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Intraspecific variability in thermal tolerance across environmental temperature gradients in the intertidal periwinkle *Littorina saxatilis*

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**UNIVERSITY OF
PLYMOUTH**

**INTRASPECIFIC VARIABILITY IN THERMAL TOLERANCE ACROSS
ENVIRONMENTAL TEMPERATURE GRADIENTS IN THE INTERTIDAL
PERIWINKLE *LITTORINA SAXATILIS***

by

CHRISTOPHER PATRICK DWANE

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

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Author's declaration

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

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

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Abstract

Intraspecific variability in thermal tolerance across environmental temperature gradients in the intertidal periwinkle *Littorina saxatilis*

Christopher Dwane

Comparisons of thermal tolerance limits of organisms living across environmental temperature gradients represent a valuable means by which to study physiological adaptation. Intertidal organisms are subject to variation in levels of thermal stress both across latitude and shore height. However, our current understanding of how this variation relates to thermal adaptation is largely based on comparisons between species, and we lack a clear picture of the degree to which different populations from the same species display local adaptation along these temperature gradients. In this thesis, I investigate the extent and nature of intraspecific local adaptation of thermal tolerance across shore height and latitude in the widely distributed, direct-developing intertidal periwinkle *Littorina saxatilis*. First, I characterise thermal tolerance differences in two ecotypes of *L. saxatilis* vertically segregated along an intertidal gradient to determine whether they exhibit local thermal adaptation in line with temperature differences across shore height. Second, I assess thermal tolerance and performance in four populations of *L. saxatilis* across a latitudinal gradient to study the extent of local thermal adaptation across latitude. Finally, I compare methodological approaches for quantifying thermal tolerance limits, to address controversy regarding how the use of different approaches may affect our ability to assess intraspecific differences across latitude. This thesis demonstrates that: 1) vertically segregated ecotypes show divergence in thermal tolerance in line with their shore position, and these differences have a developmental basis; 2) upper thermal limits of cardiac

activity are greatest in populations from higher latitudes, which appears to be a maladaptive consequence of metabolic cold adaptation; and 3) the use of different methodological approaches can substantially affect both measured upper tolerance limits and our ability to interpret trends across latitude. Overall, this thesis demonstrates that local adaptation of thermal tolerance limits in *L. saxatilis* is substantial across shore height, but conversely is less pronounced across latitude. This suggests more broadly that thermal selection across a few metres of shore height may have as great an effect on intraspecific thermal adaptation as large-scale climatic variation across latitude. At the same time, compensation of metabolic rate appears to be significant component of local adaptation across latitude in this species. This thesis also demonstrates the importance of utilizing methodological approaches which can assess changes in temperature sensitivity in response to different thermal conditions in intertidal organisms.

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Chapter 1 General Introduction

Fundamentally, an organism's ability to survive and persist in nature is determined by its ability to meet the challenges imposed by its environment. These include both biotic stress (stress imposed by interactions with other organisms) and abiotic, or environmental, stress (stress imposed by the physical or chemical properties of its environment). The field of ecophysiology concerns itself with understanding how the functional characteristics of an organism (its physiology, i.e. "how it works") relate to the environment in which it lives, and therefore how it is able to cope with stress (Feder et al. 1987; Spicer and Gaston 1999; Willmer et al. 2005). Levels of abiotic stress vary dramatically between different environments. Animal life, which is believed to have originated in thermally and osmotically stable oceans, has repeatedly evolved solutions to the challenges imposed by environments which deviate from these amenable conditions (Willmer et al. 2005). Extreme environments, in which levels of a particular abiotic stressor are particularly high, present clearly definable "challenges" to organisms living there. The study of organisms from extreme environments therefore enables researchers to understand the physiological mechanisms associated with overcoming particular forms of stress (Bartholomew 1987). At the same time, the study of such organisms in isolation risks providing little insight into the underlying evolutionary processes driving adaptation to different environments, other than demonstrating that animals "can live where they do" (Bartholomew 1987).

Typically, environments do not transition abruptly from benign to stressful. Instead, gradients in environmental stress occur over a variety of spatial scales (Willmer et al. 2005), and have broad-reaching impacts, including on the distribution and range limits of species and habitats (Spicer and Gaston 1999; Gaston et al. 2008, 2009; Bozinovic et

al. 2011; Chown and Gaston 2016). Comparing across groups of organisms at different points along large-scale environmental gradients, particularly those from extreme conditions with those from more benign conditions, reveals not only how organisms cope mechanistically with environmental stress, but also the underlying patterns governing physiological adaptation (Bartholomew 1987; Feder 1987). In particular, comparing between related lineages may reveal important insights into the evolution of physiological responses, and can also reveal evolutionary constraints upon adaptation in specific groups (Kellermann et al. 2012; Hoffmann et al. 2013; Faria et al. 2017). This process of comparing between different groups of organisms along environmental gradients may be broadly defined as the comparative approach in ecophysiology (Bartholomew 1987).

The comparative approach has its roots during the Modern Synthesis – a period in the 1930s-40s which saw the conceptual unification of the Darwinian idea of evolution by natural selection with the theory of genetic inheritance (Huxley 1942). In this period, many comparative studies were conducted with a focus upon exploring the correlation of physiological parameters with environmental conditions across different populations and species (reviewed in Garland and Carter 1994; Gaston et al. 2009).

This formative period was crucial in establishing the concept that the physiological characteristics of an organism exist in “equilibrium” with its environment, as well as revealing the enormous diversity of physiological mechanisms that exist across different taxa (Feder 1987). However, with notable exceptions (e.g.; Janzen 1967; Snyder and Weathers 1975), many studies conducted in the period following the Modern Synthesis concerned themselves with answering specific questions related to the evolution of particular physiological adaptations, instead of elucidating broad-scale

and underlying patterns governing adaptation across environmental gradients (Feder 1987; Garland and Carter 1994; Spicer and Gaston 1999; Gaston et al. 2009). However, the last two decades of the 20th century saw a redefining of the mission of comparative physiology towards asking general questions and understanding large-scale biogeographic patterns (Spicer and Gaston 1999; Chown 2004; Gaston et al. 2009; Chown and Gaston 2016). The urgent need for a change of course was expressed by Feder (1987), who argued that ecophysiolgists needed to adopt a “nomothetic” approach, much as Gould (1980) had argued for the field of palaeontology – that is to say, a move towards the description of general scientific principles or laws which underlie physiological differences between populations and species. The subsequent reinvigoration of the discipline is ongoing to this day, and is perhaps best exemplified by the emergence of “macrophysiology”, the study of physiological variation over large geographical and temporal scales and its ecological implications (Chown 2004; Gaston et al. 2009; Chown and Gaston 2016). Numerous large scale meta-analyses have been published in the past two decades, often incorporating datasets from dozens of species and seeking to characterize and understand broad-scale patterns of physiological diversity and its relationship to environmental gradients (Addo-Bediako et al. 2000, 2002; Deutsch et al. 2008; Huey et al. 2009; Sunday et al. 2011, 2019; Hoffmann et al. 2013; Gunderson and Stillman 2015; Chown and Gaston 2016). This has been accompanied by the development of new theoretical frameworks to understand physiological adaptation, providing hypotheses to test using empirical data (Huey and Kingsolver 1989; McMahon 1990; Gilchrist 1995; Pörtner 2001; Clarke 2004; Angilletta 2009; Schulte et al. 2011; Pörtner et al. 2017). At the same time, comparisons across environmental gradients in individual species and taxa have remained extremely relevant. These have played an important role in providing

empirical data to test predictions derived from theoretical work (Gaston and Spicer 1998, 2001; Marshall et al. 2015; Morley et al. 2016; Alruiz et al. 2021), and in some cases, in providing novel observations to develop new hypotheses and theoretical frameworks (e.g. Stillman and Somero 2000; Terblanche et al. 2007; Calosi et al. 2010).

Patterns of physiological variation across environmental gradients are typically assessed at either the intraspecific or interspecific levels (Spicer and Gaston 1999; Gaston et al. 2008, 2009). Intraspecific (within- species) comparisons across environmental gradients utilise spatially – separated populations from different parts of a species’ distribution (Kawecki and Ebert 2004; Gaston et al. 2008, 2009).

Interspecific (between-species) comparisons examine differences in the responses of two or more species found at different points along an environmental gradient (Bartholomew 1987; Gaston et al. 2008, 2009; Rezende and Diniz-Filho 2012).

Interspecific comparisons are often seen as the “default” level at which evolutionary adaptation is studied, because species represent biologically distinct units (Powers 1987), and because the breadth of physiological variation between species is generally greater than within species (Spicer and Gaston 1999). However, physiological differences between species are not only influenced by environmental differences, but also by their unique evolutionary histories (Keller and Seehausen 2012), and accounting for phylogenetic effects represents a substantial challenge in interspecific comparisons (Rezende and Diniz-Filho 2012). By contrast, intraspecific comparisons reduce these phylogenetic effects and enable researchers to explore how populations respond to the effects of divergent selection using a common “toolkit” of physiological mechanisms present in a single species (Feder 1987; Spicer and Gaston 1999; Gaston et al. 2008). Intraspecific comparisons can provide important insights into variation

across a species' distribution, for example whether gradients in responses precisely match environmental stress gradients, or whether responses vary away from a "peak" around the centre of a species' distribution (Gaston et al. 2008). Knowledge of these dynamics is important, because interspecific comparisons often assume a single "mean" physiological response for each species, and may overlook the extent of physiological variation within individual species (Gaston et al. 2009). Intraspecific comparisons can also examine the effects of gene flow and genetic drift on physiological adaptation (Kawecki and Ebert 2004) and may provide insights into the formation of new species, as physiological differences between populations may provide the genetic material for eventual speciation (Keller and Seehausen 2012). In summary, intraspecific comparisons across environmental gradients provide a powerful framework for studying aspects of adaptive evolution which cannot be addressed in interspecific studies.

A key concept in comparative physiology is "Krogh's principle" – the concept that for any physiological mechanism, there exists an organism especially suited for its study (Feder et al. 1987; Garland and Carter 1994). With this in mind, a suitable species for the comparative study of intraspecific physiological adaptation needs to be widespread enough to occur across a range of environmental conditions.

Unfortunately, because many species have limited ranges or only inhabit certain habitats (Gaston et al. 2008), it is often challenging to study intraspecific variation across environmental gradients, and much of our existing knowledge of how local adaptation takes place is instead inferred from interspecific comparisons. In the following review, I introduce two ecologically important gradients in which existing knowledge of the extent of intraspecific local adaptation is lacking – intertidal

temperature gradients across latitude and shore height - and outline how my thesis aims to address this knowledge gap using a suitable study species, the gastropod *Littorina saxatilis*. First, I characterize the spatiotemporal patterns of temperature variation which occur across both latitude and shore height and briefly review our existing knowledge of how the thermal tolerance of organisms varies across these gradients. I draw attention to our limited understanding of the extent of intraspecific variation across intertidal gradients and address why this is an issue. Following this, I introduce the different methodological approaches which will be used to assess thermal tolerance across these gradients in my thesis. Lastly, I state of the overall aims of my thesis, and outline the characteristics that make *L. saxatilis* an ideal model to study intraspecific local thermal adaptation.

1.1 Comparing thermal tolerance across latitude and shore height

1.1.1 Temperature gradients across latitude

Temperature is perhaps the single most important physical stressor affecting life on earth across all levels of biological organization (Cossins and Bowler 1987; Hochachka and Somero 2002). It has a pervasive impact upon the ability of organisms to carry out the basic functions of life, meaning that gradients in environmental temperature at different spatial and temporal scales play a major role in determining the distribution patterns of different species and populations (Snyder and Weathers 1975; Spicer and Gaston 1999). Perhaps the broadest scale temperature gradient occurs across latitude, where mean annual temperatures are typically highest in the tropics and decrease towards the poles. This gradient is influenced at a smaller scale by local climatic and geographical features in different parts of the world. For example, while mean annual temperatures on land drop on average by 0.73°C per degree of latitude in the northern hemisphere, they fall by only 0.57°C per degree in the southern hemisphere, a consequence of the more maritime climate experienced on the southern continents (De Frenne et al. 2013). In addition to a reduction in mean annual temperatures across latitude, terrestrial temperature variability increases at higher latitudes due to greater seasonal variation in climate (Janzen 1967; Deutsch et al. 2008; Sunday et al. 2011). Thus, the range of temperatures experienced by terrestrial organisms at high latitudes is much greater than that experienced in the tropics. Importantly, this increase in variability is mostly the result of a rapid decline in minimum temperatures moving poleward, while annual thermal maxima vary comparatively little across latitude (Snyder and Weathers 1975; Sunday et al. 2011; Huey et al. 2012). By contrast, in marine environments, both annual thermal maxima and minima decrease at

comparable rates with increasing latitude (Compton et al. 2007; Sunday et al. 2011), due to the greater thermal inertia of the oceans resulting in a buffering effect on diurnal and seasonal temperature fluctuations. An additional difference between terrestrial and marine thermal regimes lies in polar regions. While terrestrial polar regions experience high seasonal thermal variability, particularly in the northern hemisphere, marine polar environments are some of the most thermally stable on earth (Peck et al. 2009). As a result, in contrast to terrestrial environments where thermal variability increases with latitude, temperature variability in marine environments is greatest at temperate latitudes and declines both in the tropics and at the poles (Sunday et al. 2011; Morley et al. 2012).

