for calcifiers that use aragonite, a highly soluble form of calcium carbonate (CaCO_3) (Parker *et al.*, 2013; Bednaršek *et al.*, 2014). Decreases in pH also affect marine organisms by reducing the pH of internal fluids (hypercapnia), disrupting their acid-base balance (Pörtner *et al.*, 1998; Wicks & Roberts, 2012; Parker *et al.*, 2013). pH has already decreased by 0.1 units since the Industrial Revolution, representing a 30% increase in $[\text{H}^+]$, and is predicted to drop by a further 0.3-0.4 units by 2100 (Clark *et al.*, 2009; IPCC, 2013). This rate of change is unprecedented, occurring faster than at any time in the last 25 M y.

Oceanic temperature and pH changes are felt most strongly at high latitudes, such as the Southern Ocean (SO). The oceans are a significant oceanic carbon sink, responsible for an estimated 30% of annual global oceanic uptake of CO₂, and the SO absorbs CO₂ at an elevated rate, due to cold surface waters and high wind speeds maximising the absorption at the air/sea interface (Sabine *et al.*, 2004; Orr *et al.*, 2005; IPCC, 2013). This high CO₂ concentration in cold SO waters combined with the upwelling of cold, carbon-rich deepwater is hastening OA (Orr *et al.*, 2005; Clark *et al.*, 2009; Lenton *et al.*, 2009). Undersaturation of aragonite at high latitudes will occur first in winter (Feely *et al.*, 2004; Orr *et al.*, 2005) perhaps as early as 2035 (McNeill & Matear, 2008; Mattsdotter Björk *et al.*, 2014). Consequently, calcifying organisms at high latitudes are likely to be affected by OA and climate change before more temperate or tropical species (Clark *et al.*, 2009; Gonzalez-Bernat *et al.*, 2013).

Temperature is the main factor affecting species ranges and functional envelopes in marine invertebrates, particularly in Antarctica, where seawater temperatures are very cold and stable (Alcaraz *et al.*, 2014; Peck *et al.*, 2014). McMurdo Sound water temperatures vary by only 1.5°C per annum (Hunt *et al.*, 2003), and have done so for 15-25 M y (Peck *et al.*, 2014), with more thermally variable Antarctic sites typically experiencing a temperature range of just 3-4°C in a year (Peck *et al.*, 2014). Many Antarctic marine invertebrates exhibit slow rates of metabolism, growth, and reproduction as a result of the cold temperatures and extreme seasonality of resources they experience (Pearse *et al.*, 1991; Peck, 2005a; Thrush *et al.*, 2006; Barnes & Peck, 2007; Barnes & Conlan, 2008; Barnes *et al.*, 2009). Consequently, evolving in such stable, constant, cold environmental conditions, Antarctic marine organisms are stenothermal, and may be unable to adapt or acclimate to even small increases in temperature (Peck *et al.*, 2004; Morley *et al.*, 2012a). In the SO, species are therefore ill-adapted to the magnitude and speed of predicted ocean changes (Barnes & Peck, 2008; Convey *et al.*, 2009; Barnes *et al.*, 2009).

Alongside the scallop *Adamussium colbecki*, *Laternula elliptica* is one of the two largest and most abundant species in the Ross Sea coastal benthos. *L. elliptica* is a long-lived infaunal bivalve with a shell length of up to 120 mm and a maximum recorded lifespan of 36 y (Urban & Mercuri, 1998). It buries deep (5-50 cm depending on location) in soft

sand and gravel sediments, extending its siphons to the benthos/demersal interface to breathe and feed (Ahn, 1993, 1994; Clark *et al.*, 2010). This burying behaviour has the added benefit of allowing *L. elliptica* to avoid physical disturbance by icebergs and anchor ice, which regularly disturb the productive shallow Antarctic environment, allowing it to exploit food while avoiding injury (Pearse *et al.*, 1991; Ahn, 1994; Ahn *et al.*, 2001; Barnes & Conlan, 2007; Barnes & Peck, 2007). *L. elliptica* feeds on large phytoplankton species that are only available for a short period in the summer (Brockington, 2001). They must survive up to 9 mo without food over winter, experiencing high seasonality of resources (Ahn & Shim, 1998; Ahn *et al.*, 2001, 2003). Despite this, *L. elliptica* is a common species throughout the Antarctic continental shelf, with a circumpolar distribution, and often reaches particularly high densities in shallow coastal environments (e.g., ~70 to >100 ind m⁻², with an estimated biomass of ~4800 g wet weight m⁻² (Ahn, 1993, 1994; Ahn *et al.*, 2001). As a filter-feeder of high abundance and biomass that links the pelagic and benthic ecosystems, it exerts considerable influence and plays a prominent role in benthic-pelagic coupling (Ahn, 1993; Clark *et al.*, 2010). Its biodeposits may also serve as an important substrate and food source for other bivalves and deposit feeders (Ahn, 1993), thereby increasing local diversity (Gazeau *et al.*, 2013). Endemic to the Antarctic, *L. elliptica* has a low metabolic rate and a particularly narrow thermal tolerance envelope, and as a result it is unable to perform critical functions at temperatures only a few degrees above ambient (Ahn & Shim, 1998; Ahn *et al.*, 2001; Peck *et al.*, 2004; Peck, 2005a,b). It produces a thin aragonitic shell that is similar in structure to that of primitive bivalves (Sato-Okoshi *et al.*, 2010; Clark *et al.*, 2010). These characteristics mean that *L. elliptica* is at risk of metabolic and physiological stress in the near future.

Calcification in bivalves involves the nucleation of CaCO₃ crystals in an internal compartment, the extrapallial cavity, using respired CO₂ (Roleda *et al.*, 2012; Wicks & Roberts, 2012). This cavity is located between the mantle and the shell, and is separated from the surrounding seawater and filled with pallial fluid specific to calcification (Wicks & Roberts, 2012; Gazeau *et al.*, 2013). Ions are then diffused or actively pumped in and out of this cavity to maintain the correct concentration for crystal synthesis. Under decreased pH conditions the subsequent CO₂ increase in both the external seawater and the internal fluid makes maintaining this concentration increasingly

difficult (Gazeau *et al.*, 2013; Parker *et al.*, 2013). The exact chemical and molecular processes controlling biomineralisation are still poorly understood, but this process is fundamental to calcifiers, tightly controlled, and energetically costly (Wicks & Roberts, 2012). Under reduced pH, calcification rates can slow, as comparatively more energy is required to maintain the correct ionic and acid-base balance of the extrapallial cavity (Wicks & Roberts, 2012). This in turn can affect long-term growth and reproduction, with flow-on effects for population size (Pörtner *et al.*, 2004; Pörtner, 2008; Guy *et al.*, 2014). Bivalves in general have little ability for acid-base compensation (Pörtner *et al.*, 2004, 2007). Therefore *L. elliptica*, as an Antarctic bivalve, would be expected to have little capacity to compensate for hypercapnia associated with OA. *L. elliptica* grows rapidly over the austral spring/summer period when sources of food (and therefore energy) are readily accessible, but over the resource-limited austral winter, it reduces its energetic expenditure and slows calcification, resulting in a slow average growth rate (Brey & Mackensen, 1997; Ahn *et al.*, 2001, 2003; Morley *et al.*, 2007). Of concern are the effects of reduced pH on *L. elliptica*, as associated increased metabolic costs will potentially be detrimental, both during periods of active feeding necessary to build up reserves (summer), and periods of torpor necessary to survive extensive periods of food shortage (winter) (Ahn *et al.*, 2001, 2003; Brockington, 2001; Peck *et al.*, 2004; Morley *et al.*, 2007).

Increased metabolic demand as a result of increased aerobic demand is a common bivalve response to environmental changes such as temperature increase. If stress levels continue, the organism can depress other functions, such as reproduction and growth, to compensate. Maintaining basal metabolic function is also a priority. High temperatures may necessitate the diversion of energy normally allocated towards such processes in order to fulfil these needs (Clarke, 1987; Alcaraz *et al.*, 2014). The repression of reproductive output to maintain long-term metabolic function has severe implications for the long-term persistence of populations, and in *L. elliptica*, this trade-off could significantly decrease population sizes (Guy *et al.*, 2014).

Environmental changes do not occur singularly; understanding the synergistic effects of environmental changes is therefore vital, as organisms must cope with all stresses they experience in order to survive. Combined stressors can act additively, synergistically, or

antagonistically to augment or repress certain functions, either increasing or decreasing a species' performance and fitness. The magnitude of these changes, and whether they positively or negatively impact a species, is highly dependent on species-specific physiological constraints, and its resilience to the environmental variables it experiences. Ocean warming will not act in isolation upon organisms; instead, acidification will occur alongside temperature increase (Hendriks *et al.*, 2010). Other environmental challenges for Antarctic species may also include increased UV-B radiation, sedimentation, and salinity fluctuations from melting of sea ice and glaciers (Camus *et al.*, 2005; Barnes & Conlan, 2007; Barnes & Peck, 2008; Griffiths, 2010; Ingels *et al.*, 2012; Llabres *et al.*, 2013). When considering ecologically relevant responses to OA and warming, it is therefore important and useful to consider how both stressors act together as well as in isolation (Gazeau *et al.*, 2013; Byrne *et al.*, 2014).

Several laboratory studies have examined the effects of elevated temperature on *L. elliptica* adults, and these have shown lower survival and poorer burial ability in experiments ranging from 24 h to 63 d in duration (Peck *et al.*, 2002, 2004; Peck, 2005a; Morley *et al.*, 2012a). Only one study has examined the effect of reduced pH, and noted an increase in oxygen (O₂) consumption rates and chitin synthase gene expression levels at 4 mo, and heat shock protein (HSP70) gene expression levels at 3 wk (Cummings *et al.*, 2011). To date there have been no studies investigating the effects of both stressors in combination. Consequently, there is an urgent need to determine how *L. elliptica* will be affected over medium to long-term timescales by SO warming and acidification.

Adults were collected from Cape Evans, McMurdo Sound and exposed in the laboratory to a range of temperatures and pHs predicted for the region in both 2050 and 2100 (IPCC, 2013), for a 5 mo period. I hypothesised that *L. elliptica* would exhibit responses consistent with elevated stress as temperature increased and pH decreased, and that this would be most apparent with the combination of both stressors (as in Kroeker *et al.* [2013]). I did not anticipate that they would be able to acclimate over the duration of this experiment (5 mo), based on the results of previous single stressor experiments (Cummings *et al.*, 2011; Morley *et al.*, 2012a). This study fills a valuable knowledge gap

on an important species, investigating the combined effect of OA and warming on a common stenothermal Antarctic species.

2.2 – Methods

2.2.1 – Collection

Laternula elliptica adults were collected from Cape Evans, McMurdo Sound, Ross Sea, Antarctica (77.635258°S, 166.414382°E) on 14 and 17 November 2013. They were collected from coarse sand and gravel sediments at 6 m depth by SCUBA divers. Collected animals (average shell length [SL] 81.3 mm, range 65.5-99.8 mm) were transported to Wellington, New Zealand on 29 November 2013. The animals were held for 6 mo (until June 2014) in a flow-through system fed by seawater chilled to Ross Sea ambient temperature (-1.6°C), at pH ranging from ~8.00 (ambient Cape Evans level at the time of sampling) to ~8.30. These pH values are within the range experienced by these animals in their natural habitat at Cape Evans over the summer months (Kapsenberg *et al.*, 2015). *L. elliptica* specimens were housed in multiple sediment-free tanks, held with their siphons upwards in mesh baskets to mimic their natural orientation. This allowed them to extend their siphons and feed and respire freely. They were fed several times per wk with a liquid commercial mix of algae tailored to bivalves (Instant Algae Shellfish Diet 1800, Reed Mariculture Inc.).

Individuals were moved to a new specialised aquarium facility on 6 June 2014. They were re-acclimated to ambient Antarctic conditions for 10 wk at $-1.63 \pm 0.0004^{\circ}\text{C}$ and pH 8.02 ± 0.0002 units (mean \pm SE). In all cases, *L. elliptica* specimens were housed in tanks as described above, and fed twice weekly, with 3 mL of 0.5% dry weight liquid Shellfish Diet 1800 delivered via pipette into the water above each animal's siphon. Immediately following the move to the new facility, they underwent a mass spawning event, releasing both sperm and eggs into their tanks. This event lasted approximately 3 d.

2.2.2 – Experimental setup

Filtered (1 μm) seawater from Evans Bay, Wellington Harbour, was chilled to -1.6°C , then fed to separate header tanks constructed from 55 L insulated bins, with one header tank per treatment. Treatment water was adjusted to a range of temperature and pH conditions (Table 2.1) in each header tank before being fed to the experimental tanks at a rate of 120 mL min^{-1} in a flow-through system. Nine separate header tanks supplied seventy-two 4 L tanks (eight replicate tanks per treatment, arranged in a randomised block design). Both temperature and pH were directly controlled by Jumo dTrans pH 02 industrial controllers, remotely controlled and monitored by a LabView server communicating via a MODBUS serial interface. Water was circulated in each header tank with a pump, and the temperature and pH of the header tanks was constantly logged using pH and temperature probes. Analogue controller inputs from each experimental header tank were measured with precision PT100 temperature probes and Sensorex S150C pH probes. Seawater pH was adjusted in each header tank by diffusing food-grade CO_2 gas through thin-walled (0.5 mm) silicone tubing, and the temperature of the -1.6°C seawater supply stock was adjusted in the header tanks with submerged 500 W heater elements connected via solid state relays to the pH controllers. The pH probes in each header tank were calibrated fortnightly with Tris(hydroxymethyl)aminomethane (TRIS) and 2-amino-2-methyl-1-propanol (AMP) buffers, matched to the salinity of seawater. Salinity was measured every 15 minutes from chilled harbour seawater immediately prior to it entering the system.

The 4 L replicate tanks containing *L. elliptica* were insulated with a matrix of interlocking sheets of foam mat; details of the tank setup are provided in Fig. 2.1.

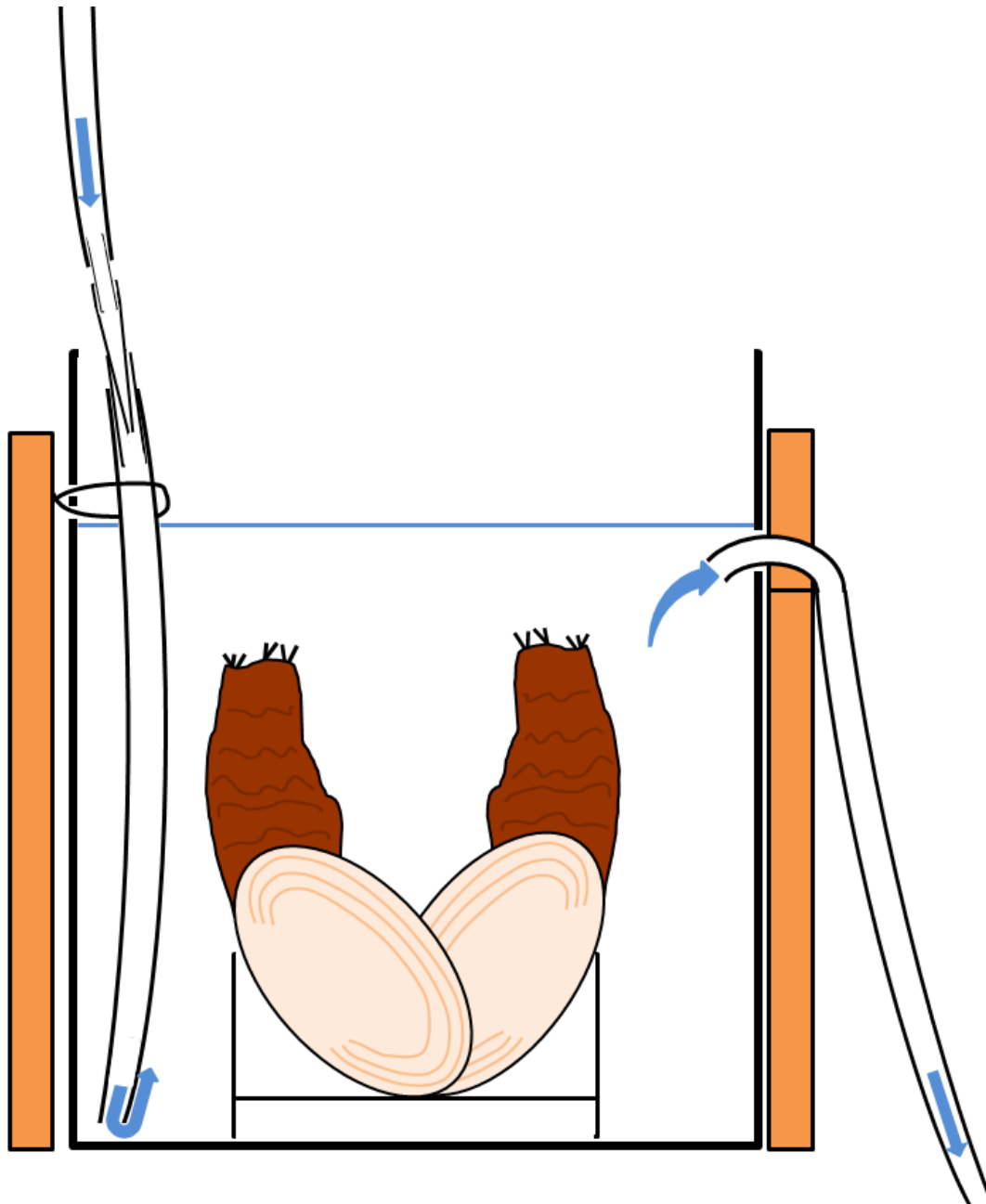


Figure 2.1 - Setup of experimental tanks. Note the mesh basket supporting *Laternula elliptica* individuals in their natural orientation, insulating foam (orange) around the tank, and details of entry and exit tubing. Flow was controlled to a rate of 120 mL min^{-1} . The position of the inflow and outflow tubing in the tank ensured that the water was well-mixed, and that animals received well-oxygenated water.

A 3×3 matrix of temperatures and pHs was chosen in a fully crossed design (Table 2.1). Temperatures included current conditions for the Ross Sea (-1.4°C ; as cold as the system allowed) and two levels of warming (-0.5 and $+0.5^{\circ}\text{C}$). The intermediate value of -0.5°C represents less severe warming predictions of $+1^{\circ}\text{C}$ for the Ross Sea, while $+0.5^{\circ}\text{C}$

(+2°C warming) is predicted for the region by 2100 (IPCC, 2013). pH levels spanned from current spring levels in the field (pH 8.00) to include two levels of decreased pH (pH 7.85 and 7.65). A pH of 7.65 (a -0.35 unit drop) is predicted for the Ross Sea by 2100 (IPCC, 2013).

Table 2.1 - Experimental treatments. Green = ambient Ross sea value; orange = intermediate value; red = predicted in the Ross Sea for 2100 (IPCC, 2013). As experimental temperatures increase and pHs decline, the amount of stress experienced is expected to increase. This is indicated by the colour gradation from green (low-stress control treatment) to red (predicted high-stress end-of-century treatment).

		Temperature (°C)		
		-1.4	-0.5	+0.5
pH	8.00	Control		
	7.85			
	7.65			

2.2.3 – Experimental acclimation

Seawater temperatures and pHs were changed gradually over 18 d prior to the start of the experiment. Once re-acclimated to ambient conditions, 144 *L. elliptica* individuals were randomly redistributed amongst multiple 50 L holding tanks to enable temperature change to the target levels. There were three tanks for each target temperature, with 16 animals per tank. Temperatures were changed as appropriate over 11 d at a rate of 0.2°C d⁻¹. Animals were held at their target temperature (-1.4°C, ambient; -0.5°C; and +0.5°C) for at least 48 h. Individuals were then randomly distributed amongst the 4 L replicate tanks, with two per tank held upright in small mesh baskets (Figures 2.1, 2.2). pH was changed over the next 7 d at a rate of 0.05 units d⁻¹, until target treatment conditions were reached (pH 8.00, ambient; pH 7.85; and pH 7.65). The experiment began on 11 September 2014, once every animal had experienced final treatment conditions for at least 48 h. The experiment concluded 143 d (5 mo) later, on 2 February 2015.

Tanks were cleaned monthly. Individuals continued to spawn sporadically throughout the experiment, and tanks were also cleaned after these events.



Figure 2.2 – *Laternula elliptica* in experimental tanks, respiring with siphons open. White material visible on the floor of the tank is a mixture of eggs and sperm from a recent spawning event.

2.2.4 – Quality control and characteristics of experimental seawater

For the first seven days of the experiment, temperature was monitored daily in each experimental tank with a handheld probe (RTD Thermometer, Center 376 Data Logger, TP-R04) to verify that temperatures recorded automatically in the header tanks were accurately representing in-tank conditions. Once it was established that temperatures were consistently stable and distinct between the three temperature levels, then

temperature was checked 1-2 times per week in four randomly identified experimental tanks per treatment, representing half the total number of tanks.

Water samples were taken from each treatment on Day 42, and at the conclusion of the experiment (Day 143). They were fixed with one drop of 1 M mercuric chloride (HgCl₂) per sample and analysed for dissolved inorganic carbon (DIC) and total alkalinity (A_T), using methods described in Cummings *et al.* (2011). Aragonite and calcite saturation state (Ω_{Ar} and Ω_{Ca} , respectively), partial pressure of CO₂ (pCO₂), and carbonate (CO₃²⁻) and bicarbonate (HCO₃⁻) ion concentrations were calculated for each treatment using measured A_T, pH, and salinity.

2.2.5 – *Laternula elliptica* sampling

L. elliptica were sacrificed at two time points during the experiment: after 5 wk and 5 mo, to ascertain their response to short- and medium-term exposure to experimental conditions, respectively. At each time point, one individual per replicate tank was used to determine various measures of organism health and function. At both time points, physical and physiological condition were determined (see below), and expression of heat shock protein (HSP70) was assessed (see Chapter 3). Prior to the last time point, individuals from seven specific treatments were used to assess respiration rates (see below). Oxygen (O₂) consumption was assessed over 9 days (from Day 133 to Day 141); the full suite of treatments and replicates could not be processed in this timeframe due to the large number of individuals and the length of time required for each assessment. Mortalities were recorded as the experiment progressed.