Temperature variation across latitude plays a critical role in determining the distribution and range limits of different species. It is especially important in ectothermic organisms, which, unlike endotherms, are unable to regulate their internal body temperature and so are “at the mercy” of environmental temperature variation (Schmidt-Nielsen 1997). Broadly, changes in the performance of ectotherms across different temperatures may be defined using thermal performance curves (TPCs; Huey and Stevenson 1979; Kingsolver 2009; Sinclair et al. 2012; Schulte 2015). These are unimodal, left skewed curves, with a peak describing the temperature at which an organism’s performance is optimal (T_{opt}). The region of the TPC in which performance is above a certain threshold - for instance 80% of maximal performance according to Angilletta (2002); 50% in Sinclair *et al.* (2016) - describes an organism’s thermal performance breadth. The upper and lower limits of the TPC (thermal tolerance limits, often referred to as CT_{max} and CT_{min} respectively) describe an organism’s thermal

tolerance breadth, which is the range of temperatures it can tolerate for at least a short period of time.

The shape and breadth of TPCs are closely linked to the range of environmental temperatures over which ectotherms can inhabit (Cossins and Bowler 1987; Huey and Kingsolver 1989; Spicer and Gaston 1999; Angilletta 2009). Therefore, ectotherms living in different thermal environments display shifts in the thermal window encompassed by their TPC in response to environmental temperature variation. This ensures that adequate performance is maintained to support long-term survival, growth and reproduction, and that extreme environmental temperatures do not regularly exceed tolerance limits (Huey and Stevenson 1979; Angilletta 2009; Schulte 2015). This thermal adaptation can involve shifts in performance breadth, T_{opt} , and upper and lower tolerance limits, and can either occur through genetic adaptation, or reversible or irreversible adjustments in response to local environmental temperature regimes via acclimation (Hochachka and Somero 2002; Angilletta 2009).

Comparisons of species and populations living at different latitudes have revealed a broad correlation between thermal physiological traits and environmental temperature variation, with both optimal performance temperature and upper and lower thermal limits being higher in species living at lower latitudes and decreasing at higher latitude (Snyder and Weathers 1975; Addo-Bediako et al. 2000; Deutsch et al. 2008; Angilletta 2009; Sunday et al. 2011, 2019). In addition to upper and lower limits, thermal performance and tolerance breadth also vary systematically in response to different levels of temperature variability across latitude. This concept was invoked by Janzen (1967) who suggested that, because thermal regimes in lowland tropical environments experience little overlap with those at high altitudes, mountain ranges

should pose a greater physiological obstacle to organisms living in tropical ecosystems. By contrast, climate variability in lowland temperate environments is much higher and hence more likely to overlap with temperatures at high altitude, making it easier for temperate organisms to cross mountain ranges. Today, this concept is referred to as the Climate Variability Hypothesis (CVH; Gaston et al. 2009; Bozinovic et al. 2011). The CVH proposes that, because climatic variability increases with latitude, populations and species from higher latitudes should possess a higher thermal tolerance breadth to cope with increased variability, while organisms from stable low latitudes should possess reduced tolerance breadth (Spicer and Gaston 1999; Bozinovic et al. 2011). Linked to the CVH is the Specialist-Generalist trade-off hypothesis, which predicts that species from more variable, high latitude environments (“generalists”, also known as “eurytherms”) should possess wider thermal performance breadth than species from more stable tropical environments (“specialists” or “stenotherms”; Kingsolver 2009; Verberk et al. 2016a)

Numerous large-scale studies and meta-analyses have provided support for both the CVH and the Specialist-Generalist trade-off hypothesis, finding that thermal tolerance and performance breadth is narrowest in species living at the equator and wider in temperate species (Deutsch et al. 2008; Sunday et al. 2011, 2014, 2019; Huey et al. 2012). Importantly, underlying trends in thermal breadth differ substantially between terrestrial and marine environments, matching climatic differences in each of these environments across latitude. In terrestrial environments, increased tolerance breadth in organisms from higher latitude are primarily driven by a shift in lower limits of thermal tolerance rather than upper limits (Addo-Bediako *et al.* 2000; Clusella-Trullas *et al.* 2011; Sunday *et al.* 2011; Huey *et al.* 2009). This may be because annual thermal

maxima vary comparatively little across latitude, as occasional extreme temperature events occur even at high latitudes (Sokolova et al. 2000; Sunday et al. 2011; Clark et al. 2021). By contrast, in marine environments both upper and lower tolerance limits decrease with increasing latitude (Sunday *et al.* 2011; Compton *et al.* 2007; Morley *et al.* 2012; 2016; Stillman and Somero 2000), meaning that latitudinal changes in thermal breadth are not as extreme as in terrestrial environments. In summary, variation in the thermal tolerance limits and thermal breadth of ectotherms broadly reflects changes in climatic variability across latitude, demonstrating the importance of both mean temperatures and thermal variability on the process of thermal adaptation.

1.1.2 Temperature gradients in the intertidal

Organisms living in intertidal habitats have long attracted the attention of ecophysiologists (Bartholomew 1987; Newell 1979; Tomanek and Helmuth 2002). Given that they primarily descend from marine lineages, the distribution of intertidal species is believed to be highly constrained by their ability to withstand physiological stress, as they must contend with extremes of stress far greater than those experienced by subtidal relatives (Newell 1979; Stillman and Somero 1996). Intertidal taxa are also often slow moving or immobile, meaning conditions experienced by individuals separated by only a few metres may differ dramatically and consistently throughout an organism's life (Dong et al. 2021). These factors, coupled with a steep gradient in intensity and variation of biotic and abiotic stress, make the intertidal an ideal environment to study physiological adaptation between populations and species living at different shore heights (Newell 1979; Bartholomew 1987; Somero 2002; Tomanek and Helmuth 2002; Stenseng et al. 2005; Bozinovic et al. 2011). At the same

time, the effects of exposure at low tide also interact with climatic differences across latitude (Helmuth et al. 2002), which, coupled with the fact that many coastlines run north-south (Schoch et al. 2006; Finke et al. 2007; Seabra et al. 2015), has led to intertidal organisms being widely utilized in comparisons across latitudinal gradients (Stillman and Somero 2000; Compton et al. 2007; Gaitán-Espitia et al. 2014; Armstrong et al. 2019; Dong et al. 2021).

Thermal stress in intertidal habitats occurs due to a sharp vertical gradient from comparatively benign marine conditions to highly variable terrestrial conditions over the course of only a few metres, with the exact transition point varying from hour to hour depending on tidal fluctuations (Newell 1979). Moving up the shore, time spent under emersion at low tide gradually increases, and because maximum temperatures are much higher in air than in water (Finke et al. 2007), both greater intensity and duration of thermal stress, and greater thermal variability, is experienced at the top of the shore (McMahon 1990; Finke et al. 2007). In particular, organisms in the splash zone at the very top of the shore may be continually exposed to terrestrial conditions for periods of several days during neap tides (McMahon 1990). Thermal stress also varies on a microhabitat scale, often over the course of only a few metres in response to local topography, rock coloration, and the orientation of the rockface with respect to the midday sun (Seabra et al. 2011; Choi et al. 2019; Wang et al. 2020).

Across latitude, mean temperatures in the intertidal generally increase towards the equator (Schoch et al. 2006; Seabra et al. 2011), mirroring patterns seen in terrestrial and marine environments. An important distinction is that, unlike tropical terrestrial environments which are extremely thermally stable, upper intertidal habitats in the tropics exhibit enormous temperature variability across tidal cycles (Marshall et al.

2015). Thus, although thermal variability increases with shore height, it does not show the same latitudinal changes as seen in marine and terrestrial environments, instead remaining high across different latitudes, although seasonal temperature variability still increases in temperate regions (Helmuth et al. 2002). Substantial differences in thermal stress regimes across different sites also occur on a local scale (Seabra et al. 2015; Choi et al. 2019; Wang et al. 2020; Dong et al. 2021). These differences occur due to regional climatic variation (Seabra et al. 2015; Choi et al. 2019) but are also influenced by the frequency of overlap between emersion at low tide and exposure to midday temperatures at different sites. For example, Finke et al. (2007) found that the extent of overlap between low tides and midday sun exposure varies systematically across coastlines in different parts of the world, with South American and East Australian coastlines consistently experiencing the greatest aerial exposure during midday summers. On a smaller scale, Helmuth et al. (2002) examined differences in the length of midday exposure at low tide across different sites along the western coastline of the USA. They found that many southern sites were protected from high temperatures by always being at high tide during midday sun exposure, while conversely, northern sites were more at risk due to being at low tide during such exposure conditions. This resulted in “hot spots” of severe thermal stress which did not match broader latitudinal patterns. In addition, they found that the location of these hot spots changed from year to year. In summary, while thermal adaptation in the intertidal is chiefly driven by broad scale differences in temperature across latitude and shore height, it is also influenced by localized or “mosaic” patterns of thermal heterogeneity (Helmuth et al. 2002, 2006), as well temporal variation in thermal stress, which are important factors influencing local thermal adaptation at smaller scales.

1.1.3 Thermal adaptation in the intertidal

It has long been established that species found at different shore heights display differences in thermal tolerance corresponding to the temperatures experienced in their respective habitats (Newell 1979; McMahon 1990). One of the most comprehensive studies into patterns of thermal adaptation across latitude and shore height was conducted by Stillman and Somero (2000). They examined the correlation between lethal temperatures and vertical shore position, as well as latitude (temperate or tropical) across 20 species of the Porcelain crab genus *Petrolisthes*, finding that maximal thermal tolerance of each species correlated with their maximal microhabitat temperature, and that this occurred both in respect to shore height and latitude. Another important finding is that upper thermal limits display a greater decline across latitude in lower compared to upper intertidal organisms (Stillman and Somero 2000; Compton et al. 2007; Dong et al. 2021). This mirrors differences in latitudinal variation in thermal tolerance between marine and terrestrial environments (Sunday et al. 2011; 2014) and probably reflects the relative influence of each environment at different shore heights, as there is a greater consistency in maximal air temperatures across latitude compared to sea temperatures (Sunday *et al.* 2011).

At the same time, species from the upper shore also possess lower thermal safety margins (TSM) than those from the lower intertidal (Stillman and Somero 2000; Miller et al. 2013; Vinagre et al. 2019). TSMs represent the difference between upper tolerance limits of an organisms and the maximum habitat temperatures it experiences (Sunday et al. 2014). TSMs are reduced towards the equator (Armstrong et al. 2019;

Vinagre et al. 2019), suggesting that elevated thermal tolerance limits do not fully compensate for increased thermal maxima experienced in different habitats (Stillman and Somero 2000). Tropical upper intertidal species may thus regularly encounter temperatures close to or exceeding their thermal limits, indicating they may be especially vulnerable to future temperature increases (see also Marshall et al. 2015).

Despite possessing broader thermal tolerance windows compared to lower intertidal species, upper intertidal organisms possess reduced capacity to adjust their thermal tolerance breadth through acclimation (Tomanek and Somero 1999; Gunderson and Stillman 2015; Armstrong et al. 2019). For example, when *Petrolisthes* crabs from different shore heights were acclimated to high temperatures for extended time periods, upper thermal limits were improved in subtidal and lower shore species to a greater extent than in upper intertidal species (Stillman and Somero 2000; Stillman 2004). Similarly, Stenseng et al. (2005) found that two subtidal and lower intertidal species of the gastropod *Tegula* displayed a greater acclimatory capacity of their upper limits of cardiac function compared to an upper intertidal species. These differences appear to reflect a trade-off in the thermal strategies of upper and lower shore species (Verberk et al. 2016a). Upper shore species display a characteristic “eurythermal” strategy, possessing wide TPCs with broad thermal tolerance and performance breadth to cope with extreme temperature fluctuations, but conversely a poor ability to adjust their TPCs via acclimation. By contrast, lower intertidal species, which display a “stenothermal” strategy, have narrower thermal performance and tolerance breadth, but conversely a greater ability for long term adjustment of their TPCs. There are several potential reasons why acclimatory capacity may be lower in upper intertidal species. Firstly, biochemical constraints may set “ceilings” upon further increases in

thermal tolerance via acclimation in species possessing high thermal limits (Hochachka and Somero 2002; van Heerwaarden and Kellermann 2020). Acclimation may also be less advantageous in species with broader TPCs, because a larger shift in the position of the TPC is required to improve performance compared to in species with narrower TPCs (Verberk et al. 2016a). Alternatively, high acclimation capacity may be less favoured in upper shore organisms because habitat temperatures experienced at any one time are likely to be poor predictors of future conditions (McMahon 1990).

Although numerous interspecific comparisons have demonstrated the importance of thermal adaptation across shore height and latitude, there is a lack of consensus regarding the extent of within-species variation in thermal tolerance across intertidal gradients (Wang et al. 2019). While some studies have identified intraspecific differences in thermal tolerance across latitude (Gaston and Spicer 1998; Sokolova et al. 2000; Kelly et al. 2012; Madeira et al. 2012; Tepolt and Somero 2014) others have failed to find evidence of substantial latitudinal variation in upper limits (Clarke et al. 2000; Lee and Boulding 2010; Logan et al. 2012; Gaitán-Espitia et al. 2014; Dong et al. 2015, 2017; Jupe et al. 2020). Similarly, only a limited number of studies have explored intraspecific variation in thermal tolerance across shore height (Sokolova et al. 2000; Diederich and Pechenik 2013; Moyen et al. 2019). Comparisons across latitude are often made more challenging by mosaic patterns of temperature variation across different sites which may obscure latitudinal climatic differences (Helmuth and Hofmann 2001; Seabra et al. 2015; Choi et al. 2019). This problem is especially great when populations from only two sites (Garland and Adolph 1994; Sokolova et al. 2000), or from a small part of a species' total distribution are considered. However, even larger scale studies have in some cases failed to identify latitudinal differences in

thermal tolerance limits. For example, Gaitán-Espitia et al. (2014) found evidence of greater performance breadth in high latitude populations of the porcelain *Petrolisthes violaceus*, but no evidence of systematic variation in upper tolerance limits, even across a 3000 km latitudinal gradient. This result is especially pertinent, given that the genus *Petrolisthes* is known to display substantial interspecific variation across latitude (Stillman and Somero 2000).

In addition, much of our knowledge of intraspecific variation in tolerance limits is limited to intertidal species with planktonic larval dispersal (Lee and Boulding 2010; Diederich and Pechenik 2013; Gaitán-Espitia et al. 2014; Tepolt and Somero 2014; Moyen et al. 2019). Since animals with high dispersal may encounter a range of environmental conditions different to those experienced by their parents, such conditions may favour physiological plasticity over local genetic adaptation (Hollander 2008; Hart and Marko 2010; Lee and Boulding 2010; Sanford and Kelly 2011), and these differences may be obscured following laboratory acclimation (Spicer and Gaston 1999). Because of this, we lack information on the extent of local adaptation of thermal tolerance in direct-developing intertidal species, many of which display a high degree of local divergence for other traits such as morphology, metabolism and reproductive strategy, often with a genetic basis (Butlin et al. 2014; Rolán-Alvarez et al. 2015; Maltseva et al. 2021). Lack of knowledge of the extent of intraspecific differences also hampers our efforts to assess how species distributions may respond to future changes in temperature (Kuo and Sanford 2009; Sanford and Kelly 2011). This is compounded by the fact that many studies, particularly meta-analyses assessing the vulnerability of different species to climate change (Deutsch et al. 2008; Sunday et al. 2011, 2014; Gunderson and Stillman 2015) frequently assume a single generalised

thermal tolerance response per species. Thus, if inter-population variability in thermal tolerance is high, the overall resilience of a species to temperature change may be underestimated (Spicer and Gaston 1999). Conversely, if interpopulation variability in thermal tolerance is low, this increases the utility of studies which assess spatial variability in microhabitat temperature regimes in the intertidal (Seabra et al. 2011, 2015; Miller and Dowd 2017; Choi et al. 2019; Wang et al. 2020), as these can then be used to more confidently predict performance and fitness outcomes of conspecifics living at different sites.

1.1.4 Methods of assessing thermal tolerance limits

Two major methods for determining thermal tolerance limits exist (Lutterschmidt and Hutchison 1997; Kingsolver and Umbanhowar 2018). The first is the static method (Lutterschmidt and Hutchison 1997; Rezende et al. 2014), in which organisms are held at a constant stressful temperature and the time taken to reach a physiological endpoint (UTL or upper tolerance limit) is measured. The second, the dynamic or ramping method, relies on exposing organisms to benign temperature at the start of a trial and then continually increasing temperature until a physiological endpoint (the critical thermal maximum, CT_{max}) is reached (Cowles and Bogert 1944; Lutterschmidt and Hutchison 1997).

Of the two, the dynamic approach has enjoyed more widespread use in comparative studies (Kingsolver and Umbanhowar 2018), thanks to its requirement for smaller replicate numbers and the fact it generates intuitive endpoints which facilitate the comparison between different taxa and datasets (Lutterschmidt and Hutchison 1997; Sunday et al. 2011; Hoffmann et al. 2013; Kingsolver and Umbanhowar 2018).

Endpoints used to assess CT_{max} under the dynamic approach vary widely across taxa and include knockdown in insects (Terblanche et al. 2011), heat coma in gastropods (Sandison 1967), and the onset of spasms in lizards (Cowles and Bogert 1944; Lutterschmidt and Hutchison 1997). The dynamic approach can also be extended to measure changes in performance-based traits such as cardiac activity or metabolic rate with increasing temperature (Stenseng et al. 2005; Jupe et al. 2020; Dong et al. 2021).

The dynamic approach has seen extensive use in the study of variation in thermal tolerance limits across latitude and shore height (Stillman and Somero 2000; Stenseng et al. 2005; Deutsch et al. 2008; Sunday et al. 2011; Hoffmann et al. 2013; Buckley and Huey 2016; Dong et al. 2017, 2021). However, a fundamental limitation of the dynamic approach has recently attracted much attention in the literature, namely that it cannot distinguish between the effects of intensity vs. duration of thermal stress on upper thermal tolerance limits (Rezende et al. 2011, 2014; Santos et al. 2011; Terblanche et al. 2011). This is because both temperature and exposure time increase simultaneously under dynamic ramping, meaning the extent to which injury accumulation and depletion of energy reserves occurs is dependent on the rate of temperature increase (Kellermann et al. 2012; Jørgensen et al. 2019). This was highlighted in a study by Terblanche et al. (2007), who studied the effect of changes in ramping rate on the CT_{max} of Tsetse flies. It was predicted that slow ramping rates would result in higher CT_{max} measurements due to acclimation and heat-hardening (Cossins and Bowler 1987; Lutterschmidt and Hutchison 1997; Angilletta 2009). However, instead they observed the opposite, as CT_{max} estimates were lower in flies subjected to slower ramping rates. Similar effects of heating rate on CT_{max} have also been demonstrated in springtails (Allen et al. 2016), fish (Mora and Maya 2006) and a variety of marine taxa (Nguyen et

al. 2011; Peck et al. 2014), indicating that estimates of thermal tolerance may differ substantially depending on the ramping rate employed, and that faster ramping rates tend to provide higher estimates of CT_{max} .

Due to these limitations, dynamic ramps offer only a “snapshot” of an organism’s thermal tolerance under a single set of conditions, and so may provide poor estimates of organism’s thermal tolerance limits under natural conditions in which both intensity and duration of thermal stress fluctuate (Rezende et al. 2011; Santos et al. 2011). To counter this, renewed interest has been placed in the thermal death time (TDT) approach, which enables the effects of duration and intensity of thermal stress to be properly distinguished (Rezende et al. 2014; Castañeda et al. 2015).

The TDT approach utilises static trials to generate tolerance times over a range of different temperatures. Then, log-transformed tolerance times are plotted against test temperatures, revealing a characteristic linear relationship between temperature and thermal tolerance – the TDT curve – which can be described using the following equation:

$$\log_{10}t = \frac{(CT_{max} - T)}{z}$$

With T corresponding to the assay temperature, t describing tolerance time, and CT_{max} describing the temperature at which tolerance times would hypothetically equal one minute, The constant z describes an organisms’ sensitivity to temperature change and corresponds to the reciprocal of the slope given when TDT curves are plotted with temperature against the logarithm of tolerance time (Rezende et al. 2014; Jørgensen et al. 2019).

The relationship between CT_{max} and z encapsulates both a populations' ability to tolerate acute exposure to high temperature, and its sensitivity to exposure at lower but still chronically stressful temperatures over longer periods. Because of this, comparisons of z and CT_{max} values from different populations and species have recently been used to generate important novel insights into how thermal strategies differ across environmental gradients (Rezende et al. 2014; Castañeda et al. 2015; Alruiz et al. 2021). Castañeda et al. (2015) exposed *Drosophila* from different populations along a latitudinal gradient in Chile to four static assay temperatures, and TDT curves were modelled for each. A trade-off between CT_{max} and z was observed across latitude, with the southernmost species (from the coldest thermal environment) having higher CT_{max} values but also higher z -values, indicating reduced survival under prolonged thermal stress. By contrast, flies from warmer populations had lower z values, indicating greater survival times at less extreme temperatures, but also lower CT_{max} indicating reduced acute tolerance. More recently, Alruiz et al. (2021) showed how variation in thermal tolerance across four species of *Drosophila*, measured using the TDT approach, corresponded to their respective distributions across a latitudinal thermal gradient, demonstrating that differences in CT_{max} and z may be highly important in determining species distribution patterns across environmental gradients. The TDT approach has recently been used to identify trade-offs in the chronic vs. acute thermal tolerance in different life history stages of an intertidal gastropod (Truebano et al. 2018); across size and age groups in *Daphnia* (Burton and Einum 2020), and across body size in ectotherms (Peralta-Maraver and Rezende 2021), demonstrating that it may provide important insights into variation in thermal tolerance which are not apparent under the dynamic approach. Intertidal environments represent a promising environment in which to utilise the TDT approach, given that duration of heat

exposure varies substantially across shore height, and the ability to resist extended periods of thermal stress represents a substantial selective pressure in the upper intertidal (McMahon 1990). Despite this, no studies have so far applied the TDT approach to comparisons in intertidal species, either across shore height or latitude, meaning we do not currently understand the extent to which a trade-off in intensity vs. duration of thermal stress may play a role in impacting thermal tolerance in this environment.

At the same time, others have argued for the continued use of dynamic approaches, given that they have heightened ecological relevance in simulating rates of warming under natural conditions (Terblanche et al. 2011; Marshall et al. 2015; Dong et al. 2021) and can incorporate the assessment of thermal performance prior to collapse, which cannot be assessed under the static approach (Kingsolver and Umbanhowar 2018). In particular, dynamic approaches involving the measurement of cardiac activity are widespread in studies of intertidal organisms (Stenseng et al. 2005; Moyen et al. 2019; Dong et al. 2021). Cardiac activity is a useful measure of performance as it is closely correlated to metabolic rate (Frederich and Pörtner 2000) and can be used to identify responses such as metabolic depression which are associated with heat stress in some intertidal species (Hui et al. 2020). In addition, the temperature at which cardiac activity ceases – cardiac flatline - is related to lethality and thus represents a good marker for upper tolerance limits (Dong et al. 2021).

In summary, both the static TDT approach, and the dynamic approach incorporating cardiac activity, provide complementary methods which are both highly relevant to the study of thermal adaptation across gradients in intertidal organisms. Both approaches go beyond describing thermal tolerance in terms of a single endpoint; instead allowing

for a more multi-faceted assessment of how thermal sensitivity responds across different thermal conditions. This aspect is particularly critical given the highly variable nature of intertidal environments. Thus, in my thesis I adopt both approaches, using each to address different questions regarding intraspecific thermal adaptation in the intertidal. At the same time, the use of a combination of methodological approaches requires consideration of the effect each approach may have on the interpretation of results (Cooper et al. 2008; Rezende et al. 2011; Santos et al. 2011), and this is an aspect I also aim to address.

1.2 Thesis aims

The central aim of my thesis is to characterise the extent and nature of intraspecific local adaptation of thermal tolerance across environmental temperature gradients in the widely distributed intertidal periwinkle *Littorina saxatilis*. Below, I briefly introduce the structure and themes of each chapter of my thesis. Following this, I outline the unique characteristics of *L. saxatilis* that make it a suitable study system for intraspecific comparisons of thermal adaptation across environmental gradients.

In Chapter 2, I investigate thermal adaptation across shore height, characterising thermal tolerance differences in two vertically segregated populations of *L. saxatilis*. To achieve this, I utilise the static TDT approach, enabling me to assess whether populations at different shore heights possess differences in their response to the effects of intensity vs. duration of thermal stress. I include both adults and embryonic stages, allowing me to assess whether differences in thermal sensitivity across shore height have an ontogenetic basis.

In Chapter 3, I investigate thermal adaptation across latitude, characterising differences in thermal performance, thermal tolerance, and physiological plasticity across four latitudinally distinct populations of *L. saxatilis* following acclimation to three temperatures (10°C, 15°C, 20°C). I measure TPCs for cardiac activity in response to a dynamic temperature ramp of 6°C h⁻¹, to investigate latitudinal differences in thermal tolerance and performance. I also assess the effect of thermal acclimation on the shape of TPCs, and in turn whether acclimation capacity varies across latitude.

In Chapter 4, I assess how the use of different methodological approaches commonly used in the literature may affect our interpretation of patterns of thermal tolerance across latitude. In three separate comparisons, I consider: a) the effects of different

ramping media (air vs. water) in dynamic assays, b) the effect of different ramping rates in dynamic assays (6°C h^{-1} vs. $12^{\circ}\text{C h}^{-1}$), and c) how predictions based upon dynamic assays differ from those generated using the static TDT approach.

In Chapter 5, I synthesise the core findings of the previous chapters, considering them in the context of previous research. I also discuss gaps in our existing knowledge and highlight avenues for future work.

1.3 Study species: *Littorina saxatilis*

The rough periwinkle, *Littorina saxatilis* is an intertidal gastropod belonging to the subgenus *Neretrina* within the Littorinid family (Reid 1996; Rolán-Alvarez et al. 2015). In general, it can be distinguished from congeners by its roughly textured shell, although the species has a highly variable morphology and coloration which differs both within and between different populations (Reid 1996). Indeed, morphological variation is so great that under traditional taxonomic methods based upon shell traits, the species was once known under 28 synonyms (Reid 1996), but has now been recognized using genetic and reproductive characters to represent a single, highly variable species (Johannesson 2016).

Littorinids have long been used in the study of thermal adaptation in the intertidal (Newell 1979), due to their high abundance (Rolán-Alvarez et al. 2015), ease of collection, and limited mobility, the latter factor making them highly reliant upon physiological mechanisms to resist environmental stress at low tide (Dong et al. 2021). In addition, Littorinids possess a number of highly tractable physiological traits, including heat coma (Sandison 1967; Clarke et al. 2000) and cardiac flatline (Marshall

et al. 2015; Dong et al. 2021), which facilitate the straightforward comparison of upper thermal tolerance limits across different populations.