2.2.5.1 – Survival

Replicate tanks were examined daily, and any mortalities were recorded. These data were summarised and presented as cumulative percentage survival after each month of the experiment. Survival curves of week-by-week cumulative survival were also constructed for each treatment. In addition, the time to 50% survival (LD₅₀) was

calculated for each treatment, or projected when survival was > 50% at the end of the experiment. Results are presented as average values (n = 8, except for -1.4°C/pH 7.85, -1.4°C/pH 7.65, -0.5°C/pH 8.00 and +0.5°C/pH 7.85, where n = 7, due to mortalities during acclimation).

2.2.5.2 – Physical and physiological condition

Immediately prior to dissection, SL (mm) and total wet weight (g) were measured (Fig. 2.3). Post-dissection, valves were air-dried to a constant weight (Fig. 2.4), and left and right valve weights were summed to give a total shell weight (SW, g) for each animal. Tissue was oven-dried at 50-60°C for 48 h to give dried flesh weight (FW, g). Pre-weighed foil packets were used to contain tissue during the drying process (Fig. 2.5). FW did not include dissected mantle and adductor tissue, which had been removed for HSP70 analyses (see Chapter 3). However, the amount of adductor and mantle tissue removed from each individual was very small in each case (average 1.01% of total tissue wet weight, range 0.41-2.09%). Dried flesh of all replicates from the 5 mo time point was then ashed at 470°C for 24 h to give ash-free dry weight (AFDW, g); AFDW = FW – ashed weight, which was used to calculate oxygen consumption (see below).



Figure 2.3 - *Laternula elliptica* individual, showing where shell length was measured on the valve: from the centre point of the anterior edge to the centre point of the posterior edge.



Figure 2.4 - *Laternula elliptica* valves air drying. Note the slightly iridescent nacreous layer on the inner surface of the valve.

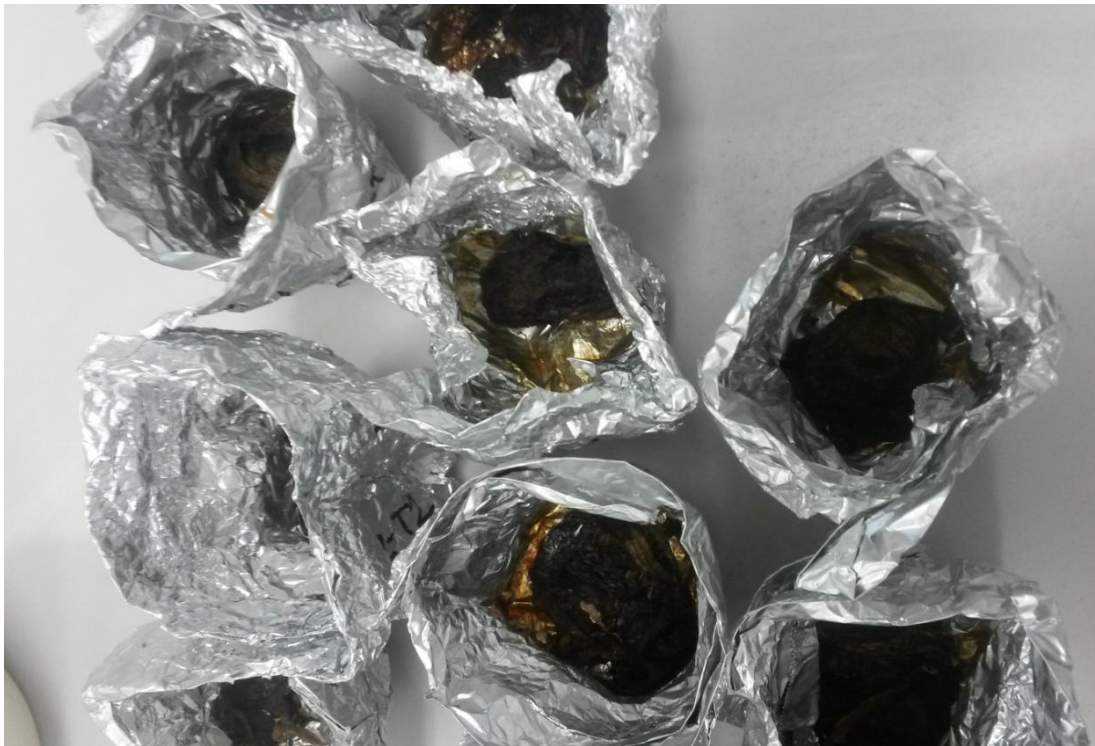


Figure 2.5 - Foil cases containing oven-dried *Laternula elliptica* body tissue.

Condition indices (CI) were calculated to measure any fluctuation in condition, potentially indicating stress (Roper *et al.*, 1991), over the course of the experiment. Both physical and physiological indices were used. Ratios between FW, SL and SW were calculated to give a measure of shell weight change (physical condition; SW:SL), and tissue weight change (physiological condition; FW:SL and FW:SW). $CI_{FW:SL}$ gives the weight change of body tissue relative to SL, and $CI_{FW:SW}$ gives tissue weight change relative to SW. $CI_{FW:SL}$, $CI_{FW:SW}$, and $CI_{SW:SL}$ were calculated for 5 wk and 5 mo, and the percentage change in each condition index over the course of the experiment (5 wk – 5 mo) was also calculated relative to condition at 5 wk.

Other condition indices used to indicate recent stress in bivalves that focus on the proportion of shell cavity capacity utilised by soft tissue (Crosby & Gale, 1990; Roper *et al.*, 1991) were not suitable for *L. elliptica*. The valves of *L. elliptica* do not shut completely, and siphon tissue extends through an opening in the valves beyond the shell

boundary. Therefore comparing body tissue volume to internal shell capacity volume was not relevant for this species.

2.2.5.3 – Oxygen consumption

Respiration in *L. elliptica* was measured as oxygen (O₂) consumption h⁻¹. Fibre optic temperature and O₂ sensors (PreSens Precision Sensing GmbH, Regensburg, Germany) were inserted through a rubber bung in the lid of a 1 L respiration chamber until they extended 5-7 cm into the chamber (Fig. 2.6). The sensors were connected to a Fibox-3 oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany) located outside the chambers. The number of animals and treatments selected for respiration measurements are detailed in Table 2.2.

Temperature and O₂ sensors were calibrated before use with water saturated with hydrogen sulphite (no dissolved O₂), and with water that had previously had air bubbled through it for 5 minutes (fully O₂ saturated).

Table 2.2 - Treatments selected for respiration measurements, and the number of *Laternula elliptica* used for that treatment.

		Temperature (°C)		
		-1.4	-0.5	+0.5
pH	8.00	2 ‡	3	2 †
	7.85			3
	7.65	2 ‡	3	1 †

Key: † = Sample size reduced by mortalities; ‡ = Sample size was initially 3 but an individual was omitted as values were unrealistic.



Figure 2.6 - Closed 1 L respiration chamber containing *Laternula elliptica*. Temperature and O₂ fibre optic probes (grey rods) are visible inserted into the chamber.

Animals were placed in the respiration chamber without a lid or probes to acclimate for 15 min. The chamber was held in a water bath at the appropriate treatment temperature with water flowing through it (Fig 2.7). After this time, individuals had extended their siphons again and had resumed normal pumping behaviour. The lid with its attached probes was then fixed in place, ensuring no air bubbles were trapped inside the chamber, and the chamber was returned to the water bath (Fig. 2.8). The animal and probes were allowed to acclimate for 30 min, and O₂ concentration (% air saturation) and temperature (°C) were recorded every minute for the following 3.5 hours. O₂ saturation was not allowed to drop below 50%. Individuals were disturbed as little as possible to ensure respiration could continue unperturbed.

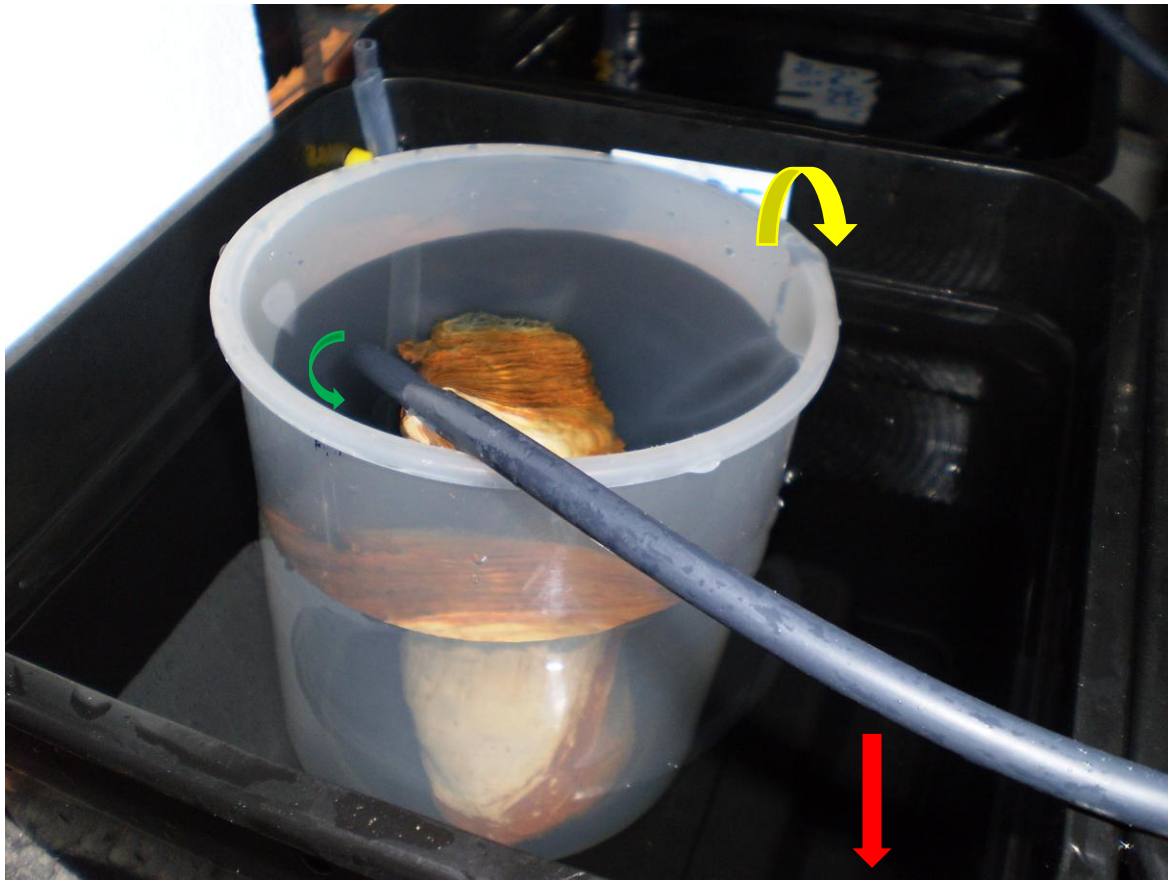


Figure 2.7 - *Laternula elliptica* acclimating to the respiration chamber in a water bath prior to the lid being attached. Treatment temperature- and pH-specific water entered the chamber (green arrow) via the grey tube at 120 mL min^{-1} , spilled over the lip of the chamber (yellow arrow) after circulating through it, replenished the chilled water in the water bath, and flowed out through the exit tubing (not pictured) (red arrow).



Figure 2.8 - Respiration chamber in a water bath, with measurements underway. Most of the respiration chamber is submerged in the water bath of treatment-specific temperature to keep its temperature stable. The water bath level extends nearly to the lip of the chamber; the water-line in the water bath is indicated by a red arrow.

The raw temperature and air saturation values at the beginning (after 30 min) and end (after 4 h) of the assessment were converted to O_2 concentration ($\mu\text{mol L}^{-1}$) and

subtracted to give total O₂ consumption. Equations used for this conversion were from Huber & Krause (2004), adapted to a saline, cold medium. First, the vapour pressure of water at temperature T ($p_w(T)$), where T is in Kelvin, was calculated as given in equation 2:

$$p_w(T) = \exp \left(A - \frac{B}{T} - C \times \ln T \right) \quad (2)$$

where T is the temperature in Kelvin and A, B, and C are the constants 52.57, 6690.9, and 4.681, respectively (Huber & Krause, 2004). Second, the Bunsen absorption coefficient at temperature T ($\alpha(T)$), where T is in °C, compensated by the salt concentration of the water, was calculated as given in equation 3.

$$\alpha(T) = \frac{\left(\exp \left[\left(A + \frac{B}{T} + C \times \ln T + D \times T \right) - [Cl^-] \left(P + \frac{Q}{T} + R \times \ln T + S \times T \right) \right] \right)}{10^3} \quad (3)$$

T is the temperature in °C, $[Cl^-]$ is the concentration of chloride ions, and the constants are: A = -7.424; B = 4.417×10^3 ; C = -2.927; D = 4.238×10^{-2} ; P = -0.1288; Q = 53.44; R = -4.442×10^{-2} ; and S = 7.145×10^{-4} (Huber & Krause, 2004). $[Cl^-]$ was calculated from the average salinity value for that day, converted to $[Cl^-]$ via equation 4, adapted from Huber & Krause (2004), where S is salinity in ‰.

$$[Cl^-] = \frac{S - 0.03}{1.805} \quad (4)$$

Finally, O₂ concentration ($\mu\text{mol L}^{-1}$) was calculated from these calculated values as given in equation 5:

$$O_2 [\mu\text{mol L}^{-1}] = \left[\frac{p_{\text{atm}} - p_w(T)}{p_N} \times \frac{\% \text{ air}}{100} \times 0.2095 \times \alpha(T) \times 1000 \times \frac{M(O_2)}{V_M} \right] \times 31.25 \quad (5)$$

where: p_{atm} (actual atmospheric pressure) = 1013.25 mbar, given the experiment was conducted near sea level; p_N (standard pressure) = 1013 mbar (constant); % air saturation = the value measured by the probe; 0.2095 = volume content of oxygen in air (constant); $M(\text{O}_2)$ (molecular mass of oxygen) = 32 g mol⁻¹ (constant); and V_M (molar volume) = 22.414 L mol⁻¹ (constant) (Huber & Krause, 2004).

Respiration assessments were conducted over 9 days, with 3-4 runs per day. The treatments and blanks sampled each day were randomly determined. One blank run, where the chamber contained only treatment water, was performed for each of the seven treatments and converted as above. Oxygen consumption values for measurements containing animals were adjusted using these blanks by subtracting the average consumption of the blanks for all treatments from the total oxygen consumption of each individual. Oxygen use was then converted to $\mu\text{mol L}^{-1} \text{ h}^{-1}$, and adjusted to the g AFDW of each individual.

2.2.6 – Statistical analyses

All survival analyses were conducted on the animals used for the 5 mo sample point. Differences in percentage survival to 5 mo between treatments were investigated using a Kaplan-Meier analysis. The effect of temperature and pH on the odds of survival was further examined with a binary logit generalised linear model. Generalised linear regression models were used to assess which of the factors (temperature, pH, and temperature*pH interaction) explained a significant amount of variation in survival. The Akaike Information Criterion (AIC) and ΔAIC were then used to select which of the significant models was the most parsimonious (Richards, 2005).

The significance of the effect of pH and temperature on survival, physical and physiological condition and respiration were tested with a between-groups factorial ANOVA when assumptions of normality and homogeneity of variances were met. When these assumptions were violated, a Kruskal-Wallis test was used. The relationships between respiration rate and each physiological condition index ($\text{CI}_{\text{FW:SL}}$ and $\text{CI}_{\text{FW:SW}}$) were also investigated. Spearman's correlation coefficient was used, as even with

transformation, data did not meet assumptions of homoscedasticity. Analyses were conducted with SPSS Version 22, and $\alpha = 0.05$ in all cases.

2.3 – Results

2.3.1 – *Experimental conditions*

Temperature, pH, and salinity data were averaged over the whole experiment (Temperature and pH: $n \geq 14,331$; salinity: $n = 12,432$). Average salinity was 34.66 ± 0.004 psu (mean \pm SE). Experimental seawater conditions are presented in Table 2.3. Temperatures were distinctly separate among all three temperature levels. While the target of the coldest temperature treatment was -1.7°C , the coldest average temperature that could be achieved was -1.4°C . However, this coldest temperature was still consistently separate from the other treatments. Each pH level was consistent with target levels and separate from the other levels (Table 2.3). Ar ranged from 2218.8 to 2286.1 on average (Table 2.3). There was a well-defined difference in Ω_{Ar} with pH treatment. $\Omega_{\text{Ar}} \approx 1$ (average Ω_{Ar} of 1.19, range = 0.91-1.06) at ambient pH (pH 8.00), while pH 7.85 produced an average Ω_{Ar} of 0.73 (range = 0.66-0.78), and Ω_{Ar} was consistently lowest at pH 7.65 (average Ω_{Ar} of 0.46, range = 0.43-0.51; Table 2.3).

Table 2.3 - Seawater chemistry of the experimental treatments over the course of the experiment.

Target Experimental Treatment	Temperature (°C) (meas.)	pH (meas.)	A _T (μmol kg ⁻¹) (meas.)	DIC (μmol kg ⁻¹) (calc.)	pCO ₂ (ppm) (calc.)	Ω _{Ar} (calc.)	Ω _{Ca} (calc.)	[CO ₃ ²⁻] (μmol kg ⁻¹) (calc.)	[HCO ₃ ⁻] (μmol kg ⁻¹) (calc.)
-1.7°C/pH 8.00	-1.440 ± 0.0006	8.000 ± 9.18 × 10 ⁻⁵	2265.3 ± 16.0	2201.2 ± 23.3	536.8 ± 37.1	0.969 ± 0.045	1.544 ± 0.072	64.2 ± 3.0	2101.3 ± 23.9
-1.7°C/pH 7.85	-1.406 ± 0.0010	7.847 ± 0.0001	2260.1 ± 26.1	2247.8 ± 7.3	841.7 ± 126.1	0.660 ± 0.104	1.053 ± 0.166	43.8 ± 6.9	2148.2 ± 8.7
-1.7°C/pH 7.65	-1.452 ± 0.0010	7.650 ± 0.0001	2253.0 ± 26.0	2295.5 ± 9.3	1312.6 ± 161.4	0.432 ± 0.059	0.689 ± 0.095	28.6 ± 3.9	2179.7 ± 16.1
-0.5°C/pH 8.00	-0.522 ± 0.0003	7.986 ± 0.0001	2251.1 ± 28.8	2197.8 ± 2.0	624.1 ± 128.7	0.906 ± 0.185	1.443 ± 0.295	60.1 ± 12.3	2097.8 ± 2.1
-0.5°C/pH 7.85	-0.512 ± 0.0003	7.845 ± 6.46 × 10 ⁻⁵	2256.8 ± 27.6	2223.5 ± 11.0	738.5 ± 93.0	0.777 ± 0.106	1.238 ± 0.169	51.5 ± 7.1	2125.4 ± 9.9
-0.5°C/pH 7.65	-0.553 ± 0.0005	7.643 ± 8.78 × 10 ⁻⁵	2250.3 ± 31.6	2288.0 ± 9.4	1317.6 ± 217.2	0.454 ± 0.082	0.723 ± 0.131	30.1 ± 5.4	2173.4 ± 17.9
+0.5°C/pH 8.00	+0.496 ± 0.0002	7.999 ± 4.26 × 10 ⁻⁵	2256.8 ± 26.7	2180.9 ± 8.5	535.7 ± 69.3	1.063 ± 0.141	1.691 ± 0.224	70.5 ± 9.4	2077.5 ± 3.4
+0.5°C/pH 7.85	+0.503 ± 0.0004	7.840 ± 8.56 × 10 ⁻⁵	2254.8 ± 30.2	2226.3 ± 4.8	803.8 ± 160.2	0.760 ± 0.155	1.210 ± 0.246	50.4 ± 10.3	2126.4 ± 4.4
+0.5°C/pH 7.65	+0.492 ± 0.014	7.650 ± 0.0004	2253.0 ± 27.7	2277.4 ± 2.8	1245.3 ± 237.3	0.508 ± 0.102	0.809 ± 0.162	33.7 ± 6.7	2167.1 ± 10.7

Data are presented as means ± SE. pH on the total hydrogen scale. (meas.) = measured value; (calc.) = calculated value.

Temperature and pH averages are based on > 14,000 records over the experiment. A_T and DIC are presented as the average of water samples taken on Days 42 and 143 (one per treatment per time point, n = 2). Calculated values used measured values of temperature, pH, and A_T for each treatment, and an average salinity of 34.7.

2.3.2 – Survival

There were four mortalities during the 11 d temperature acclimation phase of the experiment. One occurred at -1.4°C , two at -0.5°C , and one at $+0.5^{\circ}\text{C}$. Mortalities were observed in every treatment throughout the experiment. Survival percentages are shown month-by-month for each treatment in Fig. 2.9, colour-coded to elucidate patterns. At the end of the experiment, survival was highest in the $-1.4^{\circ}\text{C}/\text{pH } 7.85$ treatment, and lowest in the $+0.5^{\circ}\text{C}/\text{pH } 7.65$ treatment (85.7 and 12.5%, respectively). Control animals ($-1.4^{\circ}\text{C}/\text{pH } 8.00$) exhibited good survival, with 75% still alive at the conclusion of the experiment, while most other treatments had less than 60% survival. Of particular note are the low survival rates after 5 mo at the highest temperature (37.5% at $+0.5^{\circ}\text{C}/\text{pH } 8.00$; 57.1% at $+0.5^{\circ}\text{C}/\text{pH } 7.85$, and 12.5% at $+0.5^{\circ}\text{C}/\text{pH } 7.65$). The $-0.5^{\circ}\text{C}/\text{pH } 8.00$ and $-0.5^{\circ}\text{C}/\text{pH } 7.65$ treatments also had particularly low survival, with both at $< 45\%$. Smaller increases in temperature and decreases in pH also generally reduced survival (Fig. 2.9, 5 mo).