Aside from these general characteristics, *L. saxatilis* possesses several unique traits making it especially favourable for use in intraspecific comparisons across environmental gradients. Firstly, *L. saxatilis* has the broadest latitudinal distribution of its genus, being found along the Atlantic coast of Europe and North Africa from Svalbard to the Canary Islands, as well as Greenland and the Eastern coast of North America. It also inhabits a diverse array of intertidal habitats, with populations being found in both extremely sheltered estuarine habitats as well as highly exposed promontories (Reid 1996). In contrast to many intertidal species, *L. saxatilis* can be found inhabiting a variety of shore heights, although it is typically associated with the upper intertidal. As a result, different populations of the species experience a diverse array of thermal conditions, both across latitude and intertidal gradients.

Despite this wide distribution, the species has extremely low within-lifetime dispersal - possibly as low as 2-10 meters (Johannesson 2016). This is a consequence of its ovoviviparous (brooding) development, in which embryos are carried by adult females until gestation inside a modified capsule gland known as a brood pouch. Embryos undergo direct development and hatch as crawlways, which are miniature versions of the adults (Reid 1996). This developmental mode is unique to the species within the genus *Littorina* and contrasts with other members of the subgenus which are direct-developing egg-layers (Reid 1996). Low dispersal rates result in very high genetic heterogeneity between populations, a feature beneficial to the study of local thermal adaptation as it results in a high degree of local genetic adaptation. Phylogenetic comparisons have indicated that gene flow between sites is extremely limited,

demonstrating the status of different populations as discrete “units” suitable for the comparative study of local thermal adaptation along their latitudinal distribution (Doellman et al. 2011; Panova et al. 2011). In some locations, low dispersal has also facilitated the vertical segregation of *L. saxatilis* across shore height into distinctive morphological varieties known as ecotypes (Reid 1996). Notable examples are found in NW Spain (introduced in detail in Chapter 2), although similar ecotypes also occur in the north of England and Scotland (Reid 1996; Grahame et al. 2006; Christmas et al. 2014). Ecotypes have emerged in parallel at different locations, so share a common genetic affinity across shore height, while their distinctiveness is maintained by limited dispersal and premating isolation barriers (Butlin et al. 2014). Their existence thereby offers a rare opportunity to explore intraspecific local adaptation of thermal tolerance in distinct populations across shore height, which is otherwise challenging to study as many intertidal species are only found at a single shore height (Newell 1979), or possess planktonic larval dispersal resulting in high gene flow and limited distinction across shore height (Diederich and Pechenik 2013).

Chapter 2 Divergence in thermal physiology could contribute to vertical segregation in intertidal ecotypes of *Littorina saxatilis*

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Abstract

Thermal stress is a potentially important selective agent in intertidal marine habitats, but the role that thermal tolerance might play in local adaptation across shore height has been underexplored. Northwest Spain is home to two morphologically distinct ecotypes of the periwinkle *Littorina saxatilis*, separated by shore height and subject to substantial differences in thermal stress exposure. However, despite other biotic and abiotic drivers of ecotype segregation being well studied, their thermal tolerance has not been previously characterised. We investigated thermal tolerance across multiple life-history stages by employing the Thermal Death Time (TDT) approach, to determine: i) if the two ecotypes differ in thermal tolerance, and: ii) how any differences vary with life history stage. Adults of the two ecotypes differed in their thermal tolerance in line with their shore position: the upper-shore ecotype, which

experience more extreme temperatures, exhibited greater endurance of thermal stress compared to the lower shore ecotype. This difference was most pronounced at the highest temperatures tested. The proximate physiological basis for these differences is unknown, but likely due to a multifarious interaction of traits affecting different parts of the TDT curve. Differences in tolerance between ecotypes were less pronounced in early life-history stages, but increased with ontogeny, suggesting partial divergence of this trait during development. Thermal tolerance could potentially play an important role in maintaining population divergence and genetic segregation between the two ecotypes, since the increased thermal sensitivity of the lower-shore ecotype may limit their dispersal onto the upper shore and so restrict gene flow.

2.1 Introduction

Populations living across environmental gradients may be subject to dramatic variation in levels of biotic and abiotic stress, with the resultant divergent selection regimes driving local adaptation and potentially leading to ecological speciation (Spicer and Gaston 1999; Keller and Seehausen 2012; Nosil 2012). In intertidal marine environments, extreme temperatures represent a significant source of physiological stress, with both intensity and duration of exposure varying in concert with the length of exposure during low tide (McMahon 1990; Helmuth and Hofmann 2001; Tomanek and Helmuth 2002; Little et al. 2009). Notably, intertidal organisms frequently contend with temperatures exceeding the critical limits of aerobic performance, beyond which endurance is time-limited (Sokolova and Pörtner 2003; Pörtner et al. 2017), and so display a variety of mechanisms to prolong survival under these conditions including mobilization of the heat shock response (Tomanek and Somero 1999, 2000; Tomanek

and Helmuth 2002), antioxidant defenses (Abele et al. 2007), and anaerobic metabolic pathways (Livingstone 1983; Sokolova and Pörtner 2001). Intertidal organisms have consequently been widely utilized as a model for the study of thermal adaptation. Numerous studies have demonstrated that species living at different shore heights display clinal variation in thermal stress tolerance (Burggren and McMahon 1981; Sanders et al. 1991; Marshall and McQuaid 1992; Stillman and Somero 1996, 2000; Tomanek and Somero 1999, 2000), mirroring patterns seen across latitude (Stillman and Somero 2000; Compton et al. 2007; Somero 2010; Armstrong et al. 2019). However, between-species comparisons are far from ideal for studying the processes leading to thermal adaptation across environmental gradients, because individual species possess unique evolutionary histories which may not represent current patterns of zonation (Garland and Adolph 1994; Keller and Seehausen 2012; Rezende and Diniz-Filho 2012). Intraspecific comparisons, by contrast, reduce the influences of macroevolutionary factors and allow for the potential mechanisms underpinning local population divergence to be elucidated (Coyne and Orr 2004; Keller and Seehausen 2012; Rezende and Diniz-Filho 2012). Intraspecific comparisons have revealed local thermal adaptation leading to population divergence (Kavanagh et al. 2010; Keller and Seehausen 2012; Ohlberger et al. 2013; Gibbons et al. 2016), demonstrating that temperature gradients can promote reproductive isolation and ultimately act as a driver of ecological speciation (Coyne and Orr 2004; Keller and Seehausen 2012; Rezende and Diniz-Filho 2012). Such mechanisms could play a significant role in driving population divergence in the intertidal, where strong patterns of vertical segregation exist in response to thermal stress (Newell 1979; McMahon 1990). In particular, the combination of thermal selection acting in concert with other clinal selective forces

present within the intertidal may further enhance divergence *via* multifarious selection (Nosil et al. 2009; Nosil 2012).

The role of early life-history stages in local thermal adaptation is frequently overlooked (Radchuk et al. 2013). Early life-history stages represent a particularly critical period of vulnerability to thermal stress (Przeslawski 2004; Byrne 2012; Radchuk et al. 2013; Lockwood et al. 2018), partly because they may lack physiological and morphological defenses of adults (Andronikov 1975), and because even minor stress-induced alterations in development can have knock-on consequences in later life (Hamdoun and Epel 2007; Nord and Giroud 2020). Variation in the responses of early life-history stages to thermal stress across environmental gradients has been demonstrated in numerous species (Kuo and Sanford 2009; Zippay and Hofmann 2010; Tangwancharoen and Burton 2014), indicating the potential for these stages to play a key role in local thermal adaptation. Despite this vulnerability, early life-history stages have been shown to have both reduced (Zippay and Hofmann 2010; Lockwood et al. 2018) and enhanced (Sewell and Young 1999; Miller et al. 2013; Tangwancharoen and Burton 2014) thermal tolerance compared to adults. It has therefore been suggested that physiological responses may differ across developmental stages, representing an adaptive response to the distinct thermal environments they inhabit (Miller et al. 2013; Tangwancharoen and Burton 2014; Lockwood et al. 2018; Truebano et al. 2018). Although several studies examining within-species thermal tolerance variations across intertidal gradients have incorporated early life-history stages (Bingham et al. 1997; Diederich and Pechenik 2013), these typically utilize species with planktonic larvae that experience different thermal regimes to adults. Highly dispersive, planktonic larvae also increase population connectivity (Sanford and Kelly 2011) reducing the likelihood

of local genetic adaptation (Hollander 2008). By contrast, direct developers with poor dispersal, where early life-history stages are exposed to the same environment as their parents, may show greater capacity for local genetic adaptation of thermal tolerance with shore height, because traits which resist thermal selection during early development are also likely to promote survival in later stages.

The rough periwinkle, *Littorina saxatilis* (Olivi), is an ideal model organism for the study of the physiological mechanisms driving intraspecific population divergence, due to its presence across intertidal gradients, low dispersal ability, and direct development (Reid 1996; Galindo and Grahame 2014; Rolán-Alvarez et al. 2015; Johannesson 2016).

L. saxatilis is the only ovoviviparous species within the genus *Littorina*, the eggs developing in a brood pouch inside the female and being subsequently released as shelled crawling juveniles (Reid 1996). Populations of *L. saxatilis* found across many NE Atlantic shores are characterised by the presence of two distinct ecotypes adapted to habitats with contrasting selective forces: wave action (Wave ecotype), and crab predation (Crab ecotype). These ecotypes represent a well-characterised model for parallel divergence and speciation in the face of gene flow (Rolán-Alvarez 2007; Butlin et al. 2014; Rolán-Alvarez et al. 2015; Johannesson 2016; Fernández-Meirama et al. 2017). Strikingly divergent examples of the two ecotypes are found on exposed rocky shores in Galicia, NW Spain (Reid 1996). Here, the local form of the Wave ecotype (referred to henceforth as SU, “smooth unbanded”) is present in mussel beds on the lower shore, while the Crab ecotype (referred to henceforth as RB, “ridged banded”) is found on and above the barnacle zone on the mid and upper shore. The two ecotypes display divergent morphologies in line with their habitats: a thin shell and large, high-tenacity foot in the SU vs. a reinforced, crab-resistant shell in the RB (reviewed in

Rolán-Alvarez 2007). Gene flow between the two ecotypes is restricted by size assortative mating (Johannesson et al. 1995; Rolán-Alvarez et al. 1999; Cruz et al. 2004), differential micro-habitat choice (Otero-Schmitt et al. 1997), and the respective vulnerabilities of the RB and SU to high wave action on the lower shore (Rolán-Alvarez et al. 1997), and high predation on the upper (Boulding et al. 2017).

Crucially, segregation of the two Galician ecotypes by shore height means they are subject to differing windows of exposure to both desiccation and thermal stress at low tide (Rolán-Alvarez et al. 1997). Notably, the RB ecotype experiences more frequent and protracted periods of extremes temperature during the summer than the SU (Fig. 2.1A-B). Previous studies have suggested that the two ecotypes may differ in their thermal tolerance as an adaptive response to the distinct thermal conditions of their respective habitats (Rolán-Alvarez et al. 1997; Cruz et al. 2004) with the implication that these differences may act to further reduce gene flow between the two ecotypes (Nosil et al. 2005; Keller and Seehausen 2012). However, despite evidence that the two ecotypes differ in their respiratory physiology (Martínez-Fernández et al. 2008, 2010) and desiccation tolerance (Rolán-Alvarez et al. 1997), the prediction that the two ecotypes differ in thermal tolerance has never been tested in these populations. Accordingly, the aim of our study was to investigate differences in thermal tolerance between the RB and SU ecotypes. We investigated thermal tolerance across life-history stages using the Thermal Death Time (TDT) approach (Rezende et al. 2014), firstly to test the prediction that the RB ecotype should display increased thermal tolerance compared to the SU, and, secondly, to investigate whether such differences originate in early life-history. The TDT approach, which allows for the relative effects of intensity *versus* duration of thermal stress to be distinguished, has been applied to both marine

(Rezende et al. 2014; Semsar-kazerouni and Verberk 2018; Truebano et al. 2018; Burton and Einum 2020) and terrestrial taxa (Rezende et al. 2014; Castañeda et al. 2015; Jørgensen et al. 2019) and to comparisons across multiple life-history stages (Truebano et al. 2018), demonstrating its broad applicability across different environmental conditions. The TDT methodology has also been used to identify a trade-off across latitude in organisms' abilities to resist acute *versus* chronic thermal stress (Urban 1994; Rezende et al. 2014; Castañeda et al. 2015; but see Jørgensen et al. 2019). Based upon similarities in patterns of physiological adaptation between intertidal and latitudinal gradients (Stillman 2004; Compton et al. 2007), we anticipated that a comparable trade-off may also be apparent across the height-segregated Galician *L. saxatilis* ecotypes. Since embryos experience the same thermal environment as adults inside the brood pouch, we predicted that thermal tolerance differences between embryos of the two ecotypes would mirror those observed between adults.

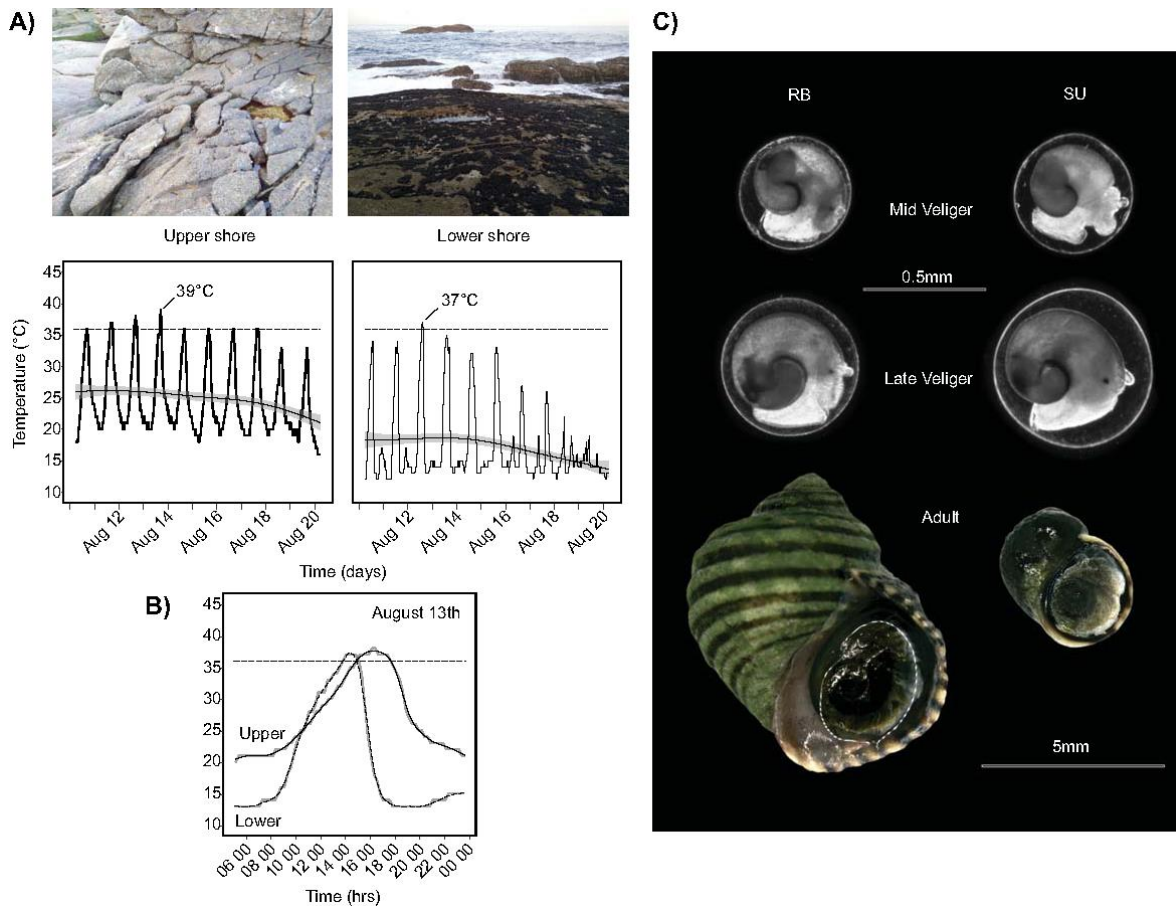


Figure 2.1: Field temperature data and life-history stages. (A) Examples of typical habitats on the upper and lower shores, Silheiro Cape, Galicia (NW Spain), accompanied by habitat temperatures recorded using robolimpet data loggers over a ten-day period from 11- 20 August 2009. Maximum temperatures experienced during the recorded period are annotated, while solid shaded lines represent average temperatures. Upper and lower shore habitats respectively correspond to the microhabitat temperature regimes experienced by the RB and SU ecotypes of *Littorina saxatilis* (see methods). Dashed line represents 36°C, the lowest assay temperature used in our study. Temperatures were recorded at 15-minute increments to the nearest °C. (B) Temperatures over the course of a single day, representing the second warmest and warmest days during the ten-day period, respectively, for the upper and lower shore. (C) Individuals of each *L. saxatilis*

ecotype – developmental stage combination used in this study. Separate scale bars for adults and embryonic stages are depicted. Images of embryos are stills derived from experimental video data captured early in static thermal tolerance trials before lethal endpoints were reached.

2.2 Methodology

2.2.1 Animal collection and husbandry

Adult *L. saxatilis* (Fig. 2.1C) were collected from Silleiro Cape in Galicia, Spain (+42° 6' 4'', -8° 53' 58'') from December 2018 to February 2019. The SU ecotype was sampled from mussel beds on the lower shore while the RB ecotype was sampled from the upper shore above the barnacle zone and close to the splash zone (following Boulding et al. 2017; Fig. 2.1A). These habitats exhibit contrasting thermal regimes which are most pronounced in the summer (Fig. 2.1A-B). Within 24 h of collection, snails were sent via express airmail to the Marine Biology and Ecology Research Centre at the University of Plymouth in plastic boxes containing dampened cardboard inside sealed polystyrene boxes. Upon arrival, they were transferred to 5 L aquaria containing aerated seawater (salinity = 35 ± 1) and held at 15°C, a temperature which approximates field sea temperatures in Galicia at the time of collection. Snails from both ecotypes were held under common laboratory conditions for at least one week prior to experiments. Water changes were conducted weekly, and snails were fed *ad libitum* on *Ulva lactuca*.

2.2.2 Acquisition of field temperature data

Field temperature recordings (Fig. 2.1A-B) were obtained at Silleiro Cape over a 17-day period (8th-25th August 2009) using individual robolimpet data loggers (Lima and Wethey 2009) positioned in microhabitats corresponding to the locations of each ecotype; directly exposed to the sun on the lower shore (corresponding to the position of the SU ecotype during low tide), and in a more protected location (shaded by rock crevicing) on the upper shore. This reflects the location of the RB ecotype within shaded refugia at low tide, a phenomenon not seen in the SU (E. Rolán-Alvarez, *pers. obs.*). Because of this, it should be noted that the disparity in temperatures between the two dataloggers are likely less extreme than they would be had both been placed in sun- exposed locations at both shore heights. Sensors recorded air temperatures at low tide and water temperatures upon immersion at high tide. Temperature data was recorded at 15-minute increments to the nearest °C.

2.2.3 Assessment of thermal tolerance

To assess thermal tolerance across all life-history stages, we utilized the static approach (Lutterschmidt and Hutchison 1997). We constructed Thermal Death Time (TDT) curves, which allow for the effects of intensity and duration of thermal stress to be distinguished (Rezende et al. 2014). Under the TDT approach, log-transformed endpoint times (lethal or sublethal) derived from static assays are plotted across a range of temperatures, and the resulting regression slopes used to calculate CT_{max} (the extrapolated temperature at which endpoint would occur after a period of 1 min) and z (the temperature change required for a tenfold change in survival time to occur (Rezende et al. 2014)).

2.2.4 Thermal tolerance in embryos

Two developmental stages - mid veliger (characterised by the presence of a larval, but not adult, heart) and late veliger (characterised by the presence of both a larval and adult heart, but still retaining a velum) - were identified and isolated under low power microscopy (Fig. 2.1C). Although all developmental stages can be found together in *L. saxatilis* brood pouches (Reid 1996), we selected these stages due to their high prevalence in the individuals we dissected, likely due to the relatively long developmental window occupied by these stages relative to earlier stages (Pelseneer 1911; C. Dwane, pers. obs.). In addition, despite the apparent morphological similarity between mid and late veligers (Fig. 2.1C), significant changes in physiological sensitivity have been demonstrated between these stages in the closely related species *Littorina obtusata* (Bitterli et al. 2012), which are linked to the timing of cardiovascular development.

Thermal assays in embryos from both ecotypes were performed using a custom-built bioimaging system (Tills et al. 2013; Truebano et al. 2018). At the start of each thermal assay, embryos were dissected out of the brood pouch from live females. These were individually added using a paintbrush to separate wells of a 48-well plate (Falcon Scientific, UK) containing 1.25 mL of filtered, autoclaved seawater preheated to one of five assay temperatures (37, 38, 39, 40 and 41°C) and held within an incubation chamber mounted on an XY motorized stage above an inverted camera and optics (Tills et al. 2013). These temperatures were selected because they represent maximal temperatures experienced by these populations (Fig. 2.1A-B), and resulted in survival timeframes (1 min – 20 h) suitable for TDT analysis (Rezende et al. 2014; Jørgensen et

al. 2019). Temperatures within the chamber were controlled using an UNO Combined controller (OKOLAB, Italy) and desktop PC running OKOLAB's UNO-TS software. Temperature fluctuated by $\pm 0.3^{\circ}\text{C}$ around the assay temperature in the first hour following the addition of embryos, after which it stabilized to $\pm 0.2^{\circ}\text{C}$. Salinity was kept at 35 ± 1 by maintaining high humidity within the chamber and by occasional topping up of water during the trials. Oxygen concentrations levels within the wells remained at normoxic levels ($>90\%$ air saturation). Due to the high density of embryos carried within the brood pouches of females (Reid 1996), it is possible that oxygen concentrations experienced by embryos in the field would actually be lower than those experienced during our trials. However, we chose to use normoxic conditions to facilitate cross-comparison between ecotypes and temperatures. It should be noted that *L. saxatilis* embryos can be raised successfully in seawater (Pelseneer 1911; C. Dwane, pers. obs.), demonstrating that exposure to normoxic conditions does not inhibit long-term survival.

Between six and 16 individuals drawn from each developmental stage were used per thermal assay, with fewer animals per assay used at higher temperatures to reduce setup times. Embryos obtained from a different female were used for each assay, and in the case of the SU ecotype, embryos from multiple females were used within each assay due to the small number of embryos at this developmental stage carried by each parent (Conde-Padín et al. 2007). A minimum of three assays per ecotype were performed for each temperature. At $37\text{-}40^{\circ}\text{C}$, videos (600x600 pixels, 30x magnification, 10 frames s^{-1}) were recorded onto hard drives (6 TB Barracuda Pro, Seagate, USA) mounted within a hard drive array (eBox TeSU, DatOptic, USA). To provide higher temporal resolution at higher temperatures (where survival times were

much shorter), different acquisition frequencies were used at different temperatures (15-20 s at 10-, 5-, or 2-min intervals). Recorded video footage was analyzed using Fiji (Schindelin et al. 2012). A range of criteria were used to define mortality, including the loss of cardiovascular function, loss of gut peristalsis, and cessation of muscular twitching in the head and mantle. Survival times were recorded based upon the last video sequence in which one of these traits could be observed. Because of the rapid cessation of activity at 41°C, video acquisition was not used, and instead embryos were added to the wells sequentially and endpoints determined through visual assessment using the live camera feed. Total sample sizes were as follows: RB mid veligers (37°C- n = 20, 38°C- n = 20, 39°C- n = 15, 40°C- n = 12, 41°C- n = 14), SU mid veligers (37°C- n = 19, 38°C- n = 17, 39°C- n = 13, 40°C- n = 15, 41°C- n = 14), RB late veligers (37°C- n = 20, 38°C- n = 22, 39°C- n = 16, 40°C- n = 15, 41°C- n = 14) and SU late veligers (37°C- n = 18, 38°C- n = 15, 39°C- n = 13, 40°C- n = 14, 41°C- n = 11). During video analysis, shell and egg sizes were measured using still images obtained from each embryo, with shell height and width measurements taken as described in Reid (1996) using Fiji.

2.2.5 Thermal tolerance in adults

Thermal tolerance in adults of the two ecotypes was assessed using time taken to enter heat coma as a sublethal proxy for survival (henceforth referred to as heat coma time), due to methodological difficulties in determining mortality in adult gastropods using a static approach. Heat coma in Gastropods is characterised by the inability of the nervous and muscular system to respond to external stimuli (Sandison 1967; Truebano et al. 2018) and represents a point of physiological compromise beyond which the animal is unable to react to external threats, such as predation (Cowles and

Bogert 1944; Lutterschmidt and Hutchison 1997). Only males were used to remove potential sex-specific differences in thermal tolerance, which also ensured that the use of only reproductively mature individuals (as females cannot be positively distinguished from immature specimens without dissection). The snails were sexed under light microscopy and males positively identified by the presence of a penis behind the right eye (Reid 1996). Prior to the trials, all experimental individuals were fitted with a small (4-6 cm) piece of fine thread attached to the center of the operculum using Super glue (Loctite). Snails were then left in seawater overnight to recover from the addition of the thread, and only individuals which subsequently displayed normal crawling behavior (> 95 % of individuals) were used.

During the trials, snails were held in individual 350 mL glass jars containing seawater preheated to one of the five test temperatures (36, 37, 38, 39 and 40°C) in a Sub Aqua Pro water bath (Grant Instruments, UK). The RB ecotype was tested at all five temperatures (36°C, n = 10; 37°C, n = 12; 38°C, n = 14; 39°C, n = 15; 40°C, n = 15) while the SU was only tested at the four lower temperatures (36°C, n = 13; 37°C, n = 13; 38°C, n = 10; 39°C, n = 13) as exposure at 40°C resulted in heat coma after a period of less than 1 min. Jars were left unsealed ensuring thermal tolerance was assessed under normoxia (> 95 % a.s.). Oxygen levels were measured using a calibrated oxygen sensor (Microx-4, PreSens, Germany) during preliminary trials.