Not only did some treatments have lower survival than others, but the path to reaching these final values was different. Survival curves for all treatments are presented in Fig. 2.10. There was generally good survival across treatments for the first two mo, with mortalities beginning to occur at 3-4 mo. The exception was an immediate reduction in survival at $+0.5^{\circ}\text{C}/\text{pH } 8.00$, declining to 75% just 1 mo into the experiment. There was a consistent decline in survival for this treatment throughout the experiment. By contrast, the $+0.5^{\circ}\text{C}/\text{pH } 7.65$ treatment demonstrated good survival until 4-5 mo, after which animals suddenly and rapidly died off. The most severe mortalities occurred between 4 and 5 mo, with the percentage of animals alive in the $-0.5^{\circ}\text{C}/\text{pH } 8.00$ treatment halving between these two times. This die-off was visible in the lowest pH at all temperatures: i.e., $-1.4^{\circ}\text{C}/\text{pH } 7.65$; $-0.5^{\circ}\text{C}/\text{pH } 7.65$; and $+0.5^{\circ}\text{C}/\text{pH } 7.65$ (Fig. 2.10c).

1 mo	pH	8.00	100	85.7	75
		7.85	100	100	100
		7.65	100	100	100
			Temp (°C)		
		-1.4	-0.5	+0.5	

2 mo	pH	8.00	100	85.7	75
		7.85	100	100	100
		7.65	100	75	100
			Temp (°C)		
		-1.4	-0.5	+0.5	

3 mo	pH	8.00	87.5	85.7	75
		7.85	85.7	87.5	100
		7.65	85.7	75	100
			Temp (°C)		
		-1.4	-0.5	+0.5	

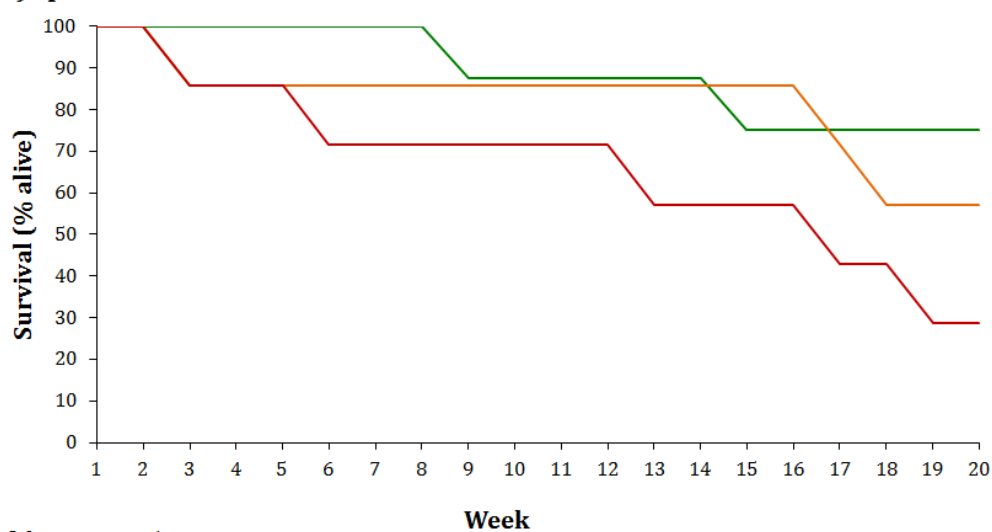
Key (% alive):	
	80-100
	60-79
	40-59
	20-39
	0-19

4 mo	pH	8.00	75	85.7	62.5
		7.85	85.7	87.5	85.7
		7.65	85.7	75	75
			Temp (°C)		
		-1.4	-0.5	+0.5	

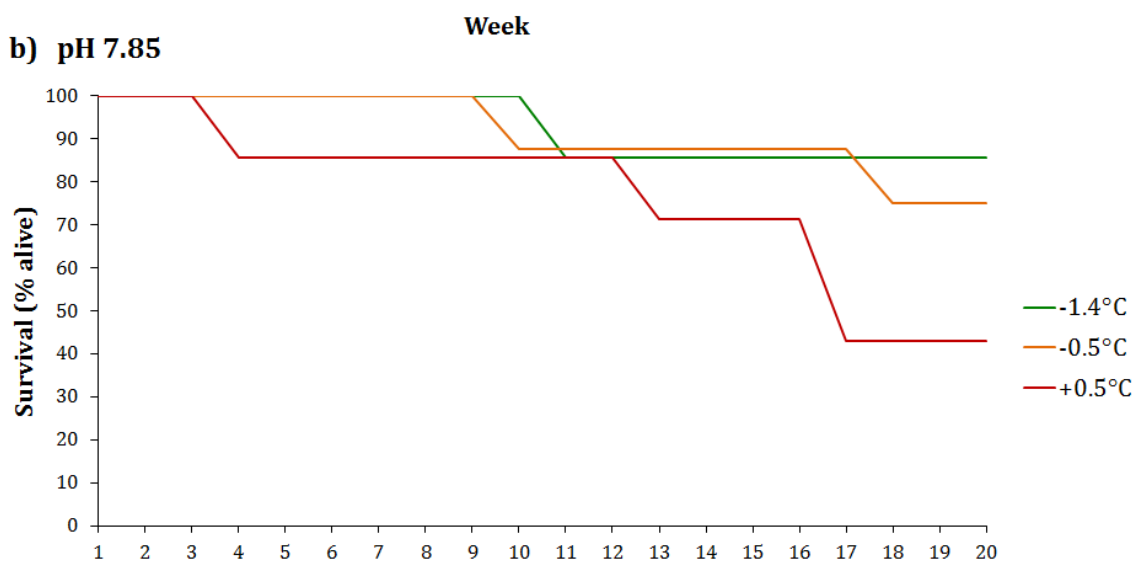
5 mo (end)	pH	8.00	75	42.9	37.5
		7.85	85.7	75	57.1
		7.65	57.1	37.5	12.5
			Temp (°C)		
		-1.4	-0.5	+0.5	

Figure 2.9 - Percent survival of *Laternula elliptica* in each treatment after each month of exposure to experimental conditions.

a) pH 8.00



b) pH 7.85



c) pH 7.65

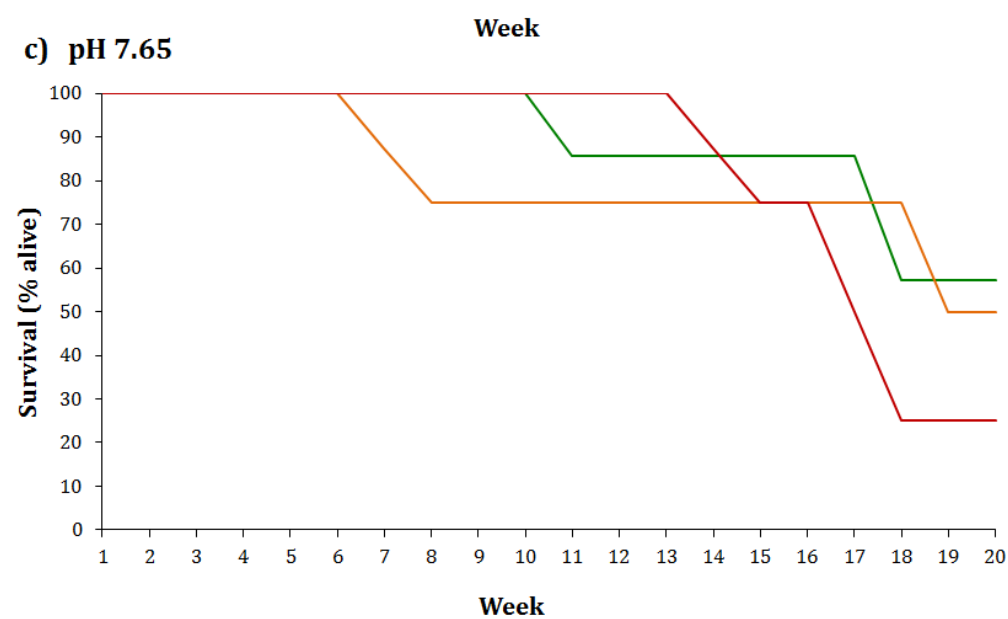


Figure 2.10 - Percent survival of *Laternula elliptica* over 5 mo at experimental conditions. Results are plotted by pH: a) pH 8.00; b) pH 7.85; and c) pH 7.65.

There were also patterns in survival with temperature. This is clearly illustrated when temperatures are considered at each pH level (Fig. 2.10); survival decreased with temperature at each pH level, being greatest at ambient temperature and least at the highest temperature. This pattern was evident throughout the experiment, and was particularly distinct for the final three wk. Animals at +0.5°C generally had an earlier onset of mortalities than those at ambient temperature at pH 8.00 and pH 7.85. *L. elliptica* at +0.5°C/pH 7.65 survived extremely well throughout much of the experiment, with 100% survival up until 13 wk, after which time survival plummeted abruptly to the lowest survival of all treatments (Fig. 2.10c). Increases in temperature negatively affected survival in *L. elliptica* adults. By contrast, pH did not have as great an effect on survival as temperature. Despite the patterns discussed above, when the survival curves were compared with a Kaplan-Meier analysis they were non-significant across treatments (log rank $\chi^2_{(8)} = 14.66$, $p = 0.066$).

2.3.2.1 – Time to 50% survival (LD_{50})

The 50% survival times (LD_{50}) for *L. elliptica* in each treatment are noted in Table 2.4. While statistical analyses could not be applied to these data ($n = 1$), there is an apparent trend of decreasing survival time with increasing temperature, at all pH levels. The lowest LD_{50} occurred in the +0.5°C treatments, with 50% survival reached in an average of 108 days. The -0.5°C treatments had the next-lowest LD_{50} , with an average LD_{50} of 136 days. All -1.4°C treatments exhibited the best survival, with none having reached 50% mortality by the end of the experiment, and projected values indicating a mean LD_{50} across all pH levels of 220 days. In contrast, there was no clear effect of pH.

Table 2.4 - Time to 50% survival (LD50) in days for *Laternula elliptica* exposed to each treatment.

		Temperature (°C)			Average
		-1.4	-0.5	+0.5	
pH	8.00	206 †	134	87	142
	7.85	288 †	141	119	183
	7.65	165 †	132	118	138
Average		220 †	136	108	

Key: † = Projected value, extrapolated because LD₅₀ had not yet been reached at the conclusion of this 5 mo-long experiment

2.3.2.2 – The odds of dying

An increase in temperature from -1.4°C to +0.5°C increased the odds of *L. elliptica* dying by 7.55 times. When a binomial probability distribution with a logit link function was fitted to the data at 5 mo, the odds of dying at ambient (-1.4°C) temperature were significantly lower than at elevated (+0.5°C) temperature ($p = 0.011$). There was no significant effect of pH on the odds of dying ($p = 0.075$).

2.3.2.3 – Modelling survival

Logistic regression models were fitted to survival data and compared using AIC values. Changes in temperature explained most of the patterns in survival observed throughout the experiment, with pH approaching significance but ultimately not affecting survival. Six models tested relevant combinations of temperature, pH, and temperature*pH against survival. Models were compared using AIC and Δ AIC values, and the four best are presented in Table 2.5. Each model's AIC was subtracted from the AIC of the reference model (chosen because it had the smallest AIC and was therefore the best fit) to give Δ AIC. Provided the model is significant overall, Δ AIC of < 2 indicates equally good fit to the reference model, Δ AIC of 4-7 indicates the model is probably not a good fit, and Δ AIC of > 10 indicates the model is highly unlikely to be the best model and can

be ignored (Richards, 2005). Consequently the reference model, with temperature as the only factor, was the best fit to *L. elliptica* survival data. A model with only pH had a ΔAIC of < 2 (Table 2.5) but was not significant overall, meaning that pH did not significantly explain patterns in survival. When other models were significant, temperature was the only significant factor, although pH was significant at the 10% level; however the large ΔAIC value for these models rendered them unsuitable (Table 2.5). Two other models poorly explained observed patterns in survival ($\Delta AIC > 20$; data not shown) and were rejected (Richards, 2005).

Table 2.5 - Summary of logistic regression models fitted to survival data. comparing combinations of temperature, pH, and temperature*pH that best explained changes in survival. The model of best fit, temperature ("Temp"), is contrasted with the other models. Models are listed in order of best fit.

Model	Akaike Information Criterion (AIC)	ΔAIC † (cf. "Temp" model)	Overall Model Significance	Factors in Model	Factor Significance
Temp (Reference model used in analysis)	16.31		0.006 **	Temp	0.011 *
pH	16.55	0.24	0.075	pH	0.087
Temp + pH	30.87	14.56	0.003 **	Temp	0.010 *
				pH	0.073
Temp*pH	38.60	22.29	0.039 *	Temp*pH	0.134

Key: * = significant at the 5% level; ** = significant at the 1% level; † = difference between the AIC of each model and the AIC of the reference model (the model with the smallest AIC).

2.3.3 – Physiological and physical condition

The *L. elliptica* adults in this study had full stomachs and full gonads when dissected at each time point, indicating that they were sufficiently fed throughout the experiment and were able to sustain gamete production. Spawning was occasionally observed in all treatments throughout the experiment. The *L. elliptica* specimens were generally large and heavy and in good health at the start of the study. Two physiological condition indices were assessed: $CI_{FW:SL}$ and $CI_{FW:SW}$. Individuals with high ratios of flesh weight to

the weight or length of their shell were considered healthy and in good condition. Physical condition concerns the weight of the shell relative to its length ($CI_{SW:SL}$), with a decrease in SW relative to shell length SL indicating dissolution, and an increase indicating calcification, provided SL remains constant.

2.3.3.1 – 5 wk

After 5 wk exposure to experimental conditions, there was no significant difference in $CI_{SW:SL}$ among treatments (Temperature: $F_{(2,62)} = 0.21$, $p = 0.813$; pH: $F_{(2,62)} = 0.36$, $p = 0.700$; Temperature*pH: $F_{(4,62)} = 0.20$; $p = 0.936$). There was also no effect of treatment on either of the physiological CIs (i.e., $CI_{FW:SL}$: Temperature: $F_{(2,62)} = 1.58$, $p = 0.215$; pH: $F_{(2,62)} = 0.64$, $p = 0.529$; Temperature*pH: $F_{(4,62)} = 0.14$; $p = 0.969$; $CI_{FW:SW}$: Temperature: $F_{(2,62)} = 1.35$, $p = 0.267$; pH: $F_{(2,62)} = 0.36$, $p = 0.703$; Temperature*pH: $F_{(4,62)} = 0.209$; $p = 0.933$).

2.3.3.2 – 5 mo

There was no significant difference in $CI_{SW:SL}$ treatments at 5 mo (Kruskal-Wallis $\chi^2_{(8)} = 9.254$, $p = 0.321$). The physiological condition of animals exposed to ambient conditions was relatively high, and there was a noticeable decline in condition at higher temperatures (Fig. 2.11). This pattern was noted for both $CI_{FW:SL}$ and $CI_{FW:SW}$, indicating that flesh weight declined relative to both shell weight and length over 5 mo. $CI_{FW:SL}$ of *L. elliptica* held at ambient temperature was significantly different to those from the -0.5 and +0.5°C treatments ($p = 0.026$ and $p = 0.004$, respectively) at 5 mo, but did not differ between -0.5 and +0.5°C treatments ($p = 0.477$) (Fig. 2.11a). pH did not significantly affect $CI_{FW:SL}$ ($F_{(2,29)} = 0.92$, $p = 0.410$), and there was no interaction between factors (Temperature*pH: $F_{(4,29)} = 0.60$, $p = 0.664$). The highest $CI_{FW:SW}$ was found in ambient temperature animals (Fig. 2.11b), and decreased significantly with temperature ($F_{(2,29)} = 6.61$, $p = 0.004$; Fig. 2.11b), again with no effect of pH ($F_{(2,29)} = 0.62$, $p = 0.544$) and no interaction ($F_{(4,29)} = 1.19$, $p = 0.338$). There were significant losses in soft tissue relative to shell weight at +0.5°C, but not at -0.5°C, as evidenced by a significant decrease in

$CI_{FW:SW}$ between the -1.4 and +0.5°C treatments ($p = 0.002$), while the -0.5°C treatments were similar to both -1.4 and +0.5°C ($p = 0.133$; Fig. 2.11b).

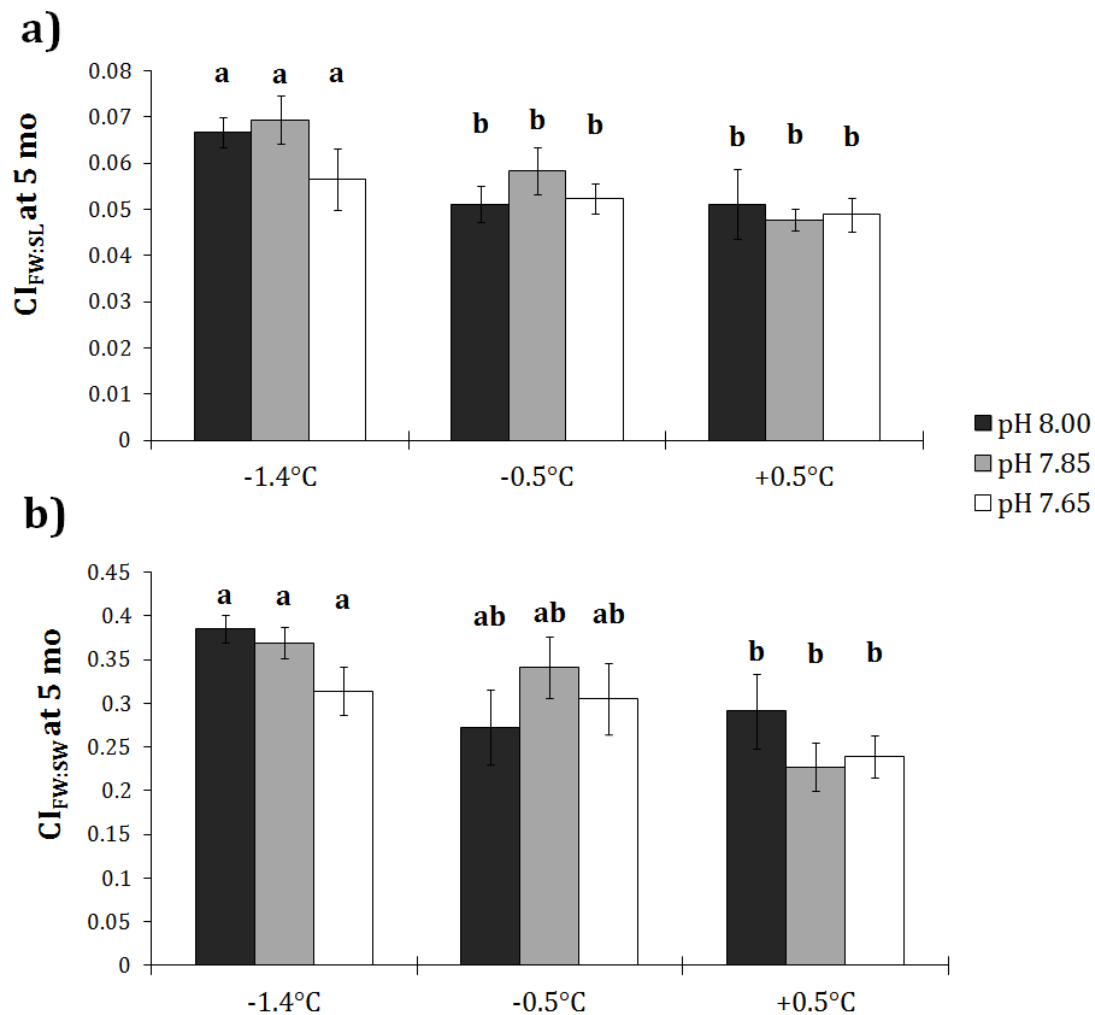


Figure 2.11 - Physiological condition of *Laternula elliptica* after 5 mo exposure to experimental conditions: a) $CI_{FW:SL}$; b) $CI_{FW:SW}$. Statistically different means at $\alpha < 0.05$ are indicated by different letters. Data are presented as means \pm SE.

2.3.3.3 – Change between 5 wk and 5 mo

In the 16 wk between the 5 wk and 5 mo time points, change in $CI_{SW:SL}$ was similar across treatments. $CI_{SW:SL}$ ranged from 0.11-0.25 in all treatments over the course of the

experiment and there was no significant percentage change in $CI_{SW:SL}$ with pH or temperature over this period (Kruskal-Wallis $\chi^2_{(8)} = 12.41$, $p = 0.134$). Consequently, neither elevated temperature nor lowered pH affected the change in the ratio of shell weight to shell length in adult *L. elliptica* during the experiment.

While there were relatively small decreases in both $CI_{FW:SL}$ and $CI_{FW:SW}$ at ambient temperature (Fig. 2.12), these indices declined significantly in individuals from elevated temperature treatments between 5 wk and 5 mo (Fig. 2.12). There was a significant percentage loss of $CI_{FW:SL}$ at increased temperature (Temperature: $F_{(2,29)} = 4.83$, $p = 0.016$) but not with pH, and nor was there any interaction between stressors (pH: $F_{(2,29)} = 1.96$, $p = 0.160$; Temperature*pH: $F_{(4,29)} = 0.68$, $p = 0.614$; Fig. 2.12a). Percent loss of $CI_{FW:SL}$ at ambient temperature was significantly different to both -0.5 and $+0.5^\circ\text{C}$ treatments ($p = 0.020$ and $p = 0.038$, respectively), while -0.5 and $+0.5^\circ\text{C}$ produced similar condition loss ($p = 0.987$; Fig. 2.12a). $CI_{FW:SL}$ declined by 15-28% at -0.5°C , and by 20-25% in the $+0.5^\circ\text{C}$ treatments (Fig. 2.12a).