At the start of each trial, snails were added to the preheated jars, and the foot tissue was gently prodded at regular intervals throughout the trial to determine heat coma as per Sandison (1967). At the point when no response to this stimulus was registered within a few seconds, snails were regarded as having reached heat coma, and time elapsed was recorded. The addition of thread to the operculum was necessary because

snails sometimes retracted fully into the shell as a response to prodding, preventing subsequent determination of heat coma in these individuals. In these cases, the thread attached to the operculum was gently pulled, and this was sufficient to re-extrude the foot tissue immediately after prodding. Immediately upon reaching heat coma, snails were removed to 200 mL jars containing sea water and held at 15°C for a minimum of 24 h. Snails which did not display full recovery after this time period (manifested by lack of an ability of the foot to attach to the jar sides) were excluded from the analysis. Following recovery assessment, snails were frozen at -20°C. Later, shells were measured (columella height and shell width across the widest part of the shell, perpendicular to the columella) using Vernier calipers, and cracked open to dissect the tissue, which was rinsed, blotted dry and weighed (± 0.01 mg) using a Cubis Semi-Micro Balance (Sartorius, Germany).

2.2.6 Statistical analysis

Statistical analysis was conducted using R version 3.5.3 (R Core Team 2019). The use of different endpoints in adults and embryos prevented direct comparison, thus data from adults was analyzed separately from data for mid and late veliger stage embryos. Differences in survival times between the four different embryonic treatment combinations (mid vs. late veliger, RB vs. SU) were analyzed independently at each of the five assessed temperatures (37, 38, 39, 40, and 41°C) using two-way ANOVA (with ecotype and developmental stage as factors). Data were log-transformed prior to analysis to meet assumption of homogeneity of variance. Similarly, differences at log-transformed heat coma times in adults from the two ecotypes were tested

independently at each of the four temperatures at which both ecotypes were assessed (36, 37, 38, and 39°C) using one-way ANOVA with ecotype as the single factor.

To generate TDT curves, we regressed log-transformed endpoint times against treatment temperature (Rezende et al. 2014). This generated slopes, intercepts, and r-squared values, with CT_{max} generated using the equation $CT_{max} = -\text{intercept/slope}$ and z generated using $z = 1/\text{slope}$. To statistically compare between regression slopes, ANCOVA with type 3 sum of squares was used. Differences between embryos were assessed in a two-way ANCOVA including both ecotype and developmental stage (mid- vs late-veliger) as factors, and temperature as a covariate. Adults were assessed using a one-way ANCOVA with ecotype as the single factor and the temperature covariate.

To determine whether there was a significant effect of size of individuals on endpoint times, we conducted MANCOVA tests for each ecotype-stage combination, regressing survival times (in embryos) or heat coma times (in adults) against both treatment temperature and size measurements (shell height in embryos, wet weight and shell height in adults).

2.3 Results

2.3.1 Embryos

Comparison of survival times at each individual temperature (Fig. 2.2A; Fig. 2.3A-D) revealed significant differences between ecotype: developmental stage combinations at three of the temperatures tested (37, 38 and 41°C), with significantly higher survival times in the RB late veligers relative to other groups at the two lowest temperatures (38 and 37°C). At 38°C, a significant effect of both stage and ecotype was observed

(Stage- $F_{1/70} = 20.867$, $p < 0.001$; Ecotype- $F_{1/70} = 17.647$, $p < 0.001$), with RB late veligers displaying survival times 2-3 times longer than the other three treatment groups ($p < 0.001$). Similarly, at 37°C, a significant effect of both stage ($F_{1/73} = 53.801$, $p < 0.001$) and ecotype ($F_{1/73} = 6.788$, $p = 0.011$) was observed, with survival times of RB late veligers being significantly longer than both RB and SU mid veligers (both $p < 0.05$), but not SU late veligers ($p = 0.159$). At 41°C, a significant effect of stage ($F_{1/49} = 6.153$, $p = 0.017$), but not ecotype ($F_{1/49} = 1.907$, $p = 0.174$), was observed, with no significant post-hoc Tukey comparisons (all $p > 0.05$).

Regression lines of semi-log transformed TDT curves generated from survival times demonstrated high goodness-of-fit for all treatments (Fig. 2.2B). Furthermore, r-squared values were improved through using only mean survival times (all r-squared > 0.96), which is an appropriate method for reducing residual variation by assuming 50 % mortality at each temperature (Rezende et al. 2014; Truebano et al. 2018). Linear regression (ANCOVA) analysis of these TDT curves indicated a significant difference in survival between embryonic stages, irrespective of ecotype (significant interaction between temperature and developmental stage; $F_{1/309} = 4.823$, $p = 0.029$), but no significant effect of ecotype ($F_{1/309} = 0.902$, $p = 0.343$; Fig. 2.2B). CT_{max} values derived from TDT curves were similar across all ecotype-developmental stage combinations, ranging between 42.00 - 42.25°C, while only slight differences in z-value were apparent between developmental stages (Table 2.1). It is notable that differences in survival times that were seen at 37- 38°C when analyzed separately at each temperature were not reflected by differences in CT_{max} and z-values in the TDT approach, likely due to being masked by its logarithmic nature. This is evident in a visual comparison of Fig. 2.2A (raw survival times) with Fig. 2.2B (log-transformed survival times).

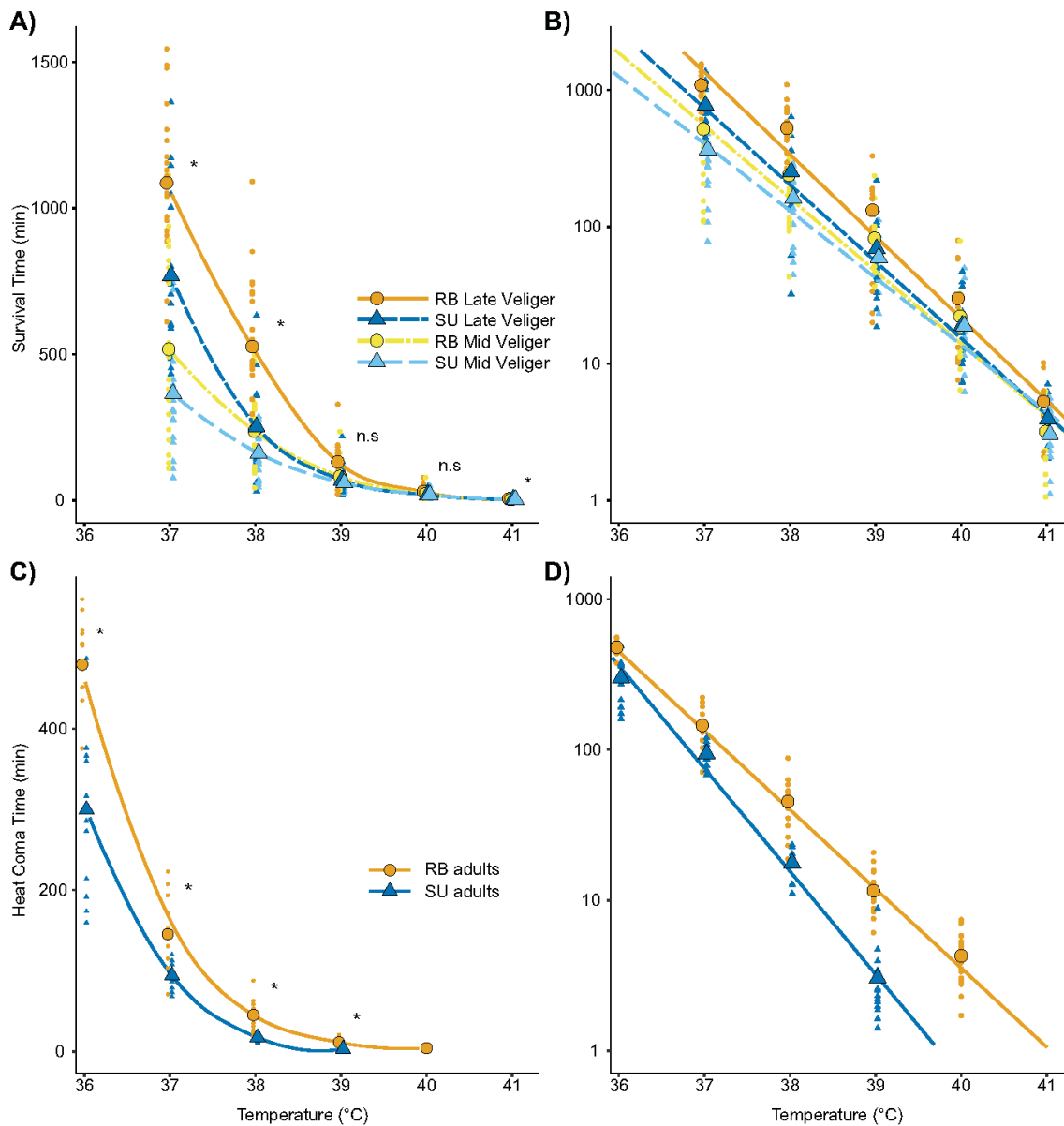


Figure 2.2: Thermal Death Time curves. (A-B) TDT curves for mid and late veliger stage embryos of two Galician *Littorina saxatilis* ecotypes (RB and SU) displaying survival times across five temperatures (37-41°C). **(C-D)** TDT curves for adults of the two ecotypes, displaying heat coma times across four (SU; 36-39°C) or five temperatures (RB; 36-40°C). Large and small data points represent mean values for each temperature, and individual endpoint times, respectively. Left-hand panels (A, C) depict raw data values, with asterisks indicating significant differences between treatment groups at the respective temperature; for details see Results. Right hand panels (B, D) depict the same data log-transformed, illustrating the semilogarithmic

relationship between temperature tolerance and exposure time associated with the TDT method. R-squared values for regression lines in (B, D) were as follows: RB Mid Veliger = 0.86; SU Mid Veliger = 0.87; RB Late Veliger = 0.91; SU Late Veliger = 0.90; RB Adult = 0.96; SU Adult = 0.96.

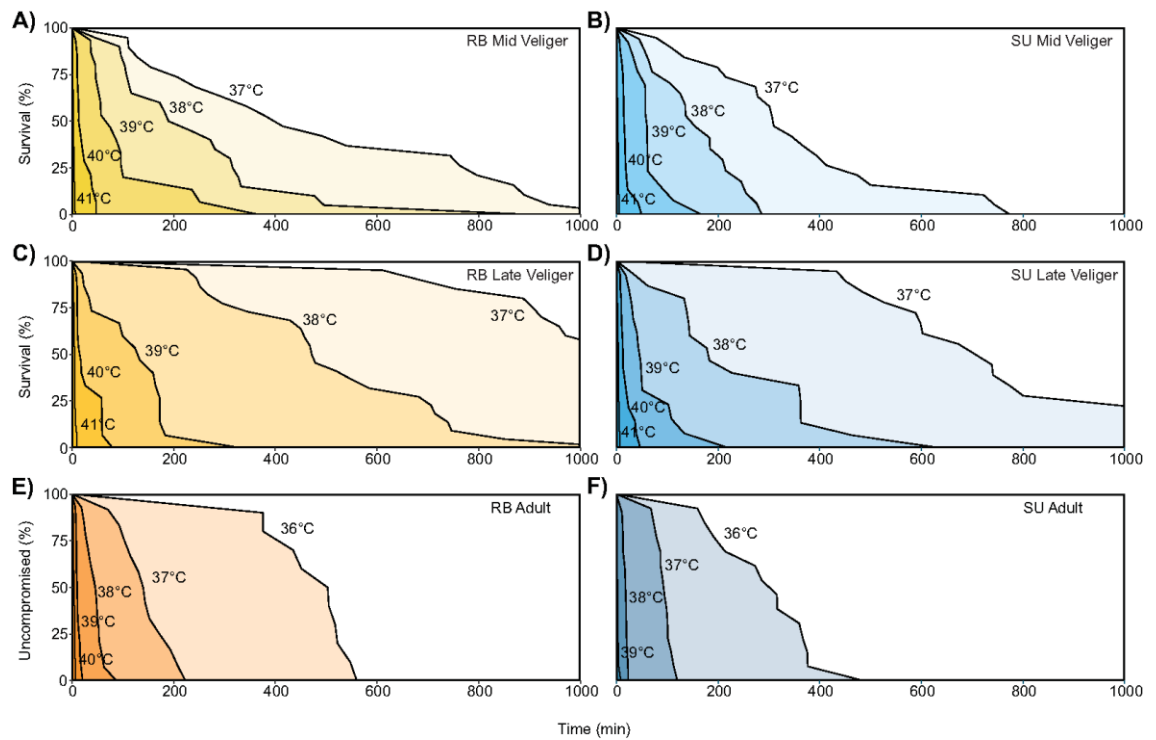


Figure 2.3: Survival plots. (A-D) Probability of survival for embryos under increasing exposure time at five temperatures for (A) RB mid veligers, (B) SU mid veligers (C) RB late veligers and (D) SU late veligers. (E-F) Probability of remaining uncompromised (not in heat coma) for adults under increasing exposure time at five temperatures and four temperatures respectively in (E) RB and (F) SU ecotypes. Note that to aid clarity of presentation, several data points representing survival times longer than 1000 minutes have been omitted from the survival plots for mid and late veligers; these data points are depicted in Fig. 2.2A.

Ecotype: Stage combination	z-value (°C)	CT _{max} (°C)
Survival:		
RB Mid Veliger	1.89	42.20
SU Mid Veliger	1.98	42.16
RB Late Veliger	1.67	42.25
SU Late Veliger	1.74	42.00
Heat Coma:		
RB Adult	1.90	41.07
SU Adult	1.46	39.72

Table 2.1: Z-values and CT_{max} values for different ecotype: stage combinations, generated by regressing log-transformed endpoint times against treatment temperature (see Methods). Estimates for embryos were derived from survival times, while estimates for adults were derived from heat coma time, a sublethal marker of physiological compromise.

2.3.2 Adults

In contrast to embryos, comparisons at each temperature in adults revealed consistently higher thermal tolerance in the RB compared to the SU ecotypes across all temperatures ($p < 0.01$; Fig. 2.2C; Fig. 2.3E-F). Unlike in embryos, these differences were most pronounced at the highest temperatures. For instance, at 39°C, almost a fourfold difference in survival time was observed between RB and SU adults ($F_{1,26} = 77.5$, $p < 0.001$). At 36°C, the lowest temperature measured, differences in survival times were less pronounced but still significant ($F_{1,21} = 19.5$, $p < 0.001$). Mirroring this, analysis of TDT curves (Fig. 2.2D) indicated that RB snails had substantially higher z and CT_{max} values than SU snails (Table 2.1). This is indicative of greater acute thermal tolerance at the highest temperature extremes in the RB ecotype, indicated by a significant interaction between ecotype and temperature ($F_{1,111} = 40.484$, $p < 0.010$) in

ANCOVA. This is apparent when heat coma times are plotted logarithmically (Fig. 2.2D) as opposed to as raw values (Fig. 2.2C). It is important to note that the lower CT_{max} values we observed in adults compared to embryos likely reflect the lower, sublethal endpoint threshold used and thus do not equate to reduced thermal tolerance in absolute terms in the adults.

2.3.3 *Controlling for size differences between ecotypes*

Shell height ($t = 46.631$, $p < 0.001$) and wet weight ($t = 21.784$, $p < 0.001$) differed between adults of the two ecotypes. RB snails were larger, with mean shell length of 9.86 ± 0.96 mm, whereas SU adults had a mean shell length of 3.44 ± 0.47 mm. We found no significant effect of either shell height or wet weight ($p > 0.05$) on time taken to enter heat coma within adults of either ecotype, suggesting size differences are not responsible for the higher thermal tolerance observed in RB compared to SU snails. In embryos, there was no significant difference in shell length between late veligers of the two ecotypes, while RB mid veligers were significantly larger than SU mid veligers (0.401 ± 0.038 mm vs. 0.380 ± 0.045 mm, respectively; $t = 3.278$ $p < 0.001$). As with adults, there was no significant relationship in embryos between shell height and survival times within each treatment group ($p > 0.05$).

2.4 Discussion

In this study we compared the thermal sensitivities across different life stages of two ecotypes of *L. saxatilis* from Galician populations (NW Spain) which are associated with different height levels of the intertidal zone and therefore exposed to substantially

different thermal environments. We found that adults of the upper shore RB ecotype, which experience higher *in situ* habitat temperatures, had greater tolerance times across the entire range of temperatures tested. This difference was most pronounced at the highest temperatures and corresponded with a shift in CT_{max} and z-values between the two ecotypes. By contrast, differences in responses between embryos were more subtle than those in adults, with increased survival times in the RB being only apparent at the lowest temperatures measured and not associated with a shift in CT_{max} and z-values. Our results support the prediction that the two ecotypes, adapted to contrasting selective forces, also differ in their thermal tolerance in line with the environmental conditions of their microhabitats (Rolán-Alvarez et al. 1997; Rolán-Alvarez 2007). We propose that the increased vulnerability of the SU ecotype to thermal stress may inhibit their small-scale dispersal into the RB habitat on the upper shore. Such limited dispersal would act a partial barrier to gene flow between the two ecotypes, reinforcing selection for other adaptive traits and contributing to the maintenance of two distinct ecotypes and the evolution of greater divergence and reproductive barriers in the Galician population.

Our employment of the TDT approach (Rezende et al. 2014) provided us with the ability to distinguish between the effects of intensity and duration of thermal stress, which is significant given the substantial variation in intensity and duration of heat exposure corresponding with shore height position (Newell 1979; McMahon 1990; Helmuth and Hofmann 2001; Tomanek and Helmuth 2002). Recent latitudinal comparisons (Rezende et al. 2014; Castañeda et al. 2015) have indicated a trade-off between CT_{max} and z across latitude (Jørgensen et al. 2019), with populations from colder, higher latitudes possessing greater acute tolerance at the highest temperatures

but increased sensitivity at less extreme temperatures. Our prediction that the RB ecotype, being exposed to higher environmental temperatures, would accordingly possess greater tolerance of prolonged exposure than the SU, but reduced acute tolerance, was not met. Instead, the RB ecotype had higher CT_{max} and z values than the SU, indicative of increased acute tolerance at the very highest temperatures without a reduction in chronic tolerance at lower temperatures, as they displayed greater thermal tolerance across the entire temperature range analyzed (36-39°C). It should be noted that if the TDT curves for the two ecotypes were extrapolated to lower temperatures, then the curves would intersect at a temperature of 35.23°C (at a survival time of 19 h 38 min) below which tolerance times in the SU ecotype would theoretically become greater than those in the RB ecotype. While this could indicate the presence of a trade-off, we do not consider differences at this end of the TDT curve to be of ecological consequence, because such long exposure times required at these temperatures would never be reached in the intertidal zone due to tidal and day/night cycles. Thus, the positive shift in both z and CT_{max} observed in the RB relative to the SU ecotypes is apparently not associated with detrimental consequences for longer term survival, as is seen in other study systems (Rezende et al. 2014; Castañeda et al. 2015).

The proximate physiological basis for greater acute tolerance in the RB is unknown and beyond the scope of this study, and could be due to a multifarious interaction of traits affecting different parts of the TDT curve (e.g., Figure S2 in Rezende et al. 2014).

Metabolic demands of tissues may differ substantially between the two ecotypes, especially due to the foot muscle of the SU ecotype being 1.4 times larger than that of the RB (Martínez-Fernández et al. 2008). Oxygen limitation plays an important role in determining thermal performance and tolerance limits in many marine ectotherms, as

the capacity of circulatory systems to meet increasing oxygen demands beyond a certain temperature threshold becomes limited, leading to a reduction in aerobic scope (Pörtner 2001, 2010; Pörtner et al. 2017). The high test temperatures used in our study, which exceed threshold temperature for the onset of anaerobiosis in water for this species (Sokolova and Pörtner 2003), may suggest that the responses we observed were driven chiefly by passive tolerance mechanisms activated beyond the critical limits for aerobic scope, beyond which survival is time-limited (Rezende et al. 2014; Pörtner et al. 2017). Anaerobic metabolic pathways play a crucial role in the passive thermal tolerance of many intertidal species (Burggren and McMahon 1981; Sokolova and Pörtner 2003; Monaco et al. 2017; Pörtner et al. 2017), and it is possible that anaerobic energy production via the succinate pathway is enhanced in the RB similarly to other upper-shore *L. saxatilis* populations (Sokolova and Pörtner 2001). However, there is also evidence of upregulation of proteins associated with rapid anaerobic production of ATP in the SU, potentially linked to increased muscle activity required to resist high wave action on the lower shore (Martínez-Fernández et al. 2008). Greater acute tolerance in the RB adults could also be linked to a more rapid ability to mobilize heat shock proteins in response to acute thermal stress relative to the SU (Rezende et al. 2014), as has been observed in upper *versus* lower shore congeners of the genus *Tegula* (Tomanek and Somero 2000). The underlying mechanisms for the observed differences in thermal tolerance between the ecotypes warrant further investigation.

Thermal sensitivity through development might be predicted to reflect environmental conditions experienced by each ontogenetic stage (Lockwood et al. 2018; Truebano et al. 2018). A previous study on the mid-shore flat periwinkle *Littorina obtusata*

demonstrated that z-values were lower in embryos compared to adult snails (Truebano et al. 2018), a difference that was attributed to the fact that *L. obtusata* embryos develop within egg masses fixed to the substrate, thus experiencing a substantially different thermal environment to adults, which are mobile and so able to escape thermal stress. Accordingly, we predicted that differences in thermal sensitivity between embryos of the two ecotypes of the brooding *L. saxatilis* would correspond closely to those observed in the adults. However, differences in CT_{max} and z values between adults of the two ecotypes were not reflected in the embryos. There are several potential explanations for this apparent mismatch. Firstly, this may be an artefact of our use of a sub-lethal endpoint in adults vs. a lethal endpoint in embryos, given that different levels of biological organization may be independently affected by thermal stress (Rezende et al. 2014; Rezende and Bozinovic 2019; Bozinovic et al. 2020). Secondly, limitations to thermal tolerance may be imposed by the need to resist other stressors associated with living in the intertidal, which are only experienced by adults. As noted, adults of the SU ecotype must cope with the physiological demands of increased wave exposure on the lower shore (Rolán-Alvarez et al. 1997); demands that are not experienced by their embryos. Also, we cannot exclude the possibility that phenotypic plasticity in the form of irreversible thermal acclimatization (Garland and Adolph 1991; Bourdeau et al. 2015) may have contributed to differences between adults, particularly as adults may have experienced exposure to extreme temperatures during summers prior to collection. However, between-ecotype differences in this species have been found to be largely invariant of environmental effects (Conde-Padín et al. 2007; Hollander and Butlin 2010; Galindo et al. 2019), including, notably, in proteome expression (Martínez-Fernández et al. 2010). In addition, the fact we observed an increase in sensitivity differences through development (i.e., between mid

and late veliger stages) further suggests that the differences we observed between ecotypes are likely to have a substantial genetic (or at least heritable) component.

It should be noted that, while the differences we identified between ecotypes and developmental stages appear subtle, even comparatively small differences in the shape of TDT curves may have substantial consequences for the survival of populations, especially when cumulative mortality over multiple successive days of thermal stress is considered (Rezende et al. 2014, 2020). For example, while mean survival times in embryos apparently exceeded maximal upper-shore habitat temperatures recorded by our temperature loggers, the high levels of variation we observed in survival times of embryos across all temperatures suggests that partial brood mortality is already likely to occur in the field during the summer, especially in the RB ecotype. This is in spite of our temperature data from the upper shore having been recorded from a shaded location, suggesting that the apparent behavioral preference of the RB ecotype for shaded refugia at low tide (as noted in our methodology) may not be completely sufficient to avoid embryo mortality. A potential strategy to further mitigate against this would be for females to breed preferentially at other times of year. However, *L. saxatilis* populations typically brood continuously year-round (Reid 1996), and while there is some evidence from Galician populations that numbers of recently-hatched juveniles are highest in the spring and autumn (Carballo et al. 2005), this could equally be explained by seasonal changes in embryo viability, or juvenile survival, rather than female fecundity. Substantially larger broods are produced by RB females compared to SU females (Saura et al. 2011), which, as well as mitigating against attrition from predation, could potentially serve to offset against partial losses of broods due to thermal stress in the summer.

We note that there are risks associated with directly inferring physiological adaptation from two-population/species comparisons, including a lack of statistical replication and the potential for confounding phylogenetic factors (Garland and Adolph 1994). However, the thermal tolerance differences we observe in this study fall within a wider context of adaptive morphological and physiological differentiation across shore height between closely related ecotypes (Rolán-Alvarez et al. 1997; Rolán-Alvarez 2007) and align closely with patterns of thermal tolerance across vertical shore gradients seen in many taxa (Newell 1979; Burggren and McMahon 1981; Sanders et al. 1991; Tomanek and Somero 1999; Stillman and Somero 2000) including in other populations of *L. saxatilis* where divergent ecotypes are not present across shore height (Sokolova et al. 2000; Sokolova and Pörtner 2001). Regardless of whether the thermal tolerance differences we describe are a direct adaptive response, or partially environmentally mediated, the presence of such differences may be significant in maintaining the vertical segregation of the two ecotypes and reinforcing selection for other traits. In particular, the increased susceptibility of the SU ecotype to thermal stress identified in our study may prevent their dispersal onto the upper shore (e.g., immigrant inviability; Nosil et al. 2005), thereby acting as an additional prezygotic isolation barrier and maintaining a selection gradient for habitat-specific traits (Keller and Seehausen 2012; Nosil 2012). It is also possible that thermal stress works in tandem with the increased susceptibility of the SU to crab predation (Boulding et al. 2017), as SU adults weakened by the effects of heat exposure may become more vulnerable to predators through their inability to escape (Lutterschmidt and Hutchison 1997). The combination of both thermal physiological stress and the predation / wave exposure gradient may therefore lead to further enhancement of reproductive barriers (via multifarious selection; Nosil et al. 2009; Nosil 2012) in the Galician RB and SU,

potentially contributing to the greater genetic divergence between the Galician ecotypes in comparison to other Wave and Crab ecotype pairs from localities where thermal stress is likely to be less significant (such as the UK; Butlin et al. 2014).

In conclusion, our study has demonstrated, for the first time, differences in thermal tolerance between the RB and SU ecotypes of *L. saxatilis* from Galician (NW Spain) populations, indicating that they may act as an important and hitherto overlooked factor in maintaining ecotype segregation and promoting the evolution of barriers to gene flow. Further research into the proximate causes of these differences may invite their use as a model for how interspecific differences in physiological traits, such as thermal tolerance, evolve along fine-scale environmental gradients. However, divergent responses in embryos and adults of the two ecotypes also suggest that thermal stress may have differing fitness and selection consequences across life-history stages, and highlight that further work is needed to explore to what extent these traits are genetic *versus* environmentally mediated in adults. Our research further highlights the importance of including multiple life-history stages in assessments of thermal tolerance and susceptibility to future climate change.