Similarly, $CI_{FW:SW}$ was deleteriously affected at elevated temperature ($F_{(2,29)} = 4.47$, $p = 0.020$; Fig. 2.12b), but pH and the interaction between stressors were both non-significant (pH: $F_{(2,29)} = 0.62$, $p = 0.546$; Temperature*pH: $F_{(4,29)} = 1.18$; $p = 0.341$). The effect of temperature on $CI_{FW:SW}$ was similar to that for $CI_{FW:SL}$, with a 15-34% loss at $+0.5^\circ\text{C}$ (Fig. 2.12b). This was due to significant differences between -1.4 and $+0.5^\circ\text{C}$ ($p = 0.015$; Fig. 2.12b). These percentage changes in $CI_{FW:SL}$ (Fig. 2.12a) and $CI_{FW:SW}$ (Fig. 2.12b) represent a ~ 4 -8% reduction in initial body weight per month relative to SL and SW. There are indications of greater loss of condition at the lowest pH (7.65) at ambient temperature for both indices (Fig. 2.12a,b). Similarly, at pH 7.65 the loss in $CI_{FW:SW}$ was greater at higher temperatures. However, neither of these trends were statistically significant, and pH ultimately had no significant impact on physiological condition in *L. elliptica*.

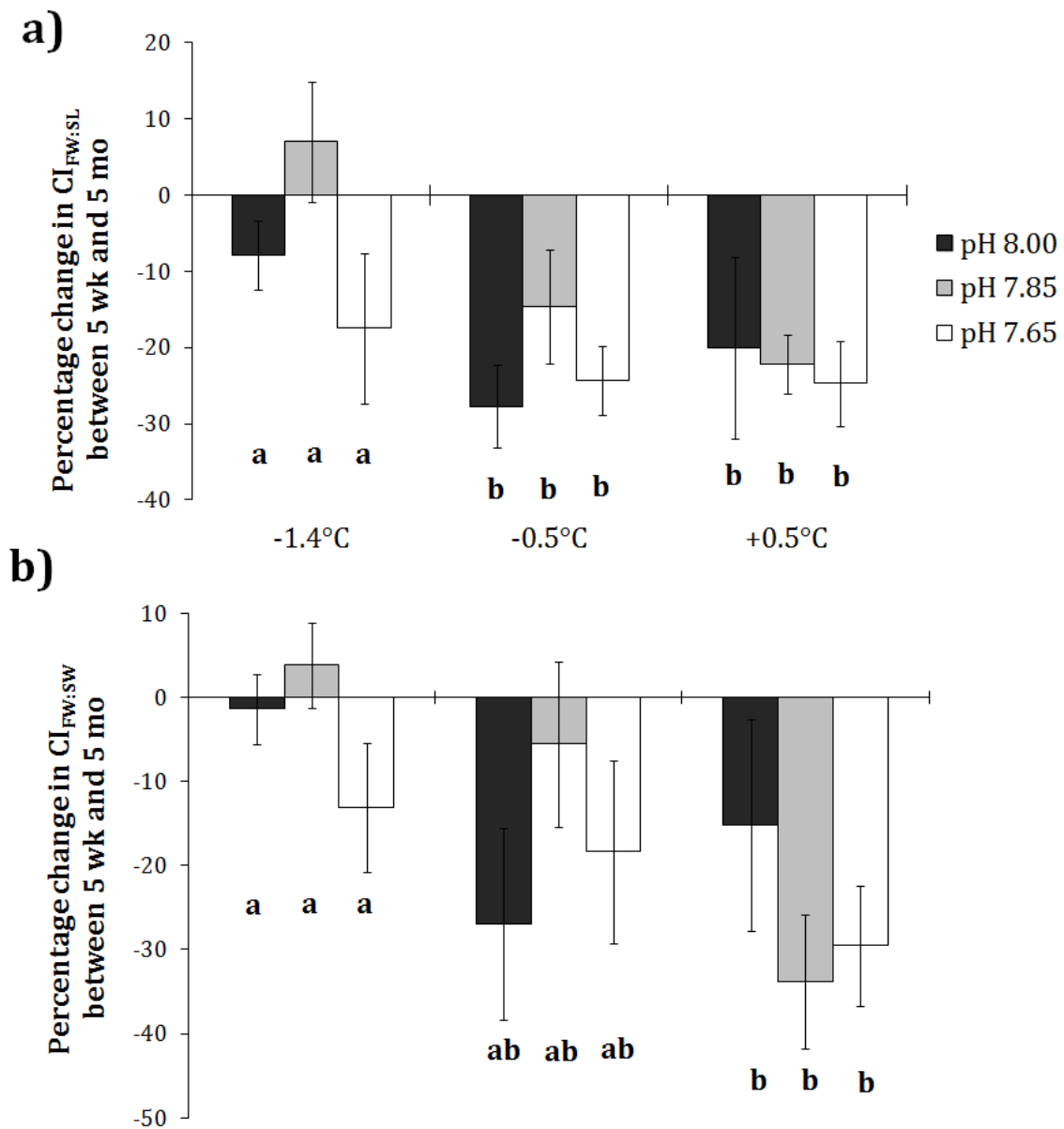


Figure 2.12 - Percentage change of physiological condition of *Laternula elliptica* between 5 wk and 5 mo: a) $CI_{FW:SL}$; b) $CI_{FW:SW}$. Statistically different means at $\alpha < 0.05$ are indicated by different letters. Data are presented as means \pm SE.

2.3.4 - O_2 consumption

By Day 133, when O_2 consumption measurements started, there had been 15 mortalities in the +0.5°C treatments (6 individuals at pH 8.00, 4 at pH 7.85, and 5 at pH 7.65) and 9 mortalities at -0.5°C (3 individuals at pH 8.00, 2 at pH 7.85, and 4 at pH

7.65). Consequently, and because of the time consuming nature of the O₂ consumption measurements, a limited number of pH treatments were targeted for these assessments: predominantly pH 8.00 and 7.65, with one 7.85 treatment also assessed (+0.5°C/pH 7.85).

Overall, O₂ consumption was not significantly affected by the experimental treatments (Kruskal-Wallis $\chi^2_{(6)} = 3.67$, $p = 0.722$). This was in part due to the high inter-individual variation within the elevated temperature and lowered pH treatments (Fig. 2.13). In contrast, control animals had extremely consistent respiration rates (note the extremely small SE for respiration rates in the ambient treatment in Fig 2.13). The +0.5°C/pH 8.00 animals had respiration rates similar to each other and comparable to those of control animals (Fig. 2.13). Individuals from all other treatments exhibited variable and relatively high rates. The exception was the +0.5°C/pH 7.65 treatment, which was represented by a single individual. Relative to the mean respiration rate of the control (-1.4°C/pH 8.00) animals, O₂ consumption of -1.4°C/pH 7.65 animals increased by 111%; -0.5°C/pH 8.00 increased by 80%; -0.5°C/pH 7.65 increased by 67%; and +0.5°C/pH 7.85 increased by 119% (Fig. 2.13).

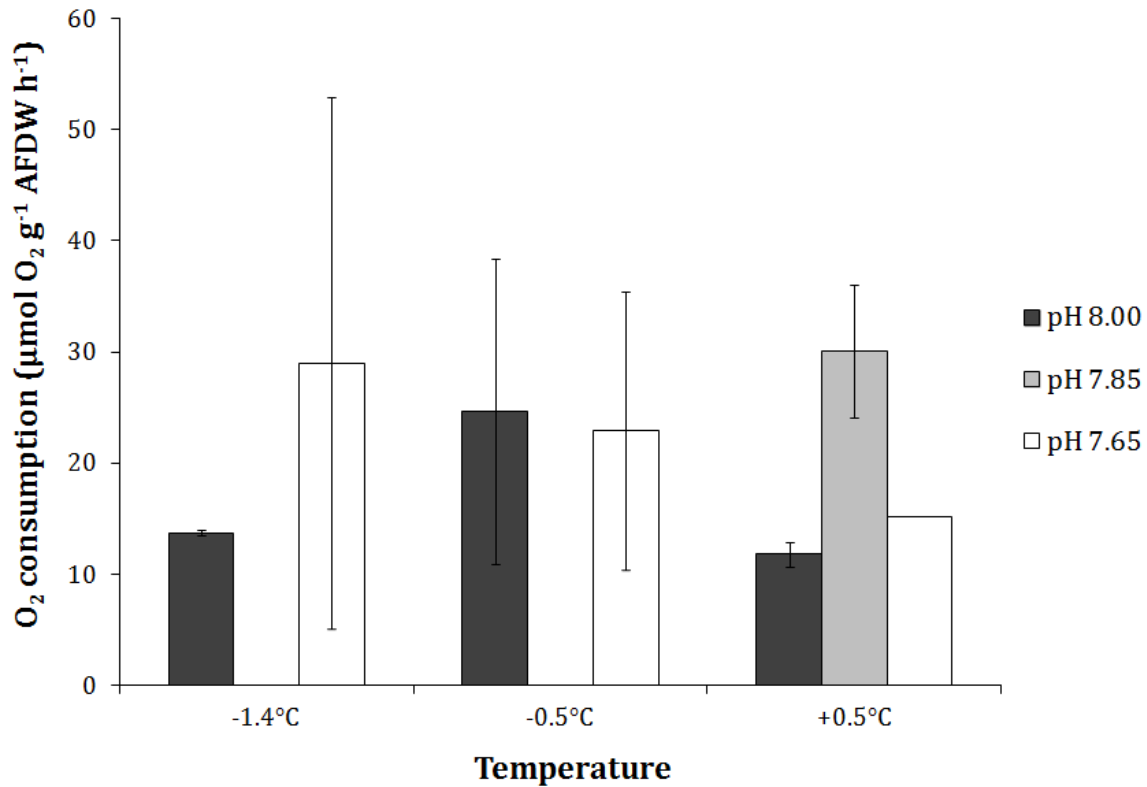


Figure 2.13 – Respiration in *Laternula elliptica* after 20 weeks at experimental conditions, expressed as $\mu\text{mol O}_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$; means \pm SE. For sample sizes, refer to Table 2.2.

2.3.4.1 – Relationship between O_2 consumption and physiological condition

O_2 consumption was significantly negatively correlated with $\text{CI}_{\text{FW:SL}}$ (Spearman's $\rho = -0.647$, $p = 0.007$, $n = 16$; Fig. 2.14a). As condition decreased, there was a strong increase in O_2 consumption, to levels $\sim 10\times$ that of the animal with the highest CI. This relationship exhibits heteroscedastic funnelling: when condition was relatively high, respiration was fairly tightly clustered and consistently low; but as $\text{CI}_{\text{FW:SL}}$ decreased, O_2 demand increased, and variability in this demand also increased (Fig. 2.14a). There was a much greater variation in O_2 consumption in lower-condition animals, regardless of treatment. Within this main relationship, not only were ambient-pH individuals in better condition generally (note circled symbols in Fig. 14a), but those at lower pHs (crosses and circles) were generally in worse condition. Furthermore, increased variability in O_2 demand with reduced condition was most often evident at pH 7.85 and 7.65. All pH 8.00 individuals except one exhibited similar (low) O_2 consumption rates,

regardless of temperature. When comparing $CI_{FW:SL}$ with respiration rate, pH patterns emerged that are more apparent than when condition indices alone are examined between treatments (see Section 2.3.3 above).

A similar relationship was observed between O_2 consumption rate and $CI_{FW:SW}$ (Fig. 2.14b). While a negative trend was also suggested, it was not statistically significant (Spearman's $\rho = -0.376$, $p = 0.151$, $n = 16$). Similar to the pattern noted for $CI_{FW:SL}$, there was an increase in O_2 consumption rate with a decrease in $CI_{FW:SW}$, and this relationship was heteroscedastic. There was also a pH-specific response in O_2 demand with condition change; pH 8.00 treatments had low, tightly-clustered O_2 consumption rates, particularly in control individuals, which were so similar they overlap in Fig. 2.14b. Individuals from the ambient-pH treatments generally retained low O_2 consumption rates across all temperatures, irrespective of $CI_{FW:SW}$.

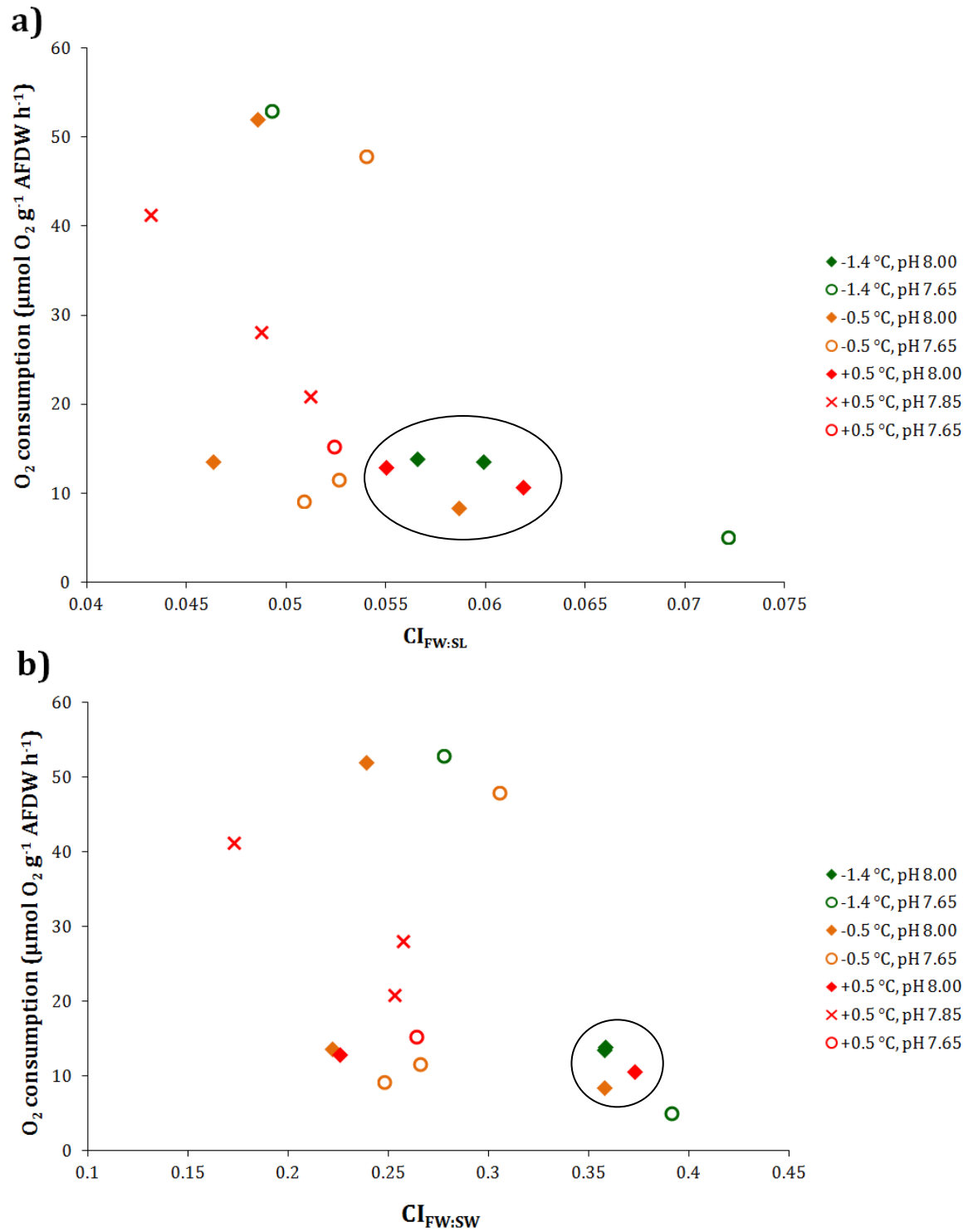


Figure 2.14 - Respiration in *Laternula elliptica* compared to physiological condition at 5 mo: a) ratio of flesh weight to shell length ($CI_{FW:SL}$); b) ratio of flesh weight to shell weight ($CI_{FW:SW}$). Respiration is measured as O_2 consumption ($\mu\text{mol } O_2 \text{ g}^{-1} \text{ AFDW h}^{-1}$). The oval and circle show the low, tight grouping of most pH 8.00 individuals.

2.4 - Discussion

Increased temperature negatively affected the survival, physiological condition, and functioning of adult *L. elliptica*. In contrast, lowered pH had a comparatively minor effect. *L. elliptica* physiological condition was not affected after 5 wk at experimentally elevated temperatures and lowered pHs. Consistent maintenance of physical condition was noted among treatments at both 5 wk and 5 mo, however by 5 mo there were strong trends of increasing mortality and a significant loss in physiological condition with elevated temperature. After 5 mo, respiration was also affected by temperature increase and a reduction in pH, with an increase in both average O₂ consumption and the variability of this consumption with increased temperature and reduced pH. O₂ consumption was negatively correlated with physiological condition, and was particularly high when temperature was elevated and pH was lowered, but stayed constant under ambient pH conditions. Multiple aspects of health and function were negatively impacted by warming in this stenothermal bivalve; temperature increases predicted for 2050 and 2100 had a greater impact on adult *L. elliptica* than pH reductions predicted for this period.

2.4.1 - Survival

Bivalves generally experience higher survival with lowered pH (Hendriks *et al.*, 2010), but in the present study, survival rates in *L. elliptica* adults did not change with pH. Elevated temperatures, however, had a negative effect on survival. Mortalities occurred earliest, and were highest and most constant at +0.5°C. In addition, *L. elliptica* were 7.55 times more likely to die when exposed to this temperature, 2°C above ambient conditions. The effect of temperature on survival is consistent with *L. elliptica* being strongly stenothermal (Peck *et al.*, 2004; 2014). Considering that a 2.5-5% decrease in survival was modelled to reduce population sizes to less than 30% of current levels in McMurdo Sound *L. elliptica* within 60 y (Guy *et al.*, 2014), the 87.5% decreases in survival found here with only +2°C warming represent a potentially catastrophic loss in

L. elliptica population sizes, not only immediately, but also beyond the end of the century as a result of a hugely reduced spawning stock.

Decreases in pH from ambient levels did not significantly affect *L. elliptica* survival (Fig. 2.9). Interestingly, in the lowest pH treatments (pH 7.65, -0.35 units below ambient), survival was initially high before rapid die-offs occurred near the end of the experiment (Fig. 2.10), indicating that reduced pH accelerated the rate of declines in survival once they occurred. Were this phenomenon to occur in wild populations in response to ocean warming and acidification, these results indicate that even if *L. elliptica* populations were initially able to withstand the effects of these stressors, over timescales of greater than 5 mo they would likely experience significantly reduced population sizes. In contrast, an intermediate decrease in pH seemed to improve survival in *L. elliptica*; at pH 7.85 (-0.15 units below ambient) survival was higher overall than at pH 8.00 or 7.65. Reductions in pH (-0.33 units below ambient) also prevented metabolic repression and improved the tolerance of blue mussels (*M. edulis*) to warming (Zittier *et al.*, 2015). The exact reasons for this are yet unknown (Zittier *et al.*, 2015), but if they are also occurring in *L. elliptica*, these mechanisms may confer the improved survival with slight pH reduction (and the associated hypercapnia) seen here. Intermediate decreases in pH may actually have allowed *L. elliptica* to compensate for negative effects of temperature, at least in the short term.

Some treatments (especially +0.5°C/pH 8.00 and +0.5°C/pH 7.85) exhibited a constant rate of mortality throughout the experiment (Fig. 2.10), as in Morley *et al.* (2012a), while others (particularly +0.5°C/pH 7.65) produced few mortalities initially before sudden heavy losses occurred after 3 mo of exposure (Figs. 2.9, 2.10). Survival patterns similar to the latter trend were associated with decreased pH in the clam *Ruditapes decussatus* (Range *et al.*, 2011). For *R. decussatus*, survival in reduced pH treatments declined after energy output could no longer be maintained. This occurred suddenly, following an energetically costly spawning event (Range *et al.*, 2011). *R. decussatus* showed a constant rate of mortality at pH 7.53 (an experimental -0.7 change in pH units) and good initial survival at pH 8.16 and 7.82 (ambient pH and a -0.4 unit change, respectively; Range *et al.*, 2011). These two latter treatments produced noticeably reduced survival after spawning occurred partway through the experiment. The

differences in ultimate survival and the sudden onset of mortalities (for both pH 8.16 and 7.82) was linked to the energetic cost of spawning observed in these two treatments (Range *et al.*, 2011). *L. elliptica* in this study experienced comparable patterns but in response to different stressors: consistently reduced survival occurred at elevated temperatures (+0.5°C), while pH 7.65 (a -0.35 unit pH change) produced good initial survival and sudden declines later in the experiment (Figs. 2.9, 2.10). The fact that *L. elliptica* in this study spawned before and during the experiment represents a potential drain on energy resources. However, as spawning occurred throughout all treatments, the costs of spawning alone do not fully explain the sudden onset of mortalities.

2.4.2 – Physical and physiological condition

2.4.2.1 – Physical condition

L. elliptica adults maintained physical condition throughout the experiment, with no significant change with pH and temperature. *L. elliptica* calcifies slowly, and minimal if any net shell weight gain or length increase would typically occur over this period (Brey & Mackensen, 1997; Ahn *et al.*, 2001, 2003; Peck, 2005b; Sleight *et al.*, 2015), particularly in the adults used here. Mussels and sea urchins can dissolve in response to internal acidification (Doney *et al.*, 2009), and dissolution rates in excess of calcification rates can potentially result in thinner and lighter shells with the same surface area (Gazeau *et al.*, 2013). However, *L. elliptica* adults maintained their relative shell weight under reduced pH in this experiment. Since empty *L. elliptica* valves exhibited significant dissolution after 4 wk at pH 7.4 (McClintock *et al.*, 2009), constant shell mass here is evidence that the live animal is successfully compensating when exposed to reduced pH. This resilience may be partially due to the protection afforded by the 10 µm-thick periostracum surrounding the outer surface of the thin shell in *L. elliptica* (Clark *et al.*, 2010; Sato-Okoshi *et al.*, 2010). This retention of physical condition was also found by Cummings *et al.* (2011) after exposing *L. elliptica* to pHs both above and below current ambient levels for 4 mo, and has also been found in other species (Range *et al.*, 2011; Langer *et al.*, 2014).

All treatments with ambient pH (pH 8.00) were supersaturated with respect to aragonite at one time point and only just undersaturated at the other, resulting in a mean Ω_{Ar} close to 1, and signifying that aragonite supersaturation occurred for periods during the experiment (Table 2.3). On the contrary, both reduced pH treatments were consistently undersaturated, with a mean Ω_{Ar} of 0.73 and 0.46, at pH 7.85 and 7.65, respectively (Table 2.3). Consequently, *L. elliptica* adults were able to maintain physical condition under extended exposure to conditions considered stressful for calcifiers; undersaturation requires additional energy for shell maintenance, as shell dissolution can occur at $\Omega_{Ar} < 1$ (Green *et al.*, 2004; Miller *et al.*, 2009; Roleda *et al.*, 2012).

While *L. elliptica* adults maintained shell mass in response to both OA and warming, there is likely an associated energetic cost for the organism. Bivalves, as extracellular calcifiers, rely on the active transport of ions to produce conditions suitable for calcification (Miller *et al.*, 2009; Thomsen *et al.*, 2015). Calcification becomes more energetically costly under acidification as the correct ionic balance becomes harder to maintain with internal pH change (Guinotte & Fabry, 2008; Miller *et al.*, 2009; Pan *et al.*, 2015). Nonetheless, some adult and larval bivalves can calcify even in acidic (pH < 7) estuarine environments, indicating species-specific calcification abilities (Miller *et al.*, 2009; Ries *et al.*, 2004; Hendriks *et al.*, 2010). In some cases, acidification even promotes calcification, as $[H^+]$ increase in the surrounding seawater can make ion transport across cell membranes, and calcification, easier (Ries *et al.*, 2009; Hendriks *et al.*, 2010; Christensen *et al.*, 2011). Temperature increase, provided it is within a species' tolerance level, can reduce negative effects of acidification, such as hypercapnia (Byrne *et al.*, 2014). However, the response of *L. elliptica* in this study to both warming and acidification involved the maintenance of shell condition, likely due to continued biomineralisation. Preserving physical condition in this way potentially comes at the cost of physiological condition, as detailed below.

2.4.2.2 – Physiological condition

While *L. elliptica* grow relatively rapidly when resources are available, they are typically resource-limited (the summer burst in primary productivity is short in duration), growing very slowly and only laying down growth rings annually (Brey & Mackensen, 1997; Ahn *et al.*, 2001, 2003). Consistent with this, SL was constant throughout the experiment; therefore any change in physiological condition is due to a change in mass, not length, of an individual. The lack of change in condition among treatments at 5 wk is not particularly surprising, as animals had only been exposed to experimental conditions for a relatively short time. *L. elliptica* adults proved resilient over 5 wk, but it is their response to longer-term 5-mo exposure that is more biologically meaningful and of more interest in this study.

Temperature increases resulted in significant loss of physiological condition in *L. elliptica*. $CI_{FW:SW}$ and $CI_{FW:SL}$ are valuable indices for comparing metabolism used for calcification (shell weight) with that used for the maintenance and growth of gametic and somatic tissue (flesh weight) (Crosby & Gale, 1990) and are an indication of relative physiological stress and health in bivalves (Roper *et al.*, 1991). Both CIs decreased by 15-30% with warming (Fig. 2.12). This tissue loss is potentially due to the utilisation and reduction of muscle proteins, particularly from the siphons, which form the bulk of the muscle tissue in *L. elliptica* (Ahn *et al.*, 2003). Conversely, pH did not significantly affect physiological condition in *L. elliptica* in this experiment (Fig. 2.12). Similarly, Cummings *et al.* (2011) found no significant change in $CI_{FW:SL}$ in *L. elliptica* with a decrease in pH to 7.78. Warming is therefore the main driver of reduced physiological condition in *L. elliptica* in this study. This loss of soft tissue indicates that *L. elliptica* are losing crucial sources of protein (Brockington, 2001; Ahn *et al.*, 2003), and are not able to acclimate to increased temperature. With longer-term exposure to warmed conditions, *L. elliptica* would likely continue to lose body mass (here, at the rate of ~4-8% reduction in initial body weight relative to SL per month) and eventually die.

This significant weight loss may be due in part to the physical cost of maintaining the aragonite shell in undersaturated conditions. This rationale is supported by Wood *et al.* (2008), who found that a brittle star burned calories to maintain $CaCO_3$ structures

under acidification, and lost muscle mass as a result. Other studies found no change in weight with acidification (clam *Ruditapes decussatus*, Range *et al.*, 2011; gastropod *Subnivalia undulata*, Coleman *et al.*, 2014). However, other species have experienced weight loss with warming (bivalve *Macoma balthica*, Beukema *et al.*, 2009; gastropod *Austrocochlea porcata*, Coleman *et al.*, 2014). The ability of a species to maintain the weight of their soft tissue, typically by keeping levels of energy expenditure below energy gain, is variable, depending on species and environmental context, and has consequences for long-term survival (Beukema *et al.*, 2011).

2.4.3 – O₂ consumption

L. elliptica exposed to elevated temperature and reduced pH exhibited higher respiration rates and increased variability in O₂ demand (Figs. 2.13, 2.14). The individual variation in respiration rate with thermal stress found here in *L. elliptica* was also found by Morley *et al.* (2009) and Peck *et al.* (2015). Comparatively, hypercapnic blue mussels (*Mytilus edulis*) maintained their O₂ consumption rates at control temperatures, but had slightly greater increases in O₂ demand with temperature rise than normocapnic mussels (Zittier *et al.*, 2015). The fact that *L. elliptica* in two of the +0.5°C treatments had O₂ consumption rates similar to control animals may be a result of all individuals with higher O₂ requirements dying off earlier in the experiment. The remaining individuals may also have been stressed and moribund. Alternatively, decreases in pH may have a narcotic effect, and this may have reduced respiration rate in some individuals (Christensen *et al.*, 2011), although this was not noted for *L. elliptica* in this study, or when exposed to pH 7.78 in Cummings *et al.* (2011). However, marine invertebrates, including *L. elliptica*, may also repress metabolic demand in the face of environmental insult (Morley *et al.*, 2009; Christensen *et al.*, 2011; Navarro *et al.*, 2013; Zittier *et al.*, 2015); this may have occurred here with the two individuals in the +0.5°C treatments.

O₂ consumption rates can be used as a proxy for energy use and therefore metabolic demand (as in Brockington [2001]; Peck *et al.* [2002]; Heilmayer *et al.* [2004]; Morley *et al.* [2007, 2009]; Wood *et al.* [2008]; Zittier *et al.* [2015]). O₂ consumption in *L. elliptica*

varies by season, with a $\sim 3\times$ seasonal variation in metabolic rate between the active summer period and the repressed winter period (Brockington, 2001; Morley *et al.*, 2007). Animals in this study were acclimated to summer conditions, and similar respiration rates have been found in *L. elliptica* in summer (Peck *et al.*, 2002; Brockington, 2001; Morley *et al.*, 2007). Morley *et al.* (2007) found a 35% increase in *L. elliptica* respiration rate with warming to -0.4°C , and Cummings *et al.* (2011) found a 150% increase with lowered pH (pH 7.78); both are consistent in pattern to the 67-119% increase in mean respiration rate found here with elevated temperature and lowered pH. Interestingly, Ahn & Shim (1998) found respiration rates higher ($159\ \mu\text{mol O}_2\ \text{g}^{-1}\ \text{AFDW h}^{-1}$) and Morley *et al.* (2009) found rates lower (i.e., $\leq 14\ \mu\text{mol O}_2\ \text{g}^{-1}\ \text{AFDW h}^{-1}$) than those observed here ($\sim 12\text{-}30\ \mu\text{mol O}_2\ \text{g}^{-1}\ \text{AFDW h}^{-1}$); these differences may be due to site-specificity in response in *L. elliptica* (Morley *et al.*, 2012b).

High individual variation in O_2 consumption rates (Figs. 2.13, 2.14) has ramifications for population-level survival (Peck *et al.*, 2015). It is highly likely that amplified respiration leads to increased nutrient requirements, dramatic loss in condition, reduced growth, or even death (Doney *et al.*, 2012; Alcaraz *et al.*, 2014). In fact, *L. elliptica* adults experienced significant ($\sim 30\%$) loss in condition at -0.5 and $+0.5^{\circ}\text{C}$ (Fig. 2.12). Oxygen consumption also increased with a decrease in body weight in *L. elliptica* (Ahn & Shim, 1998), so the relationship found here between the loss of physiological condition and increased respiratory demand (Fig. 2.14) likely holds for *L. elliptica* populations in general. High pH buffered the effect of physiological condition loss on respiration demand. Almost all individuals at ambient pH (8.00) had low O_2 demand even when their condition was comparatively low, while individuals in similar condition but exposed to lower pH and/or warmer temperatures, had highly variable and generally elevated respiration rates (Fig. 2.14). As discussed above, this result may be due to elevated activity in response to undersaturated conditions ($\Omega_{\text{Ar}} < 1$; Table 2.3).

The stress response of *L. elliptica*, particularly to thermal stress, seems to involve an increase in respiration and heartbeat rates until the stress is too much to withstand, at which point both activities rapidly fall to nearly zero and the animal dies (Peck *et al.*, 2002). This response is very similar to the hierarchical stepwise behavioural response model, whereby processes such as movement and respiration are initially increased in

response to a stressor, then decreased once they become unsustainable, followed by death (Gerhardt *et al.*, 2006; Zhang *et al.*, 2012). This stepwise stress response occurred in *L. elliptica* adults at 5-10°C (Peck *et al.*, 2002; Peck, 2005a,b), and also seems to have occurred here, with respiration rates in experimental treatments either much higher than control rates, or much lower in treatments with high mortalities. Similarly, Pörtner (2010; 2012) proposed the oxygen- and capacity-limited thermal response behavioural model, whereby an organism has an optimal temperature range determined by its aerobic scope. An increase in temperature beyond optimum levels triggers the progressive repression of growth, reproduction, and other processes (Pörtner, 2010; 2012). This repression is similar to the deliberate metabolic repression and reduced siphon activity of *L. elliptica* in winter (Brockington, 2001; Morley *et al.*, 2007). At the animal's critical temperature, there is a shift to anaerobic metabolism as aerobic metabolism becomes too energetically costly (Pörtner, 2010; 2012). This may have occurred here in adult *L. elliptica* with both warming and acidification. Given the consistent mortalities and loss in physiological condition with elevated temperature, it is most likely that the animals were unable to regulate their energetic expenditure, and died as a result of this imbalance.

2.4.4 – Implications of sensitivity to warming in Laternula elliptica

Loss of body mass and reduced survival of *L. elliptica* occurred at levels of warming predicted for 2050 and 2100. These changes, occurring with even relatively minor temperature increase, have negative implications for its survival in a warming environment. This carries negative consequences for the health of individuals and the persistence of *L. elliptica* populations with SO warming.

Survival in the face of environmental stress depends on animals surviving multiple breeding seasons; even if they cannot breed one year, they can breed the next (Peck, 2005b). This was not the case here. Adults spawned throughout the course of this study, but some also died rapidly in less than half the time it would take gonads to ripen. These results are from individuals in the laboratory supplied with non-Antarctic water and must be interpreted with caution. However, they do indicate that even with relatively

minor temperature changes experienced over only a small proportion of its lifetime, *L. elliptica* may be unable to cope with associated metabolic costs, at least over the timescale of this investigation.

2.4.5 – Concluding comments

As a keystone species important to the Antarctic benthos, wide-scale depletions of *L. elliptica* would affect benthic-pelagic coupling and biodeposition throughout the coastal environments of Antarctica (Ahn, 1993; Guy *et al.*, 2014). In this study, *L. elliptica* was severely affected by only 1-2°C warming above summer ambient conditions, with considerably reduced survival, significant loss of body tissue, and, in some individuals, highly elevated respiration rates. These responses occurred at smaller degrees of warming than are typically associated with temperature-related mortality in *L. elliptica* (i.e., +7.5-12.5°C; Peck, 2005a,b; Morley *et al.*, 2009), confirming that thermal limits are narrower for successful long-term maintenance than for short-term tolerance (Pörtner, 2010; Alcaraz *et al.*, 2014). *L. elliptica* adults were unable to acclimate to experimental conditions after 5 mo. Other Antarctic marine fauna take longer (2.5-5 mo and 6-9 mo) to acclimate to increases in temperature (Peck *et al.* [2014] and Suckling *et al.*, [2015], respectively). However, some are unable to acclimate at all (Peck *et al.*, 2014).

The waters of McMurdo Sound have very stable year-round temperatures, and a pH range that is stable in winter, increases considerably in summer, and shows some daily fluctuation, but is consistently higher than the experimental pHs tested here (Gonzalez-Bernat *et al.*, 2013; Kapsenberg *et al.*, 2015). Therefore it is not surprising that *L. elliptica* specimens originating from Cape Evans were unable to withstand even minor warming for any extended period of time, but had some resistance to pH change. Warming is also found to be more important than decreased pH in other bivalves (Zittier *et al.*, 2015). The temperature- and pH-specific seascape that *L. elliptica*, as an ectothermic marine invertebrate, is typically exposed to dictates its ability to cope with environmental conditions outside of these norms (Morley *et al.*, 2009). The results of this Chapter have clearly shown that warming is more important than OA in terms of its effects on the function, condition, and continued survival of *L. elliptica*.

Chapter 3

HSP70 expression in *Laternula elliptica* in response to ocean acidification and warming: Preliminary results

3.1 – Introduction

The distribution of a species is determined by its ability to tolerate a range of environmental conditions, termed its tolerance ‘envelope’ or ‘window’ (Pörtner, 2008). Environmental changes that push one or more of these variables beyond an organism's tolerance envelope can become stressful if they disturb homeostasis (Clark *et al.*, 2008a; Fabbri *et al.*, 2008). Environmental stresses can result in loss of performance, changes in species interactions, population declines, and range shifts or shrinkage (Pörtner, 2008, 2010; Pörtner *et al.*, 2007). Understanding factors affecting species persistence is essential when predicting likely future species range shifts in the face of global anthropogenic climate change and ocean acidification (OA) (Tomanek, 2010; IPCC, 2013). Consequently, understanding the amount of variation in environmental variables that is tolerable to each species is vital, as it informs these predictions.

An organism's ability to survive disruptions to homeostasis by environmental change is greatly assisted by heat shock proteins (HSPs). Discovered in the context of temperature elevation, HSP induction may increase with stressors other than temperature, is related to cell signalling, and is part of the general physiological stress response of an organism (Feder & Hofmann, 1999; Fabbri *et al.*, 2008; Clark *et al.*, 2009b). While HSP production is energetically costly, it protects the cell (Feder & Hofmann, 1999; Clark & Peck, 2009b; Tomanek, 2010). HSPs act as molecular chaperones, refolding and stabilising denatured proteins, preventing them from interacting with each other in an unwanted way, and preventing the formation of detrimental cytotoxic compounds (Feder & Hofmann, 1999; Clark *et al.*, 2008a; Clark & Peck, 2009b). HSPs are classified by their weight in kilodaltons. The 70 kDa HSP family is the most well-described and includes constitutively expressed genes (HSC70),

glucose-regulated proteins, and most importantly, the inducible form (HSP70) (Clark *et al.*, 2008a,b; Fabbri *et al.*, 2008). HSP70 is a highly conserved protein that is present in all taxa, signifying the importance of HSPs to cell function (Feder & Hofmann, 1999; Fabbri *et al.*, 2008).

HSP70 is up-regulated by organisms in response to environmental stress, and is therefore of interest in heat shock response studies (Clark *et al.*, 2008a). HSP70 induction indicates that an organism is not only able to make adjustments to cellular concentration of molecular chaperones to protect proteins from damage, but that conditions are stressful enough to warrant this expense (Feder & Hoffman, 1991; Clark & Peck, 2009; Tomanek, 2010). Therefore, an increase in HSP70 expression signals that an organism is experiencing environmental stress. HSP70 up-regulation in response to elevated temperatures, the classical heat shock response, has been observed in the majority of taxa that have been examined so far, except for a number of Antarctic species (Clark *et al.*, 2008a,b).

Antarctic marine fauna are stenothermal and have evolved low metabolic rates, slow growth, longer lifespan, slow movement, and some gigantism, due to low temperatures and environmental stability over past millennia (Barnes & Peck, 2007; Clark & Peck, 2009b). These cold-water conditions may cause slow rates of protein synthesis and elevated rates of protein degradation (Barnes & Peck, 2007), so that not only could proteins such as HSP be created slowly, but they may also be degraded quickly and have a limited lifespan within the cell. Having low metabolic rates reduces the amount of cellular energy consumed, and even small increases in temperature may therefore represent a comparatively large thermal stress to Antarctic marine invertebrates. It is of interest in an evolutionary context whether Antarctic species display a heat shock response, and whether they can sustain it for any period of time. An ability to up-regulate HSP70 suggests a capacity to protect against protein denaturation and cell damage in the face of environmental change, while a down-regulation over time would signify an ability to acclimate to this change (Feder & Hoffman, 1991; Clark & Peck, 2009; Tomanek, 2010). The ability of Antarctic marine species to up-regulate levels of HSP70 expression considering their energy-limited, cold environment is relevant considering the long-term changes in ocean temperature and chemistry predicted for

the next century and beyond as a result of anthropogenic climate change and OA (IPCC, 2013).

Understanding a species' heat shock response helps identify its tolerance limits to specific environmental variables. These variables, particularly temperature and pH, set the boundaries for a species' range, and understanding a species' tolerance limits shows how this range may change with ocean warming and acidification (Pörtner *et al.*, 2007; Clark & Peck, 2009b). HSP70 and related genes have been sequenced in *L. elliptica* (Clark *et al.*, 2008a; Park *et al.*, 2007). *L. elliptica* displays a heat shock response under high temperatures (+10°C; Clark *et al.*, 2008a; Park *et al.*, 2007) and up-regulates production of HSP70 with pH increase and decrease (to 8.32 and 7.78, respectively; Cummings *et al.* [2011]). However its response to smaller, more environmentally realistic temperature increases is unknown. Furthermore, HSP70 expression has not been measured in response to both elevated temperature and decreased pH in this species.

A keystone species with a circumpolar distribution, *L. elliptica* is a common coastal suspension-feeder with a significant role in benthic-pelagic coupling (Ahn, 1993; Clark *et al.*, 2010). Understanding the response of *L. elliptica* to combined stressors, simulating predicted effects of OA and warming on the Southern Ocean (SO), is important given its ecological significance. As with other sessile bivalves, *L. elliptica* may rely heavily on HSP70 in combination with other mechanisms to adapt to environmental variation and stress, such as when colonising the productive coastal benthos after ice scour events (Pearse *et al.*, 1991; Ahn, 1994; Ahn *et al.*, 2001; Barnes & Conlan, 2007; Fabbri *et al.*, 2008). Ascertaining whether this species is likely to experience stress from ocean conditions predicted for the near future will inform predictions of population and ecosystem dynamics (as in Guy *et al.* [2014]).

3.2 – Methods

See Chapter 2 *Methods* for details on *L. elliptica* collection, acclimation procedure, and experimental setup.

3.2.1 – Tissue collection

At 5 wk and 5 mo, one individual per replicate tank was sacrificed. Mantle tissue was dissected out and used for analysis of HSP70 gene expression. Mantle tissue was dissected from ~20 mm from the base of the siphon to ~10 mm from the foot (Fig. 3.1). Tissue was weighed and snap-frozen in liquid nitrogen immediately after dissection, then stored at -80°C until analysis.

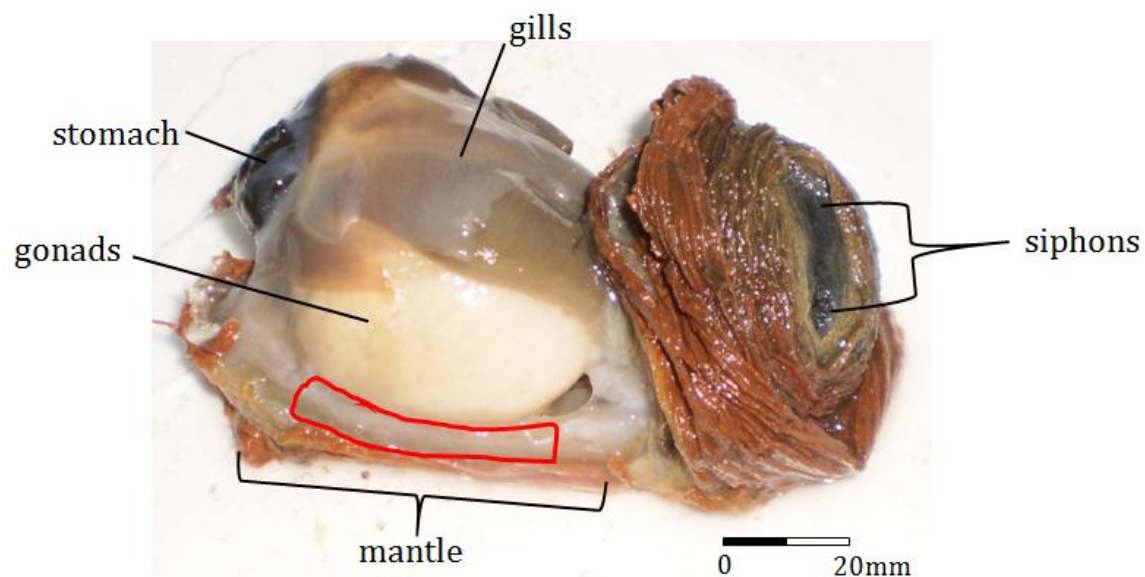


Figure 3.1 - *Laternula elliptica* mantle tissue dissection in progress. The region of mantle tissue to be dissected is outlined in red.

3.2.2 – Gene expression analysis

As this was a preliminary study mainly focussing on method development, this analysis was performed on 1 replicate of each treatment at each time point. Thus in most cases statistical analyses were not appropriate, and only preliminary trends are presented. Due to the time-consuming nature of this analysis, and the duration of the experiment, it was not possible to process all samples within the timeframe required for the completion of this thesis.

The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to compare relative expression levels of messenger RNA (mRNA) of the HSP70 gene to mRNA levels of β -actin, an internal reference gene. β -actin was used as a 'housekeeping' gene as it is expressed consistently in *L. elliptica* tissue and has been successfully used before as a reference gene in *L. elliptica* and in qPCR work (Park *et al.*, 2007; Clark *et al.*, 2008a; Cummings *et al.*, 2011). Mantle tissue was selected for analysis as it is the site of highest HSP gene expression and constituent expression of HSP70 in *L. elliptica* (Clark *et al.*, 2008a), and to allow comparison with the work of Cummings *et al.* (2011).

Total RNA was extracted from a subsample of mantle (mean wet mass 26.0 g, range 9.1-84.4 g) using Trizol reagent (Invitrogen, Life Technologies, Auckland, New Zealand). The RNA was purified by binding it to a silicate column, then washed with binding buffers and eluted into nuclease-free water (GeneJET RNA Cleanup and Concentration Micro Kit, Thermo Scientific, Auckland, New Zealand). This combined technique of Trizol extraction and silicate column purification has been used before on bivalve tissue and gives high-quality RNA yields (see Mcleod *et al.* [2009] for a similar protocol). Tissue samples were homogenised in 900 μ L of Trizol, ensuring Trizol was supersaturated to guarantee full RNA extraction. The aqueous phase containing RNA was isolated by the addition of 0.2 μ L of chloroform per 1 μ L Trizol, followed by chilled centrifugation (13,000 \times g, 4°C, 10 min). The clear aqueous phase was isolated, 0.5 μ L of 100% ethanol per 1 μ L of isolate was added, and the solution was transferred to a RNA Cleanup and Concentration Micro Kit column (GeneJET, Thermo Scientific, Auckland, New Zealand). The RNA was bound to the column and washed as per the manufacturer's instructions, then suspended in 12 μ L of nuclease-free water. The concentration and

quality of extracted RNA was spectrophotometrically assessed, with total RNA concentration measured at 260 nm and RNA purity assessed with the A_{260}/A_{280} ratio. The extracted RNA was then frozen at -20°C until cDNA synthesis.

Immediately prior to cDNA synthesis, RNA was cleaned of genomic DNA contamination with Deoxyribonuclease I as per the manufacturer's instructions (Thermo Scientific, Auckland, New Zealand). cDNA was synthesised from 1 μg RNA in a final volume of 20 μL using random hexamers and oligo $(\text{dT})_{18}$ as in the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Auckland, New Zealand). Samples were incubated at 25°C for 10 min, then at 50°C for 15 min, and terminated by heating to 85°C for 5 min. cDNA was stored at -20°C until the PCR step.

PCR reactions for each target gene (HSP70 and β -actin) consisted of 1 μL cDNA, 2 μL 0.25 μM gene-specific primers, 9.5 μL nuclease-free water, and 12.5 μL SensiMix SYBR & Fluorescein Kit (BioLine, Total Lab Systems, Auckland, New Zealand) to make a final volume of 25 μL , and analysed in duplicate. The primers used (Park *et al.*, 2007) are given in Table 3.1. Amplifications were performed and monitored in a Bio Rad CFX 96 Real-Time PCR Machine with the following conditions: initial denaturation and enzyme activation at 95°C for 10 min; 40 amplification cycles of 95°C for 10 sec, 53°C for 15 sec and 72°C for 15 sec; followed by a melt curve analysis. Temperatures and primer concentrations were determined from earlier testing, based on those conditions that yielded the lowest cycle threshold (C_t) values and highest efficiencies. Fluorescence levels were recorded (SensiMix contains Sybr Green) as C_t values, and relative expression of the HSP70 gene was compared to that of β -actin for each individual. Results are expressed as the relative x-fold increase of the HSP70 gene relative to β -actin.

Table 3.1 - Oligonucleotide primers used for qPCR of genes of interest in *Laternula elliptica*.
Primers developed by *Park et al.* (2007).

Gene	Direction	Primer nucleotide sequence (5'-3')	Amplicon size (bp)
HSP70	Forward (+)	AGATGAGGCTGTTGCATACG	97
	Reverse (-)	GGTGACGTCAAGAAGAAGCA	
β-actin	Forward (+)	GGTCGTACCACAGGTATTGT	135
	Reverse (-)	CATCAGGTAGTCGGTCAAAT	

HSP70 Genbank accession number: EF198332, β -actin Genbank accession number: EF198331

3.2.3 – Statistical analyses

As $n = 1$, only trends in the data and correlations with some of the measures made in Chapter 2 could be examined, and only preliminary trends are presented. The relationships between HSP70 gene expression at both 5 wk and 5 mo and a) physiological condition ($CI_{FW:SL}$) and b) O_2 consumption determined in Chapter 2 at the same time points, were investigated with Spearman's correlation coefficient. The data did not meet assumptions of homoscedasticity, rendering parametric tests inappropriate. These analyses were conducted with SPSS Version 22, and $\alpha = 0.05$ in all cases.

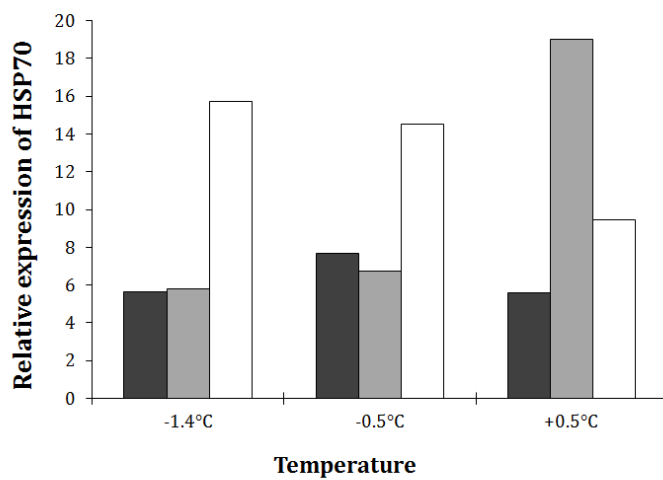
3.3 – Results and Discussion

3.3.1 – HSP70 expression in *Laternula elliptica*

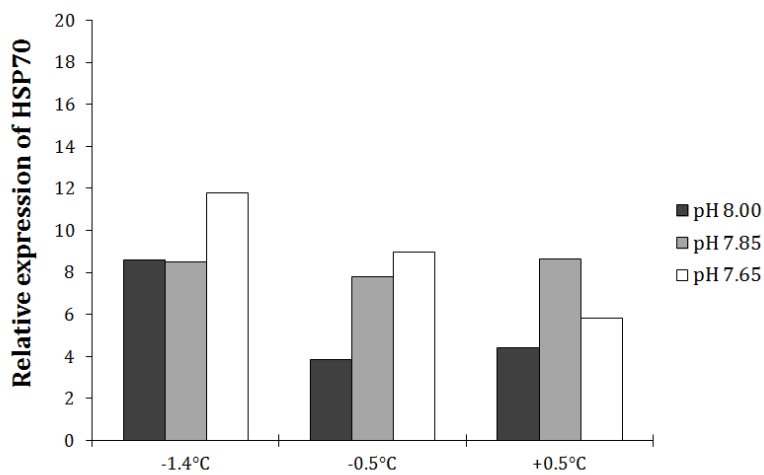
HSP70 was consistently expressed more than β -actin in all treatments, confirming its importance and deliberate expression in *L. elliptica* (Fig. 3.2). Preliminary (5 wk) levels of HSP70 gene expression were similar between ambient and mid-range pH (pH 8.00 and 7.85, respectively) at both ambient and mid-range temperatures (-1.4 and -0.5°C). However pH 7.65 produced a ~10-fold increase at both -1.4°C and -0.5°C relative to ambient pH (15-16× the internal reference gene; Fig. 3.2a). An effect of pH on HSP70 gene expression was still present at +0.5°C, but at this temperature, pH 7.85 stimulated the greatest expression, with pH 8.00 and 7.65 having lower levels. Similar levels of HSP70 gene expression relative to β -actin were found after 48 h at +10°C in *L. elliptica* (i.e., between 1.9 and 3.6-fold; Park *et al.* [2007]) as were found here with increased temperature (-0.5 and +0.5°C) and at pH 8.00 and 7.85, but the magnitude of up-regulation at pH 7.65 and +0.5°C/pH 7.85 in this study was considerably greater.

At 5 mo, the magnitude of the up-regulation of HSP70 gene expression levels in the lowest pH treatment had decreased (Fig. 3.2b), especially at elevated temperatures (-0.5 and +0.5°C; Fig. 3.2b,c). This may have occurred to save energy and resources at the cellular level. Over time, HSP70 gene expression appeared to reduce in most treatments (except for the control [-1.4°C/pH 8.00], -1.4°C/pH 7.85, and -0.5°C/pH 7.85; Fig. 3.3b,c). Consequently these results suggest that after several months at one or two degrees above summer ambient conditions, *L. elliptica* adults may have a reduced heat shock response. Similarly, reduced pH (pH 7.65) initially stimulated increased HSP70 gene expression levels, but this appears to have been downgraded over time.

a) 5 wk



b) 5 mo



c) Change between 5 wk and 5 mo

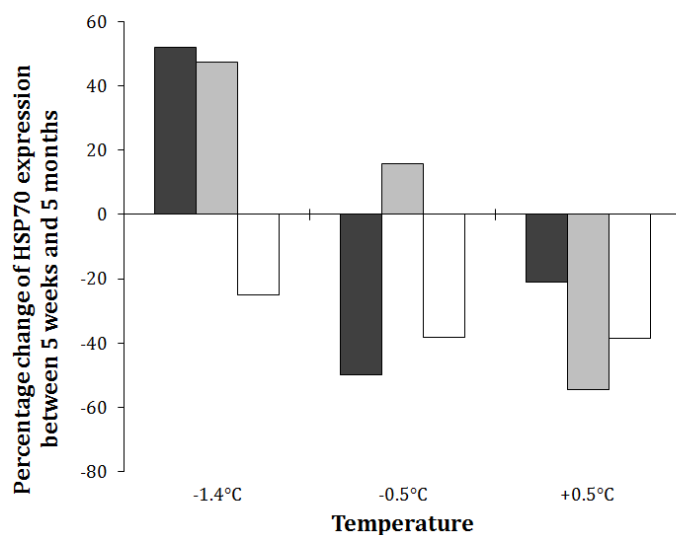


Figure 3.2 - HSP70 expression in *Laternula elliptica*. HSP70 gene expression levels are expressed as x fold induction relative to β -actin: a) after 5 wk at experimental conditions; b) after 5 mo at experimental conditions; and c) percentage change at 5 mo relative to 5 wk; n = 1.

Cummings *et al.* (2011) found an increase in HSP70 gene expression in *L. elliptica* with both an increase and decrease in pH (pH 7.78 and 8.30, relative to ambient pH of 7.99) at ambient temperatures after 3 wk. While smaller in magnitude (~0.6-1.6-fold) than the up-regulation in HSP70 gene expression levels found in this study after 5 wk (5.6-19.0-fold; Fig. 3.2a), both of these studies suggest that HSP70 gene expression levels are up-regulated in adult *L. elliptica* in response to a change in pH. This thesis work extends this understanding to longer timescales, and indicates that although this initial pH-related heat shock response persists over longer time periods, it may be subsequently downgraded (i.e., between 5 wk and 5 mo). Whether this is beneficial or not remains to be seen, considering HSPs can be either beneficial or detrimental to overall fitness (Feder & Hofmann, 1999).

There was very little effect of temperature elevation on HSP70 gene expression at ambient pH (8.00); expression patterns with 1°C warming were the same as at ambient temperature, and at 2°C warming there was still little difference at 5 wk (Fig. 3.2a,b). It is interesting in this case to note the seeming lack of a classical heat shock response (i.e., up-regulation of HSP70 gene expression as a result of temperature increase). Stenothermal Antarctic bivalves live close to their thermal limits, and despite the fact that their low-energy lifestyle and temperature limitation might be expected to contribute to a lack of heat shock response, adult *L. elliptica* have previously induced HSP70 production under higher magnitude thermal stress (i.e., increases to +10°C; Clark *et al.*, 2008a,b; Tomanek, 2010). A lack of heat shock response at ecologically realistic temperatures is suggested by the results of this study. However, a larger sample size is needed to confirm whether this is the case.

At 5 mo, control animals up-regulated HSP70 gene expression levels relative to levels at 5 wk by 48-52% at ambient and 7.85 pH, and up-regulation was also noted in the -1.4°C/pH 7.85 treatment (by 47%), and in the -0.5°C/pH 7.85 treatment (by 16%; Fig. 3.2c). However in all other treatments, HSP70 gene expression levels were 21-55% lower than at 5 wk (Fig. 3.2c). Previous studies have demonstrated that HSP70 gene expression is rapidly up-regulated, but also swiftly down-regulated, both over week-

long timescales and even in a matter of hours. This has been observed in response to +10°C thermal shock in *L. elliptica* (Park *et al.*, 2007) and +3°C warming at both ambient and reduced pH (8.10 and 7.70, respectively) in the oyster *Pinctada fucata* (Liu *et al.*, 2012). At +10°C, a similar response was also observed for gene expression levels of HSP90, a longer form of HSP, in *L. elliptica* (Kim *et al.*, 2009).

It is likely advantageous for animals to down-regulate the heat shock response when conditions are not stressful, and a compromise of intermediate HSP gene expression is usually evolutionarily favourable (Feder & Hofmann, 1999; Tomanek, 2010). As individuals were experiencing the same conditions (and stresses) at 5 mo as those that had stimulated up-regulation of HSP70 after 5 wk in the same treatments, the lesser response observed here after 5 mo may instead be a result of phenological plasticity and successful acclimation to conditions. Comparatively, sea urchin (*Psammechinus miliaris* and *Sterechinus neumayeri*) adults acclimated to reduced pH (pH 7.70) conditions after > 6 wk (Suckling *et al.*, 2014, 2015). Alternatively, it may be an acquiescence to the energetic costs of maintaining increased HSP70 induction. Individuals still experiencing reduced pH and elevated temperature may have down-regulated HSP70 gene expression, sacrificing cellular protection, to maintain their basal energetic output. This may have occurred to save energy and resources at the cellular level. The energy may have been diverted to other aspects of the heat shock response that were not measured here; for a discussion of other genes activated by heat shock in *L. elliptica*, see Truebano *et al.* (2010).

Considering that their function is to stabilise and refold proteins that are degraded by stressors such as heat, and that they must be able to withstand heat stress to do this, HSP70 proteins are likely very stable and persist in the cell for some time after their synthesis. Similarly, small (20-30 kDa) HSPs in *Toxoplasma gondii* are thermally stable up to high temperatures (between 45 and 73°C) (de Miguel *et al.*, 2009). In prokaryotes and eukaryotes, pre-exposure to an oxidative stress conveys resistance to subsequent exposure to lethal levels of that stress (or another kind of stress, such as heat) (Crawford & Davies, 1994). This process, 'adaptive response', indicates that the persistence of protective proteins, that accumulated in response to an initial stress, provides the ability to survive a second instance of stress (Crawford & Davies, 1994). A

similar concept, 'adaptive thermotolerance', has been observed in mammalian cells, involving the induction of a 72 kDa HSP after pre-exposure to thermal stress (Mizzen & Welch, 1988). Once formed, the persistence of HSPs in a cell would be consistent with an adaptive thermotolerance response, and convey protection over time.

Consequently, once a cell has induced HSP70 production long enough for suitable amounts of these proteins to accumulate within the cell, it would be beneficial to switch off HSP70 gene expression to avoid wasting cellular resources. A stenothermal Antarctic ciliate with naturally high levels of HSP70 in its macronucleus did not strongly upregulate HSP70 production with heat shock to +20°C, indicating the level of HSP70 already present was sufficient cellular protection against temperature elevation (La Terza *et al.*, 2001). HSP regulation is tightly controlled and its induction can swiftly change after heat stress (Mizzen & Welch, 1988), including in *L. elliptica* (Park *et al.*, 2007). Therefore, the down-regulation in HSP70 gene expression levels found here in *L. elliptica* in some treatments at 5 mo may be a result of the reduced requirement for new HSP70, as cellular protein levels may have remained sufficiently high from the initial up-regulation at 5 wk to combat subsequent cellular stress.

The lowered pH treatments yielded the greatest up-regulation in HSP70 induction at 5 wk (Fig. 3.2a), and all had relatively reduced HSP70 gene expression levels by 5 mo (Fig. 3.2b). Animals from the pH 7.65 treatments were generally unable to acclimate to reduced pH; their survival dropped particularly rapidly in the weeks before HSP70 was quantified at 5 mo (see Chapter 2). These animals may have suffered from a reduced expression of HSP70 and reduced molecular chaperoning, which would have resulted in proteins denaturing. Alternatively, individuals who continued to maintain high HSP70 gene expression may have been unable to maintain this metabolic demand, and may have died as a result of unsustainable energetic expenditure, leaving only those individuals with comparatively lower HSP70 gene expression. It is therefore useful to examine this hypothesis by evaluating the relationship between HSP70 gene expression levels and both physiological condition and O₂ consumption rates presented in Chapter 2.

3.3.2 – HSP70 expression, physiological condition, and O₂ consumption in Laternula elliptica

The overall relationship between HSP70 gene expression levels and physiological condition was non-significant (Spearman's $\rho = 0.038$, $p = 0.880$, $n = 18$), though trends were suggested with pH and physiological condition (i.e., $CI_{FW:SL}$; Fig. 3.3). HSP70 gene expression levels stayed relatively stable and low under ambient pH conditions, while low pH induced a greater up-regulation of HSP70. Note that pH 8.00 (ambient, circled green in Fig. 3.3) conditions produced lower induction of HSP70 for each animal's $CI_{FW:SL}$ value than reduced pH and higher temperature treatments (Fig. 3.3, circled orange and dashed-red). This suggests that hypercapnia exacerbates the magnitude and variability of the heat shock response compared to normocapnic animals in the same physiological condition. A positive relationship between levels of HSP70 gene expression and physiological condition is indicated at -0.5°C (triangles), but not at -1.4°C (crosses) and $+0.5^{\circ}\text{C}$ (open circles) (Fig. 3.3). Factor analysis would likely elucidate trends suggested here and confirm whether pH groupings are significant. Since only one replicate individual per treatment could be analysed, the collection of further HSP70 data from remaining samples, as is currently planned, will clarify these patterns.

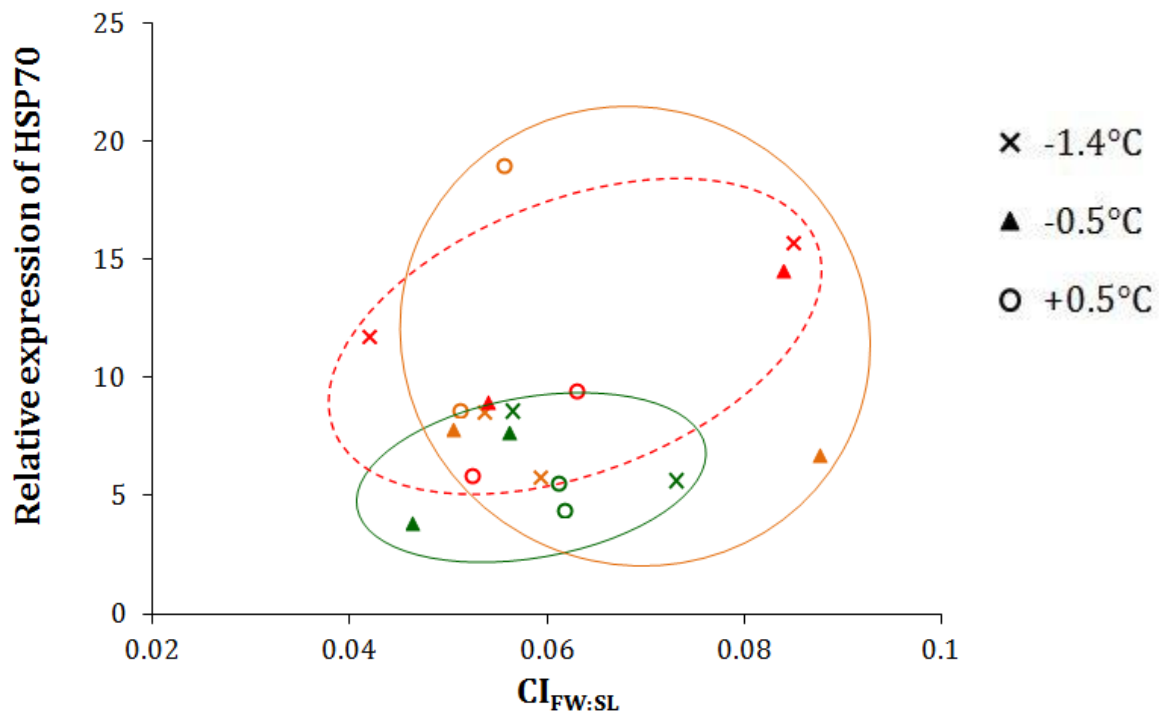


Figure 3.3 - HSP70 expression compared to physiological condition ($CI_{FW:SL}$) in *Laternula elliptica*. Data for both time points (5 wk and 5 mo) are presented. Results are grouped by pH: pH 8.00 (green symbols and circle); pH 7.85 (orange symbols and circle), and pH 7.65 (red symbols and dashed red circle). Note the clustered, low HSP70 expression of individuals from ambient pH treatments, and the high, variable range of individuals from the reduced pH treatments.

Expression of HSP70 was positively correlated with O_2 consumption (Spearman's $\rho = 0.886$, $p = 0.019$, $n = 6$; Fig. 3.4). This was largely due to high O_2 consumption in one individual exposed to $+0.5^\circ\text{C}/\text{pH } 7.85$ (Fig. 3.4). The high individual variability observed in O_2 demand with increased temperatures and reduced pH (see Chapter 2) also influenced the relationship with HSP70 gene expression levels here. The positive relationship between HSP70 gene expression and O_2 consumption shows that both processes are increased at the same time by *L. elliptica*. Consequently, both are regulated by the organism in response to continued exposure to environmental conditions at the outer range of what they usually experience, that are potentially stressful. This indicates that both respiration rate and HSP70 gene expression levels are valuable markers of physiological change in this species. This result also suggests that higher expression of the HSP70 gene increased the energetic costs at the cellular level, with higher respiration rates reflecting increased metabolic cost of exposure to these

conditions at the whole-organism level. Despite the small sample size, the correlation between HSP70 expression and O₂ consumption was significant, and additional work could potentially confirm this relationship.

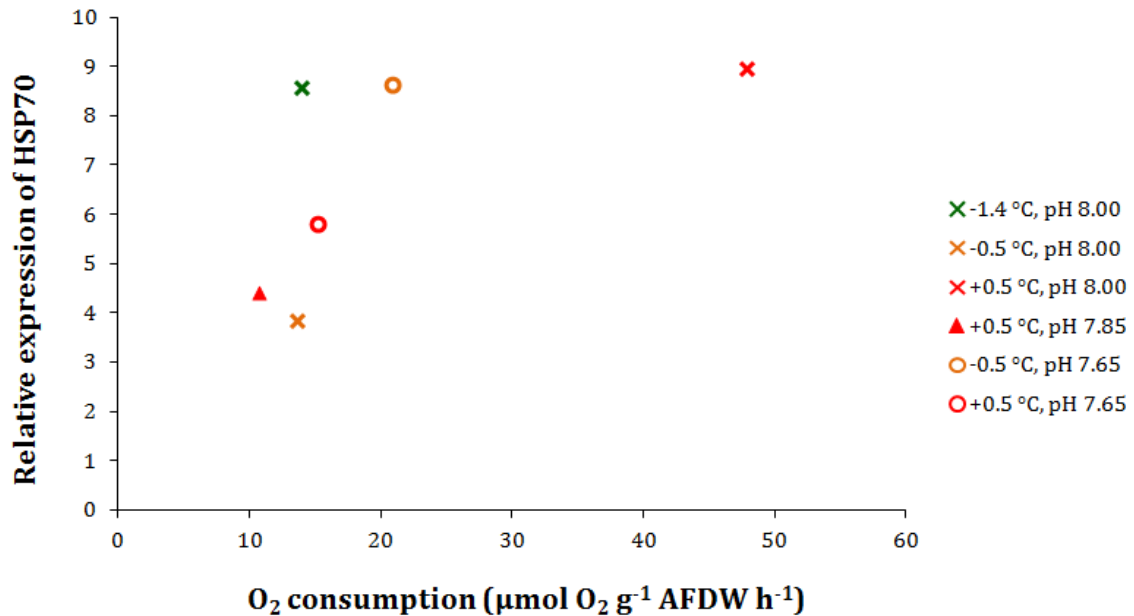


Figure 3.4 - HSP70 expression compared to O₂ consumption in *Laternula elliptica*. Data for both time points (5 wk and 5 mo) are presented. HSP70 production is expressed as x-fold induction relative to β-actin, and O₂ consumption is expressed as μmol O₂ g⁻¹ AFDW h⁻¹.

3.3.3 – Preliminary conclusions

Preliminary trends indicate that, after a short-term (5 wk) exposure to experimental treatments, adult *L. elliptica* increases HSP70 gene expression levels in response to lowered pH (pH 7.65, -0.35 units below ambient). This up-regulation, while still apparent, was less obvious at 5 mo. In contrast, levels were similar across temperatures at ambient pH after both 5 wk and 5 mo. These results suggest that *L. elliptica* does not exhibit a heat shock response at temperature increases (1 and 2°C above ambient) predicted for the near future.

After 5 mo, HSP70 gene expression levels had increased further in the control treatment (by 48-52% relative to 5 wk; Fig. 3.2c), but had declined in the elevated temperature treatments (by 21-55% relative to 5 wk; Fig. 3.2c), particularly at the highest temperature (+0.5°C). In contrast to the lack of HSP70 response with warming, reduced pH (7.65) produced an up-regulation of HSP70 gene expression at 5 wk that was less apparent at 5 mo, suggesting down-regulation over time with pH. This longer-term response to both elevated temperature and decreased pH could be a result of acclimation, repression of induction in order to maintain the energy budget, or a result of all higher-expressing individuals dying before sampling. Alternatively, down-regulation may occur once sufficient levels of HSP70 have been expressed within the cell. There was a significant positive relationship between O₂ demand and levels of HSP70 gene expression, indicating that both are increased by the organism, and that the response at the cellular level may have influenced energetic demand, consequently resulting in the higher respiration rates with higher levels of HSP70 gene expression.

There is insufficient evidence at this time to conclude which of these processes (acclimation, active repression, or die-off) is occurring. Additional work is needed to fully explore the effect of the combination of reduced pH and elevated temperature on the heat shock response of this stenothermal species. Analysis of the remaining available samples is planned, to enable quantification of the variability and the consistency of the response observed here. HSP70 concentration in these tissues could also be measured directly, to determine whether adaptive thermotolerance is occurring in adult *L. elliptica*. Both mantle and adductor tissue samples are available for future work on HSP expression in *L. elliptica* exposed to elevated temperatures and reduced pH.

Chapter 4

General overview and conclusions

4.1 – Synthesis of findings

Anthropogenic ocean warming and acidification are currently changing the temperature and chemistry of the oceans at an unprecedented pace, particularly the Southern Ocean. To address the effect that these processes will have on a vulnerable, common Antarctic bivalve in the near future, the response of the infaunal bivalve *Laternula elliptica* was investigated under elevated temperature and reduced pH. In Chapter 2, the survival and function of adult *L. elliptica* was tested in response to both lowered pH and elevated temperature over 5 mo (21 wk). Patterns of survival over time, time to 50% survival (LD₅₀), and overall survival at 5 mo were examined. Physical and physiological condition indices were calculated at both 5 wk and 5 mo, and O₂ consumption was assessed at 20 wk. Chapter 3 concerned the development of gene expression protocols for extracting HSP70 mRNA in *L. elliptica*, and presented preliminary results of HSP70 gene expression levels over time in response to lowered pH and elevated temperature. The combined results of these chapters are presented in Fig. 4.1.

Temperature elevation significantly affected several aspects of *L. elliptica* function studied; in fact almost all significant effects were noted at the highest temperature (+0.5°C) after 5 mo (Fig. 4.1). Increasing the temperature by ~1°C, to -0.5°C, caused a significant decrease in CI_{FW:SL}. Further warming to +0.5°C resulted in a significant decline in both CI_{FW:SL} and CI_{FW:SW}. In addition, the odds of dying were significantly and severely affected at the high-temperature treatment, with an almost 8-fold increase in the odds of dying observed when the temperature was raised from ambient to +0.5°C. Mortalities were noted earlier, and while not significant, survival was lower at the conclusion of the experiment at +0.5°C than at ambient (-1.4°C). A similar pattern was evident at each pH when pooled by temperature, and was likely influenced by the effect of the +0.5°C treatment on mortalities. Temperature increase did not appear to affect

HSP70 production at either 5 wk or 5 mo. $CI_{FW:SL}$ was negatively correlated with O_2 consumption. In contrast, reduction in pH had no significant effect on any of the variables measured, although there are indications of influences on HSP70 gene expression levels that require further analyses. HSP70 gene expression levels were highest at pH 7.65 at both -1.4 and -0.5°C at 5 wk, yet this up-regulation pattern was reduced by 5 mo. While decreases in pH appeared to increase overall survival and LD_{50} , increase O_2 consumption and variability, and exacerbate the degree by which O_2 consumption increased with reduced physiological condition, statistical analyses were unable to identify any differences. Overall, there was ultimately no significant interaction between temperature and pH for any of the variables measured.

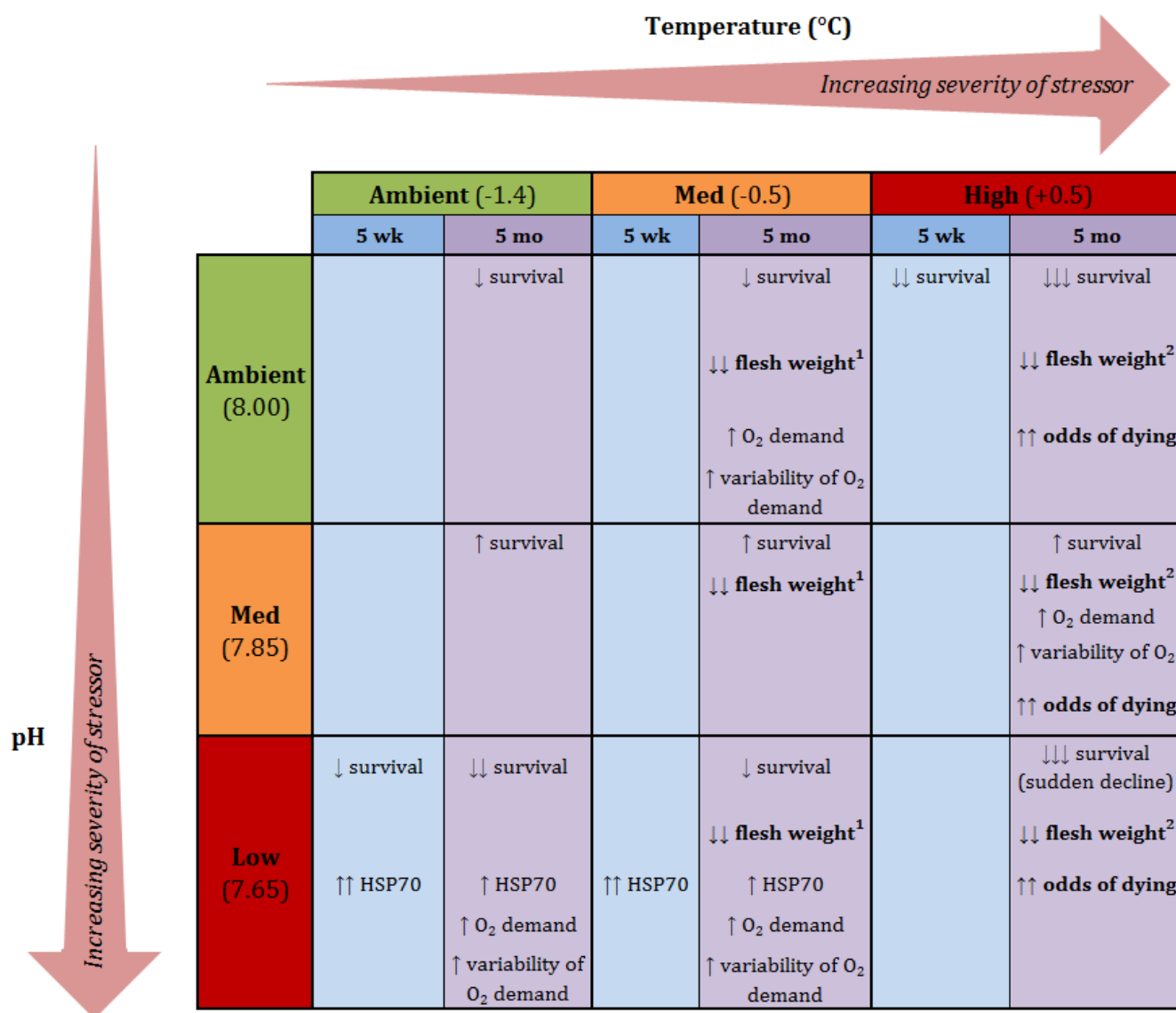


Figure 4.1 - Synthesis of the main responses of *Laternula elliptica* exposed to elevated temperature and decreased pH at both 5 wk and 5 mo. Statistically significant results ($p < 0.05$) are bolded. The number of arrows indicates the relative magnitude of the response: one = small; two = medium; three = large. “Flesh weight¹” = ratio of flesh weight to shell length ($CI_{FW:SL}$). “Flesh weight²” = both $CI_{FW:SL}$ and the ratio of flesh weight to shell weight ($CI_{FW:SW}$). HSP70 = heat shock protein 70.

HSP70 expression patterns less clear after 5 mo. However, changes in physiological condition were apparent by 5 mo. At +0.5°C, *L. elliptica* adults exhibited a significant loss in physiological condition relative to individuals at ambient temperature, losing body mass relative to both shell length and shell weight at 5 mo. The loss of tissue mass is a strong indication that individuals were stressed, with adults likely utilising

resources such as their own proteins to maintain increased metabolic demand (Ahn *et al.*, 2003). This loss in condition affected O₂ consumption, with individuals of lower condition exhibiting not only significantly increased respiration rates, but also an increase in the variability of these rates. High individual variability indicates that some individuals were coping with experimental conditions and had O₂ consumption rates on par with control animals, while others were not, and were significantly elevating their O₂ consumption. This increased respiratory demand may be in part to synthesise new tissue to replace lost body mass (Clarke, 1987). It may also be a result of an increased level of protein synthesis, as preliminary trends indicate that HSP70 gene expression levels increased in conjunction with increased respiration rates. The loss of body mass is a clear negative response to the stress of treatment conditions. This has negative implications for the long-term survival of *L. elliptica* adults, especially over winter, when resources are limited. An increase in metabolic demand could increase physiological condition loss over this period, contributing to reduced reproductive output and reduced population size (Guy *et al.*, 2014).

A ~2°C rise in temperature was enough to seriously affect *L. elliptica* function, with potential consequences for survival of the population. In the SO, pH lowering and aragonite undersaturation are expected first over winter as early as 2035, and the strongest warming will also happen in winter (Feely *et al.*, 2004; Orr *et al.*, 2005; McNeill & Matear, 2008; Mattsdotter Björk *et al.*, 2014). Aragonite saturation states are already at their lowest in winter at Cape Evans (Kapsenberg *et al.*, 2015). What does this mean for *L. elliptica* in the future living in a warming and acidifying ocean? *L. elliptica* is presumably at its most vulnerable in winter, when it must reduce periods of active respiration to reduce energy expenditure, down-regulate metabolic activity, and subsist on muscle proteins (Brockington, 2001; Ahn *et al.*, 2003; Morley *et al.*, 2007). Ahn *et al.* (2003) found that *L. elliptica* decreased body mass by ~24% over winter. This is comparable in magnitude to the body mass decreases relative to shell length reported here over 4 mo (between 5 wk and 5 mo) while well-fed under experimental conditions; though Brockington (2001) found no such loss. These results indicate that, under future conditions, *L. elliptica* will have to survive increased metabolic demand and loss of physiological condition in addition to other stresses.

The exact physiological mechanisms underlying the loss of condition in *L. elliptica* found here are unknown. However, the loss in physiological condition and reduced survival exhibited here by *L. elliptica* in response to small amounts of warming are consistent with the observations of Beukema *et al.* (2009) of reduced body mass and survival in the tellinid bivalve *Macoma balthica* in warmer-than-average years in the Wadden Sea. As with *L. elliptica*, in order to survive months of harsh conditions with limited resources, *M. balthica* relies on growth during periods of food availability over spring and summer and metabolic suppression over winter. The significant loss in body mass observed here in *L. elliptica* at increased temperature is likely a result of its inability to depress energy usage, resulting in reduced body mass. This may convey a reduced ability to successfully over-winter, or to successfully reproduce come spring, as proposed by Beukema *et al.* (2009). This is further supported by the negative correlation between physiological condition and O₂ consumption observed here in animals exposed to temperatures both +1 and +2°C above ambient. The reduced survival of *L. elliptica* in treatments that also produced condition loss indicates that the loss of physiological condition is associated with an increase in mortalities. Consequently, it is very likely that in conditions of environmental stress (e.g., winter), *L. elliptica* would continue to lose body weight relative to shell length, continue to respire more intensely, and ultimately suffer the consequences of reduced physiological condition, such as an inability to ripen gonads for spawning in summer (Kang *et al.*, 2009).

Although temperature stress is known to affect *L. elliptica* (Peck *et al.*, 2002, 2004), the magnitude of the response (i.e., survival, LD₅₀, respiratory demand, and the maintenance of body mass) to the relatively small temperature increase investigated here was considerable over 5 mo. This confirms the importance of conducting longer-term studies. These negative impacts occurred at lower levels of warming than are typically considered acutely stressful for *L. elliptica* (Peck *et al.*, 2002; Peck, 2005a,b; Morley *et al.*, 2009; Morley *et al.*, 2012a), indicating that thermal limits for successful long-term maintenance are narrower than those of short-term tolerance in this species (Pörtner, 2010; Alcaraz *et al.*, 2014), at least over a 5 mo period. Future predictions of population success (such as Guy *et al.* [2014]) should consider that *L. elliptica* may exhibit significant reductions in survival at lower temperatures than previously

thought. These results are consistent with the prediction that a +1°C increase in sea temperature may cause significant loss of biological function in many Antarctic marine species, and a +2°C warming has the potential to cause many populations to reduce or vanish altogether (Peck *et al.*, 2004). While *L. elliptica* adults appeared unable to maintain their energy demand over 5 mo timescales, urchin *Sterechinus neumayeri* adults successfully acclimated to the combined effects of elevated temperature and reduced pH over 6-9 mo timescales (Suckling *et al.*, 2015). This may be expected considering the respective susceptibility of these taxa; survival and calcification are more negatively impacted by acidification in molluscs than in echinoderms (Kroeker *et al.*, 2013).

L. elliptica maintained shell condition, with no change in shell weight relative to length of shells over the experiment. Considering that empty *L. elliptica* valves rapidly begin to dissolve under reduced pH (McClintock *et al.*, 2009), and that chitin synthase, an enzyme crucial to bivalve biomineralisation, is up-regulated in response to pH decrease in live *L. elliptica* (Cummings *et al.*, 2011), this indicates that *L. elliptica* adults were able to successfully calcify enough to keep shells at a stable weight in this experiment. While calcification is generally more difficult under acidified conditions (but see Roleda *et al.* [2012]), responses are species-specific (Ries *et al.*, 2009; Hendriks *et al.*, 2010; Kroeker *et al.*, 2013). Decreased pH combined with elevated temperature can change the structure of calcified shells, with the mussel *Mytilus edulis* producing softer, less stiff aragonite under these conditions (Fitzer *et al.*, 2014). As *L. elliptica* already has a thin, brittle shell, if a structural change of this kind were to occur with warming and acidification, it could increase its vulnerability to damage from iceberg scour or other disturbance. Other calcifying species have also exhibited stable CaCO₃ structures with reduced pH. Sea urchin CaCO₃ structures were not affected by low pH while supersaturated ($\Omega_{Ar} = 1.60$; Hazan *et al.* [2014]), and at pH 7.7 (Ω_{Ar} not given) there was no significant dissolution of the dead arms of the burrowing brittle star *Amphiura filiformis* (Wood *et al.*, 2008); however, the latter experienced dissolution at pH 6.8. *L. elliptica* was also able to maintain its shell condition with reduced pH, even at the low Ω_{Ar} found in both reduced pH treatments, especially in the lowest-pH treatment. However, this may have come at the cost of tissue loss and an associated decline in physiological condition.

L. elliptica was not significantly affected by reduced pH conditions predicted under future OA. Trends apparent with reduced pH are discussed here, but were ultimately non-significant. Reduced pH increased O₂ consumption. In combination with warming, intermediate pH decrease slightly improved rates of survival, but increased average O₂ demand and the variability of this demand (Figs. 2.13, 4.1), as well as exacerbating the effect of physiological condition loss on O₂ consumption (Fig. 2.14). Furthermore, reduced pH appeared to initially induce up-regulation in HSP70 expression at both -1.4 and -0.5°C at 5 wk, followed later by an up-regulation of smaller magnitude in these treatments at 5 mo. Down-regulation in levels of HSP70 gene expression was observed in most elevated temperature treatments at 5 mo relative to 5 wk. The comparatively minor effect of reduced pH relative to elevated temperature must be interpreted in the context of the site-specific pH-seascape normally experienced by Cape Evans *L. elliptica*, which experience rapid increases in pH (but not decreases) over short time periods in association with spring phytoplankton blooms (Kapsenberg *et al.*, 2015; Schram *et al.*, 2015). Considering the lack of statistically significant functional responses seen here in *L. elliptica* in response to reduced pH, adults may continue to withstand these seasonal pH fluctuations in the future.

When interpreted in light of these environmental conditions, the response of *L. elliptica* to pH decrease found here suggests a certain ability to cope with future acidification. This supports previous work showing change in basal metabolic function and protein expression with pH change in this species (Cummings *et al.*, 2011). However, while less striking in magnitude than those associated with warming, the negative effects of pH decrease still represent a threat to *L. elliptica* function and survival. Low pH still poses a threat to the functioning of *L. elliptica*, and reflects a seascape not experienced by these bivalves over evolutionary timescales. As *L. elliptica* display some site-specificity in temperature acclimation and burial ability (Morley *et al.*, 2012b), it would be beneficial to investigate the effect of ocean warming and acidification on animals from other sites.

Despite their infaunal mode of life, *L. elliptica* adults are exposed to overlying water conditions as they draw demersal water into their internal spaces through their siphons. Consequently, their internal tissues will still be exposed to any future warming

or acidification of the water column. Animals in this experiment experienced artificial laboratory conditions, such as a lack of sediment in tanks. As a consequence, their entire outer surface was exposed to experimental conditions. This may have artificially changed the magnitude of the observed response, considering sediment pH is often considerably lower than that of the water column (Green *et al.*, 2004; Widdicombe & Spicer, 2008; Wood *et al.*, 2008). Despite this, the impact of pH on the condition and functioning of *L. elliptica* was considerably smaller than that of temperature, with most effects of hypercapnia visible as trends that were biologically relevant, yet small in magnitude. *L. elliptica*'s apparent higher tolerance to hypercapnia is possibly due to this reduced pH of the infaunal sediment environment relative to overlying water conditions (Green *et al.*, 2004; Widdicombe & Spicer, 2008; Wood *et al.*, 2008), thus supporting the theory in Widdicombe & Spicer (2008) of increased resistance by infaunal species. Future studies could bring animals from the field in a core of *in situ* sediment (Wood *et al.*, 2008), however logistical constraints likely limit this.

The increase in respiration rate of *L. elliptica* represented increased metabolic demand. The highest respiration rate was expected to occur in the +0.5°C/pH 7.65 treatment, combining the highest temperature and the lowest pH, a pattern observed in the mussel *M. galloprovincialis* (Chinellato *et al.*, 2010). This was not the case with *L. elliptica* in this study. While both increased temperature and lowered pH produced increased O₂ consumption rates, combining stressors did not noticeably increase this rate (see next paragraph). The pattern of O₂ demand found here fits with the stepwise stress model, where behavioural responses to a stressor are increased until they are unsustainable, at which point the behaviour is rapidly decreased or stopped and survival decreases (Gerhardt *et al.*, 2006; Zhang *et al.*, 2012). High O₂ consumption in certain individuals at elevated temperature or reduced pH was likely a symptom of greatly increased energy requirements under these conditions. Had the experiment continued or the stressor increased in severity, energetic demand, already indicated by the loss of tissue mass, may have become unsustainable, and O₂ demand may have crashed, with an associated decrease in survival (but see Suckling *et al.*, 2015). This pattern would be consistent with the peak, then crash and subsequent deaths, seen in *L. elliptica* respiration and heartbeat rates with stepwise temperature increases beyond the species' tolerance limits (Peck *et al.*, 2002; Peck, 2005a,b).

L. elliptica in this study, especially those exposed to +0.5°C/pH 7.65, were able to withstand these conditions for a short period of time, but following a 13 wk threshold, they were rapidly and seriously affected, and could no longer maintain themselves under those conditions. Survival dropped precipitously over the following 5 weeks to just 12.5%, the lowest in the study. Individuals with low O₂ consumption, found with both elevated temperature and lowered pH, and particularly when these stressors were combined, may have persisted because they were able to maintain a low total O₂ consumption rate for their physiological condition, while individuals unable to cope may have all died. This may be an early indication of selection pressure. Interestingly, one individual from the -1.4°C/pH 7.65 treatment assessed for O₂ consumption indicated an extremely elevated O₂ demand (20× that of other animals, even allowing for a considerable margin of error). This individual died several days later and the unrealistic values were excluded from the analyses. This elevated rate, followed days later by the death of the individual, is consistent with the spike then crash in respiration followed by death found in *L. elliptica* (Peck *et al.*, 2002; Peck, 2005a,b).

A similar pattern is evident in the preliminary results of HSP70 expression. As previously discussed here, pH decrease to 7.65 at ambient and intermediate temperatures stimulated an up-regulation in HSP70 gene expression. However, at high temperatures, HSP70 expression was similar (and low) at both pH 8.00 and 7.65. Only one individual from the +0.5°C/pH 7.65 treatment survived to the end of the experiment. The +0.5°C/pH 8.00 was also a high-mortality treatment, with only two animals surviving, and a low level of HSP70 gene expression was measured for these individuals. Therefore the low levels of HSP70 expression measured in the +0.5°C/pH 8.00 and +0.5°C/pH 7.65 treatments relative to the +0.5°C/pH 7.85 treatment may not be a genuine signal of reduced stress, but instead a sign of selection pressure where stressed individuals who continued to up-regulate their response died, and only those few individuals requiring a small increase in response survived. This artificially low signal may be masking the fact that for *L. elliptica*, an HSP70 expression rate outside of the norm at high temperatures is potentially lethal.

As environmental stresses increase, organisms may: a) move to a new site (physical migration); b) modify internal processes to withstand change, either in the short-term or over weeks to months (physiological plasticity); or c) genetically adapt to the conditions (Peck, 2005a; Barnes & Peck, 2008; Barnes *et al.*, 2009; Peck *et al.*, 2014). *L. elliptica*, as a sessile, deep-burrowing organism (Ahn, 1993), has little ability to physically migrate elsewhere in the SO. Additionally, the coastline of Antarctica offers a limited latitudinal range, so opportunities for migration to avoid predicted changes in ocean pH and temperature are few (Peck, 2005a; Barnes *et al.*, 2009). *L. elliptica* is a long-lived species with long generation times; rebuilding gonads post-spawning takes at least 12 mo, and juveniles take ~3 y to reach reproductive size (Kang *et al.*, 2009; Ahn *et al.*, 2001; Peck, 2005a,b; Guy *et al.*, 2014). Therefore any genetic adaptation would take time to propagate through the population (Peck, 2005). *L. elliptica* has encapsulated lecithotrophic larvae that are dispersed on or near the benthos (Pearse *et al.*, 1986; Berkman *et al.*, 1991; Kang *et al.*, 2009). Depending on local water currents, the dispersal of genetic material may be restricted to the vicinity of the parent population, further slowing allele transfer between populations. Successful genetic adaptation is also complicated by a lack of physiological plasticity, which affects the ability of adults to survive long enough to reproduce. *L. elliptica* adults could not acclimate to experimentally elevated temperatures here, as even a +1°C temperature change resulted in a significant loss of body mass after 5 mo, along with increased respiratory demand and decreased survival. A similar physiological inflexibility with temperature increase has been noted in this species, and this is true for Antarctic marine invertebrates in general (Peck *et al.*, 2002, 2014). As a cold-adapted species with a narrow thermal window and a long generation time, *L. elliptica* will likely be negatively affected by climate change and OA (Pörtner *et al.*, 2007; Pörtner & Farrell, 2008).

4.2 – Future directions

This research concerned the effect of elevated temperature and lowered pH, conditions that are predicted to occur in the Ross Sea in the near future, on the metabolism, survival, condition, and heat shock response of adult *L. elliptica*. In doing so it filled a key knowledge gap pertaining to infaunal benthic molluscs in the evolutionarily interesting SO ecosystem. Understanding the response of benthic species such as *L. elliptica* is important because the seafloor hosts many marine species, and any decrease in biodiversity could affect key ecosystem services such as nutrient cycling, especially in disproportionately productive shelf environments (Widdicombe & Spicer, 2008). The response of *L. elliptica* in particular to OA and warming, such as potential reductions of growth, respiration, metabolic rate and calcification, is of interest as this species plays an important role in benthic-pelagic coupling in shallow Antarctic coastal environments (Widdicombe & Spicer, 2008; Ahn, 1993; Clark *et al.*, 2010). Over the course of this research, further questions arose that may provide direction for future research into SO OA and warming.

In *L. elliptica*, adults are particularly vulnerable to ocean warming, less so to low pH (Peck *et al.*, 2002, 2004; Peck, 2005a,b; this study). Understanding the response of juveniles forms an important complement to that of larvae and adults, informing understanding of the effect of ocean warming and acidification on the entire life cycle. Larvae are expected to be the most vulnerable bivalve life stage under OA, especially when forming shells (Kroeker *et al.*, 2013; Parker *et al.*, 2013). In some cases juveniles are more resistant to warming than adults (Beukema *et al.*, 2009), including in *L. elliptica* (Peck *et al.*, 2013). The response of juvenile *L. elliptica* to ocean warming and acidification is at present poorly understood. This needs to be explored further, as early and mid-life stages are vital for the persistence of populations in *L. elliptica* (Guy *et al.*, 2014). Environmental stressors that severely affect juveniles affect the proportion surviving to reproductive size, and the time to reproduction, with consequences for the biomass and size of *L. elliptica* populations in the future (Guy *et al.*, 2014). Stresses that particularly threaten pre-reproductive-age *L. elliptica* could create a bottle-neck, further reducing numbers of viable adults (Wicks & Roberts, 2012).

The *L. elliptica* used in this study were large and in good condition at the beginning of the experiment. They demonstrated a significant and considerable loss of physiological condition, with a ~30% decrease in tissue weight relative to both shell weight and shell length. Might younger or smaller individuals, or those in poorer condition, fare even worse in a future ocean? In fact, larger *L. elliptica* utilise comparatively greater amounts of protein to fuel metabolism than smaller individuals (Brockington, 2001), so smaller animals may lose a lower proportion of tissue under thermal stress, and actually do better than larger individuals. Larger animals are also more affected by thermal extremes, and larger *L. elliptica* are less able to re-bury themselves following temperature stress (Peck, 2005a; Pörtner, 2008). Smaller animals provide comparatively greater biodeposition rates than larger individuals (Ahn, 1993), indicating the ecological value of their persistence. Smaller individuals may be selected for under future OA and warming, provided they have wider thermal windows (Pörtner, 2008), but this should be examined further in stenothermal *L. elliptica*. Increased basal metabolic demands may necessitate the sacrifice of energy usually used for growth, potentially resulting in slower growth, smaller overall sizes, and a delay in the onset of reproduction, which occurs at ~40 mm SL in *L. elliptica* (Ahn *et al.*, 2001). Juveniles are considered to be more vulnerable to environmental stressors than adults (Parker *et al.*, 2013). Juveniles' smaller size could be disadvantageous, creating comparatively higher energetic demand, especially when considering their faster growth rate. Juveniles reach 60 mm SL in 4-5 y, while it takes another ~15-20 y for adults to reach ~100 mm SL (Ahn *et al.*, 2001; Guy *et al.*, 2014). Husmann *et al.* (2012, 2014) classified young and old *L. elliptica* by SL (< 50 mm and > 70 mm, respectively), while Peck *et al.* (2013) used juveniles of SL < 20 mm. Similar differentiations between juvenile and mature animals could apply to future work.

The improved resistance to increased temperature in smaller individuals, if present, may come at the cost of growth rates and future productivity. There is considerable ecological value in including studying the response of *L. elliptica* to ocean acidification and warming both before and after gamete production; i.e., pre-reproductive vs. mature reproductive individuals. Gamete production is a large drain on bivalve resources; survival in acidified conditions decreased noticeably post-spawning in juveniles of the

clam *Ruditapes decussatus*, associated with the increased energy demanded by this process (Range *et al.*, 2011). Almost all animals in the present study had very large gonads, taking up a large part of their body (Fig. 3.1), and animals spawned throughout the experiment. Keeping these large organs functional is a drain on resources, particularly for high-latitude organisms (Clarke, 1987). However, pre-reproductive age juveniles that have not yet formed gonads may have more energy available to divert to metabolic functions, and resist the negative influence of OA and warming that way. The energy used for reproduction, usually a consistent proportion of total energy budget, could fully be diverted to growth (Clarke, 1987; Wicks & Roberts, 2012). However, the stresses of ocean warming and acidification could still affect these juveniles.

The animals in this study were well-fed and upon dissection had consistently large, full stomachs, indicating that they were receiving sufficient food. What happens when food supply is limited? Incorporating food availability into future OA and climate change research would be an interesting addition. After initial feeding was stopped in sea urchin larvae, metabolic rates increased in response to acidification (Pan *et al.*, 2015). The ability of molluscs to withstand and adapt to the physiological stresses of future ocean changes is likely dependent on food availability; when food is low, they must use internal energy reserves to compensate, or else repress metabolism to prolong survival (Parker *et al.*, 2013). Low food concentrations and reduced pH conditions lowered physical condition in several bivalve species, with shells reaching shorter lengths and losing internal mass in individuals with low physiological condition (Gazeau *et al.*, 2013). Physiological condition clearly affects the ability of bivalves to maintain their condition in the face of OA, with starved organisms unable to compensate (Gazeau *et al.*, 2013). Feeding bivalve larvae are able to compensate for increased energetic costs of calcification when there is sufficient food available, indicating that reduced calcification is mainly due to reduced scope for growth (Thomsen *et al.*, 2015), and this likely applies to adults as well.

Food availability already strongly constrains the growth and distribution of Antarctic marine species (Chiantore *et al.*, 2002; Thrush *et al.*, 2006); food limitation is also likely to limit their ability to overcome increases in aerobic output with ocean warming and acidification. Daily fluctuations in pH observed under sea-ice at sites like Cape Evans are

linked to biological processes such as spring phytoplankton blooms (Matson *et al.*, 2014; Kapsenberg *et al.*, 2015; Schram *et al.*, 2015). In the future, pH change and phytoplankton communities will continue to interact, modifying plankton community structure and accentuating or diminishing pH variability over short timescales. As temperature increases in the ocean, smaller picophytoplanktonic species may become more abundant (Doney *et al.*, 2012). If C/N ratios in these organisms increase, there may be a reduction in food quality, with a subsequent reduction in energy available to higher trophic levels (Doney *et al.*, 2009, 2012). Similarly, reductions in sea ice extent due to warming could result in a shift in composition away from diatoms and large-celled species towards smaller phytoplankton (Montes-Hugo *et al.*, 2009). Considering *L. elliptica* feeds on large diatom species and is most common where diatoms are abundant (Ahn, 1994; Ahn *et al.*, 2001), the likelihood of populations becoming food-limited in the future is largely dependent on which of these scenarios occur.

Diatom species compositions have changed in response to past climate change (Barnes & Peck, 2008), so a switch in dominant diatom species in response to future climate change is possible, and may seriously affect *L. elliptica* unless it can switch prey species to match this change. Effects of food shortages take time to propagate through benthic communities, but would significantly reduce population sizes, with associated demographic changes (Thrush & Cummings, 2011). Larger *L. elliptica* would be expected to lose body weight first under energy-limited conditions due to comparatively greater utilisation of protein reserves, and would therefore be expected to suffer negative consequences sooner than smaller animals (Brockington, 2001). Future experiments should consider incorporating food-limited treatments to determine the effect of food stress on the response of *L. elliptica* to ocean change.

Samples already gathered as part of this research could be analysed in additional ways to enhance current understanding. Firstly, regarding gene expression work, increasing the sample size of the HSP70 gene expression analyses presented here would clarify patterns observed in this preliminary work. Measuring HSP70 concentration levels within the cell would complement gene expression levels and clarify whether HSP70 accumulates and remains in the cell after it is expressed, as discussed in Chapter 3. Further work on HSP70 gene expression in *L. elliptica* adductor tissue could show

whether HSP70 expression varies between the two types of tissue. HSP90 expression levels could also be analysed, as the HSP90 gene has been sequenced for *L. elliptica* (Kim *et al.*, 2009). This would serve as a complement to HSP70 gene expression patterns and assist further exploration of the heat shock response of *L. elliptica*. Total RNA could also be measured (as described in Cummings *et al.* [2011]) to indicate whether general protein synthesis is up-regulated within cells in response to reduced pH and/or elevated temperature. In addition, the shell structure of *L. elliptica* valves from this study could be examined using scanning electron microscopy, and the calcium content could be analysed. This would identify changes in the shell structure, and provide a more exact measure of net calcification or dissolution. Finally, antioxidant production and calcium signalling in *L. elliptica* could further indicate functional response to elevated ocean temperatures, as they appear to signify physiological stress under increased temperature (Truebano *et al.*, 2010). While beyond the scope of this thesis, empty valves from animals in this study have been collected, and additional mantle and adductor samples gathered during the course of this experiment are currently stored at -80°C and available for future analyses of this nature.

Future work should incorporate more respiration measurements at several time points throughout in the experiment. This will enable greater temporal resolution in respiration ‘snapshots’, and may reveal relationships between longer-term O₂ demand and survival. Following individuals more closely with repeated monthly assessments of O₂ consumption would show any change in O₂ demand over time, and would allow individual responses to be linked to that individual’s ability to survive the experiment. High inter-individual variability in O₂ consumption reduced the clarity and hindered the interpretation of the trends found in this study, and it would be valuable to ascertain whether certain individuals are able to maintain consistently high (or low) O₂ consumption rates over time. It would be interesting to explore whether the moribundity of individuals can be predicted from their respiration rate, and how this responds over time to a treatment. Any individuals with consistently high O₂ consumption rate are likely using a lot of energy to maintain their metabolic condition, which is likely to be a considerable drain on their resources. Will these individuals lose more condition than an individual with lower O₂ demand? Will they then die as a result of this? Conversely, if an individual has a spike in O₂ demand over time and then dies,

that would show how respiration rate is affected by experimental conditions, and how effective an indicator it is of serious organism stress, as in Peck *et al.* (2002) and Peck (2005a,b). Alternatively, are *L. elliptica* with reduced O₂ demand able to maintain this decreased rate consistently, and is there an energetic and condition loss associated with this metabolic depression? Addressing these questions would inform the understanding of the metabolic demands of *L. elliptica* with environmental change, and whether maintaining this increased metabolic demand is feasible over longer timeframes.

Ocean warming appears to be a more significant stressor than acidification for *L. elliptica*. Future studies should reflect this and feature a range of temperatures extending to +3°C or even +4°C warming, to enable more detailed study of the response of *L. elliptica* to temperature stress. It would also be beneficial to test the response of smaller temperature increases, such as the +1-2°C warming tested here, over longer timescales than 5 mo, which has been understudied compared to more acute temperature stress in *L. elliptica*. The choice of temperatures used should be tailored to the site of origin of *L. elliptica*. High-latitude Antarctic sites such as Cape Evans are cold and stable year-round, and experimental increases of a few degrees in temperature represent a considerable temperature stress for these animals. Accordingly, for Cape Evans animals, +2-2.6°C increases are suitable as an experimental treatment (Kapsenberg & Hofmann, 2014; this study). Conversely, animals from the Western Antarctic Peninsula are experiencing the fastest warming in Antarctica and among the most rapid rates warming on the planet (Meredith & King, 2005; Montes-Hugo *et al.*, 2009), and consequently ≥ 3°C experimental warming is appropriate for these animals (Morley *et al.*, 2012a). Environmentally realistic warming should be the dominant focus of future studies on the response of *L. elliptica* to future environmental change.

4.3 – Concluding comments

Upon exposure to elevated temperature and lowered pH for 5 mo, the dominant Antarctic filter-feeding infaunal bivalve *L. elliptica* experienced an increase in respiration rate, increased variability of respiration rate, decreased physiological condition, and strong trends of decreased survival at temperatures both +1 and +2°C above ambient temperatures. These results support the prediction of that a +2°C warming of the SO could cause the disappearance of populations and even entire species (Peck *et al.*, 2004).

Adults were able to maintain shell (physical) condition, indicating that mechanisms were in place to compensate for dissolution in undersaturated conditions. The considerable tissue loss relative to shell length and weight (physiological condition) suggests that *L. elliptica* were unable to reduce their energetic expenditure, possibly as a result of maintaining physical condition or up-regulating HSP70 gene expression levels. The increased O₂ consumption observed with elevated temperature or lowered pH was consistent with this hypothesis. This decrease in physiological condition would likely be particularly detrimental over winter, exacerbating previously observed physiological condition loss due to food limitation (Ahn *et al.*, 2003), and would presumably be difficult for *L. elliptica* to sustain for extended periods of time.

Lowered pH conditions exacerbated the rate and variability of O₂ consumption with loss of physiological condition. Hypercapnia also appeared to decrease survival, stimulate a short-term increase in HSP70 expression, and reduce HSP70 expression in lower-condition animals. These pH trends were biologically relevant, but small in effect compared to the impact of warming on metabolic demand, function, and survival. Warming has a greater influence than acidification on the survival and functioning of *L. elliptica*, at least over the medium-term 5 mo timescale studied here.

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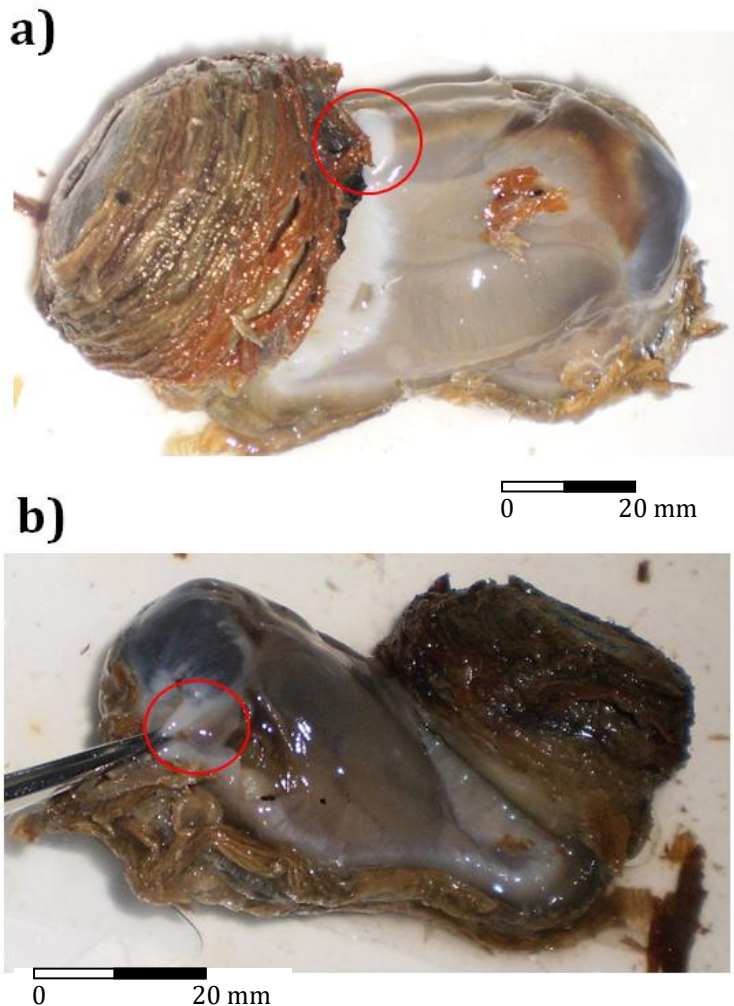
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Appendices

Appendix A – Adductor muscle dissection

Adductor muscle samples were dissected from the two attachment points to the valves: at the base of the siphon near the hinge, and at the posterior end near the foot (Sup. Fig. 1). Tissue was weighed and snap-frozen in liquid nitrogen immediately after dissection, then stored at -80°C.



Supplementary Figure 1 - *Laternula elliptica* adductor muscle dissection. The location of the adductor muscles are shown: a) anterior adductor muscle; b) posterior adductor muscle. Muscles were detached from the valves during valve removal.

Appendix B – Supplementary data

Chapter 3

Supplementary Table 1 - Annealing temperature results of *Laternula elliptica* HSP70 and β -actin qPCR gene amplification. Data presented are the number of cycles at which annealing occurred, means \pm SE.

Temperature (°C)	HSP70	β -actin	Negative control
58.0	17.29 \pm 0.11	18.81 \pm 0.66	N/A
56.3	17.41 \pm 0.34	18.69 \pm 0.66	N/A
51.9	17.69 \pm 0.19	18.87 \pm 0.69	N/A
50.0	18.28 \pm 0.15	19.68 \pm 0.61	N/A
48.0	18.39 \pm 0.21	20.03 \pm 0.69	N/A

Supplementary Table 2 - Melt curve profile data of HSP70 and β -actin qPCR gene amplification products. Data are presented as number of cycles, and peak height in reading frame units (mean \pm SE).

<i>L. elliptica</i> gene	Cycles	Temperature (°C)	Peak height (Reading frame units)
HSP70	21.02 \pm 0.24	80.00	323.93 \pm 6.10
β -actin	24.19 \pm 0.35	82.00	216.21 \pm 4.62