Chapter 3 Thermodynamic effects drive

countergradient responses in the thermal performance

of *Littorina saxatilis* across latitude

Abstract

Thermal performance curves (TPCs) provide a powerful framework to assess the evolution of thermal sensitivity across divergent selection regimes. However, there is a lack of consensus regarding how the shape of TPCs may vary across latitude, and the influence this may have on thermal limits. To explore this, we compared TPCs for cardiac activity in latitudinally-separated populations of the intertidal periwinkle *Littorina saxatilis*. We applied a non-linear TPC modelling approach, enabling us to explore how different metrics affecting the shape of TPCs varied systematically due to local adaptation and thermal acclimation. Surprisingly, both thermal optima and upper limits of cardiac activity were higher in the northernmost (cold-adapted) population and associated with greater maximal performance. This response was greatest in 10°C - acclimated individuals and declined under 15° and 20°C acclimation. We interpret this countergradient response as maladaptive and driven by the thermodynamic consequences of high basal heart rate in cold-adapted populations leading to correlated effects on the shape of TPCs. Our study highlights the danger of assuming that variation in any one aspect of the TPC is adaptive without appropriate ecological context and suggests that selection on standard metabolic rate has important repercussions on upper thermal limits.

3.1 Introduction

Latitudinal variation in temperature has a pervasive impact upon the performance of ectotherms (Huey and Kingsolver 1989; Hochachka and Somero 2002; Kingsolver 2009). Comparisons between populations and species from different latitudes therefore represent an important means to study the process of thermal adaptation (Spicer and Gaston 1999; Somero 2005; Angilletta 2009). Intertidal ectotherms represent a widely utilized model for the study of thermal adaptation across latitude, with numerous studies demonstrating variation in the upper thermal limits of closely related species living at different latitudes (Urban 1994; Stillman and Somero 2000; Compton et al. 2007). Intraspecific comparisons, however, have presented a mixed picture (Logan et al. 2012; Gaitán-Espitia et al. 2014; Tepolt and Somero 2014), possibly due to phylogenetic constraints and site-specific temperature variation (Helmuth and Hofmann 2001).

In addition to upper thermal limits, temperature differences across latitude are also predicted to drive metabolic adaptation (Hochachka and Somero 2002). Because the regulation of metabolic rate is vital to ensure cell function, feeding, growth and other traits, organisms from high latitude populations often compensate for the reduction in biochemical reaction rates experienced in colder climates by elevating their metabolism (Clarke 1993; Conover and Schultz 1995; Addo-Bediako et al. 2002; Hochachka and Somero 2002; Bozinovic et al. 2011). This process, a form of physiological compensation known as 'metabolic cold adaptation' (MCA), may be underpinned by increases in the concentration of enzymes, or a switch towards enzyme isoforms with higher catalytic activity, ultimately increasing metabolic rates in cold-adapted relative to warm-adapted populations when kept at equivalent

temperatures (Hochachka and Somero 2002). Although many studies have found evidence of MCA across latitude (Addo-Bediako et al. 2002; Sommer and Pörtner 2002; Gardiner et al. 2010; Thyrring et al. 2015), the hypothesis remains controversial (Clarke 1993; Bozinovic et al. 2011). In addition, it is unclear whether physiological compensation of metabolism at low temperatures may have knock-on consequences for differences in upper thermal limits across latitude, a factor which may be particularly relevant in intertidal species given that their upper tolerance limits occur at temperatures much higher than those at which they are behaviourally active (McMahon 1990; Monaco et al. 2017).

Thermal performance curves (TPCs) represent a powerful tool for the systematic assessment of changes in the physiological performance of organisms across temperatures. TPCs are unimodal, left-skewed curves consisting of an initial rising slope, a plateau region around the thermal optimum (T_{opt}) and a rapid decline towards the critical thermal maximum (CT_{max}) (Schulte 2015). Understanding how the shape of TPCs vary between populations adapted to different temperature regimes across latitude, as well as how organisms may adjust their TPCs to respond to changes in temperature (thermal acclimation) is vital to understanding the process of local thermal adaptation (Angilletta 2009). However, constraints upon local adaptation of the shape of TPCs exist due to trade-offs in resource allocation (Levins 1968; Huey and Kingsolver 1989) and because of the unavoidable thermodynamic consequences of increasing temperature on metabolism and enzyme stability (Gillooly et al. 2001). Numerous competing theories exist to explain how these constraints may affect the evolution of TPCs (Huey and Kingsolver 1989; Clarke 2004; Clarke and Fraser 2004; Frazier et al. 2006; Angilletta et al. 2010). Changes in standard metabolic rate due to

physiological compensation may also have knock-on consequences for the shape of TPCs across latitude, although the implications for upper thermal limits are unclear. For instance, if thermal optima evolve in direct response to environmental temperatures (Angilletta 2009), cold adaptation may be directly associated with a downward shift in T_{opt} , meaning the entirety of the TPC would be shifted towards lower temperatures in cold-adapted populations. Under a Specialist-Generalist trade-off, higher metabolic rates at low temperatures in cold-adapted populations would be associated with shallower but broader TPCs, lower max performance and lower T_{opt} compared to warm-adapted populations (Huey and Kingsolver 1989; Gilchrist 1995; Phillips et al. 2014). Alternatively, the MCA hypothesis predicts that elevated metabolic rates in cold-adapted populations might be sustained across the entirety of the TPC, leading to countergradient response across latitude and therefore greater max performance and T_{opt} in cold-adapted populations (Conover and Schultz 1995; Angilletta 2009; Gardiner et al. 2010). As even small changes in single parameters of the TPC could have important knock-on consequences on thermal breadth and upper limits (Rezende and Bozinovic 2019), it is vital to adopt approaches which allow for all parameters governing the shape of TPCs to be incorporated into an integrated framework.

To determine whether the shape of TPCs varies across latitude in a predictable manner, and whether this shape is modified by thermal acclimation, we assessed systematic variation in TPCs for cardiac activity during an acute thermal ramp across four latitudinally distinct populations of the intertidal periwinkle *Littorina saxatilis* following acclimation to three different temperatures. We assessed TPCs by measuring cardiac activity, which is often used as a proxy for metabolic rate in intertidal molluscs

(Dong et al. 2021). We utilized a non-linear regression approach to fit TPCs (Schoolfield et al. 1981; Rezende and Bozinovic 2019; Bozinovic et al. 2020), which provides an integrated framework to assess how multiple characteristics vary in tandem to affect the shape of TPCs. We selected *L. saxatilis* because it has a wide latitudinal distribution (Reid 1996), coupled with poor larval dispersal, resulting in a high degree of local genetic differentiation between populations (Johannesson 2016). Thus, we anticipated that the species would display a significant degree of latitudinal variation in thermal performance. Based on the assumption that divergence in the position and shape of TPCs across latitude directly corresponds to environmental temperature differences, we predicted that TPCs would be shifted towards lower temperatures in high-latitude populations, meaning they would exhibit higher performance at low temperatures, but conversely lower T_{opt} and CT_{max} , compared to low latitude populations.

3.2 Methodology

3.2.1 Animal collection and husbandry

We obtained adult populations of *Littorina saxatilis* from four sites across a latitudinal gradient spanning 23° along the northeast Atlantic coastline (Table 3.1). These localities were chosen as they experience contrasting temperature regimes (Fig. A1). In addition, collection sites were chosen based upon the reported absence of the *Littorina arcana* at each locality, which is identical in appearance (Reid 1996; C Dwane, pers. obs.). Species identification as *L. saxatilis* was confirmed via dissection of specimens following the study. Collection dates at different sites were staggered from August to December 2019 to reduce variation in seawater temperatures at time of collection (~13-15° C in the three southern populations). Seawater temperatures in

northern Iceland do not reach this temperature, thus the northernmost (Skagaströnd) population was collected at the maximum seawater temperatures experienced annually ($\sim 8^{\circ}\text{C}$). Snails were placed in plastic containers with dampened cardboard, transported to the University of Plymouth, and distributed randomly between 5L aquaria containing aerated sea water (salinity = 35 ± 1 , stocking density ~ 10 snails L^{-1}). All populations were held in at 15°C for > 1 week to recover from handling stress, except for the Skagaströnd population (10°C). Water changes were conducted weekly, and snails were fed *ad libitum* on *Ulva lactuca*.

Table 3.1: *Littorina saxatilis* populations used in this study.

Collection Site	Latitude	Longitude	Collection date	Shell height (mm \pm se)
Skagaströnd, Norðurland vestra, Iceland	+65°49'34"	-20°19'28"	13/08/19	13.43 \pm 0.26
St Ninian's Cave, Galloway, Scotland, UK	+54°41'37"	-4°26'57"	23/09/19	9.10 \pm 0.14
Mount Batten, Devon, England, UK	+50°21'23"	-4°7'33"	3/12/19	9.83 \pm 0.26
Cabo Silleiro, Galicia, Spain	+42°6'4"	-8°53'58"	6/12/19	9.32 \pm 0.11

Following recovery, populations were acclimated to 10, 15 and 20°C ($n \geq 3$ aquaria per treatment) for a minimum of five weeks, which is sufficient to limit differences in metabolism arising from acclimatization in the field (Newell 1979; Hawkins 1995; Sokolova and Pörtner 2001, 2003). All other conditions were maintained as per the recovery period. Mortality during the acclimation period was $<10\%$, except in 20°C -acclimated Mount Batten snails ($\sim 20\%$), 15°C -acclimated St Ninian's Cave snails ($\sim 20\%$) and 20°C -acclimated St Ninian's Cave snails ($\sim 45\%$).

3.2.2 *Ramping protocol*

Cardiac performance (as a proxy of metabolic rate) in response to acute thermal ramping was measured in air using non-invasive photoplethysmography (Burnett et al. 2013), a method extensively utilized in intertidal gastropods (Dong et al. 2017). Plethysmographic signals were detected using an infrared sensor (CNY70, Vishay semiconductors, Germany) positioned over the heart of the snail and held in place using plastic mesh. This prevented the snails from freely moving during the trial, while not restricting their ability to respire or emerge from their shells. The infrared sensor was connected to an amplifier unit (AMP03, Newshift Lda., Portugal) and data acquisition system (USB-6001, National Instruments, USA) connected to a laptop running NI-DAQExpress (National Instruments, USA). Amplified signals were recorded at a sample frequency of 40 HZ.

After being fitted with sensors, snails ($n = \text{five per trial}$) were transferred to individual plastic bags placed inside a water bath (Grant R5, Grant Instruments, UK) preheated to the required acclimation temperature (10, 15, or 20°C). The unsealed bags were weighted with marbles and submerged just above the waterline to facilitate gas exchange. Preliminary trials confirmed snails experienced normoxic conditions ($> 95\%$ a.s.) throughout the ramp. Following addition of the snails, bags were left for 15-20 min to equilibrate with water bath temperatures. Temperature during the trials was monitored using a k-type thermocouple inserted inside an empty snail shell filled with blu-tak (Bostic, UK), placed inside an additional bag. Following equilibration, temperature was increased at a rate of 6°C h^{-1} until after all snails reached cardiac flatline ($\sim 47\text{-}50^{\circ}\text{C}$), at which point the experiment was terminated. Shells were measured (columella height and shell width across the widest part of the shell,

perpendicular to the columella), and cracked open to dissect the soft tissue, which was rinsed, blotted dry and weighed (± 0.01 mg) using a Cubis Semi-Micro Balance (Sartorius, Germany). At least three trials were run per population per acclimation temperature. Faint or inconsistent heartbeat traces were discarded from further analysis.

3.2.3 Data analysis

Raw signals were converted into heartbeat interval data using the MATLAB-based ARTiiFACT package (Kaufmann et al. 2011). Heartbeats were counted for 1 min periods at 1°C ($\pm 0.1^\circ\text{C}$) intervals through the course of the temperature ramp. This was done by detecting voltage peaks in the raw signals using global peak thresholds, followed by screening to correct miscounted sections of the heartbeat signal (e.g. due to the presence of doubly- counted peaks; see Burnett et al. 2013). The normality of interbeat intervals was verified using quantile-quantile plots within Artiifact, following which heartrate in BPM was calculated.

We fitted TPCs to heartrate data separately for each individual using the “fit.thermal.curve” function (Rezende and Bozinovic 2019; also available in the r package “rTPC”; Padfield et al. 2021). This non-linear function fits curves to thermal performance data based on four descriptive parameters: a constant that shifts the curve up and down everything else being equal (C), the fold-increase in performance expected for a 10°C rise in temperature (Q_{10}), the threshold temperature in which denaturation effects are observed (T_{th}) and the thermal sensitivity of denaturation to temperatures above this value (d). Based on these parameters, the descriptors T_{opt} , CT_{max} , P_{max} (heartrate at T_{opt}), $P_{initial}$ (heart rate at the respective acclimation

temperature of the individual), and $T_{breadth}$ (the thermal breadth in which performance is a 50 % of its maximum) were derived (Fig. 3.1A). In cases where initial convergence could not be achieved for some individuals, we instead employed the “boot.thermal.curve” function (Bozinovic et al. 2020). This method accounts for slight deviations from the expected TPC shape present in the raw data by iteratively fitting the nonlinear model to bootstrapped data values from a normal distribution \pm s.d. around the mean raw data at each temperature, with standard deviation values of 1-5 bpm used depending on the individual in question. The bootstrap analysis was run iteratively 20 times, with median Q_{10} , C , T_{th} and $decay$ values used to generate TPCs for these individuals. TPCs were assessed visually for goodness-of-fit to raw data. In total, successful convergence of the non-linear regression model to heartrate data was achieved across 128 individuals, of which 50 required the use of bootstrapping. Fits of the non-linear model to the raw data were high across both non-bootstrapped and bootstrapped individuals ($r^2 = 0.88 \pm 0.094$, mean \pm sd).

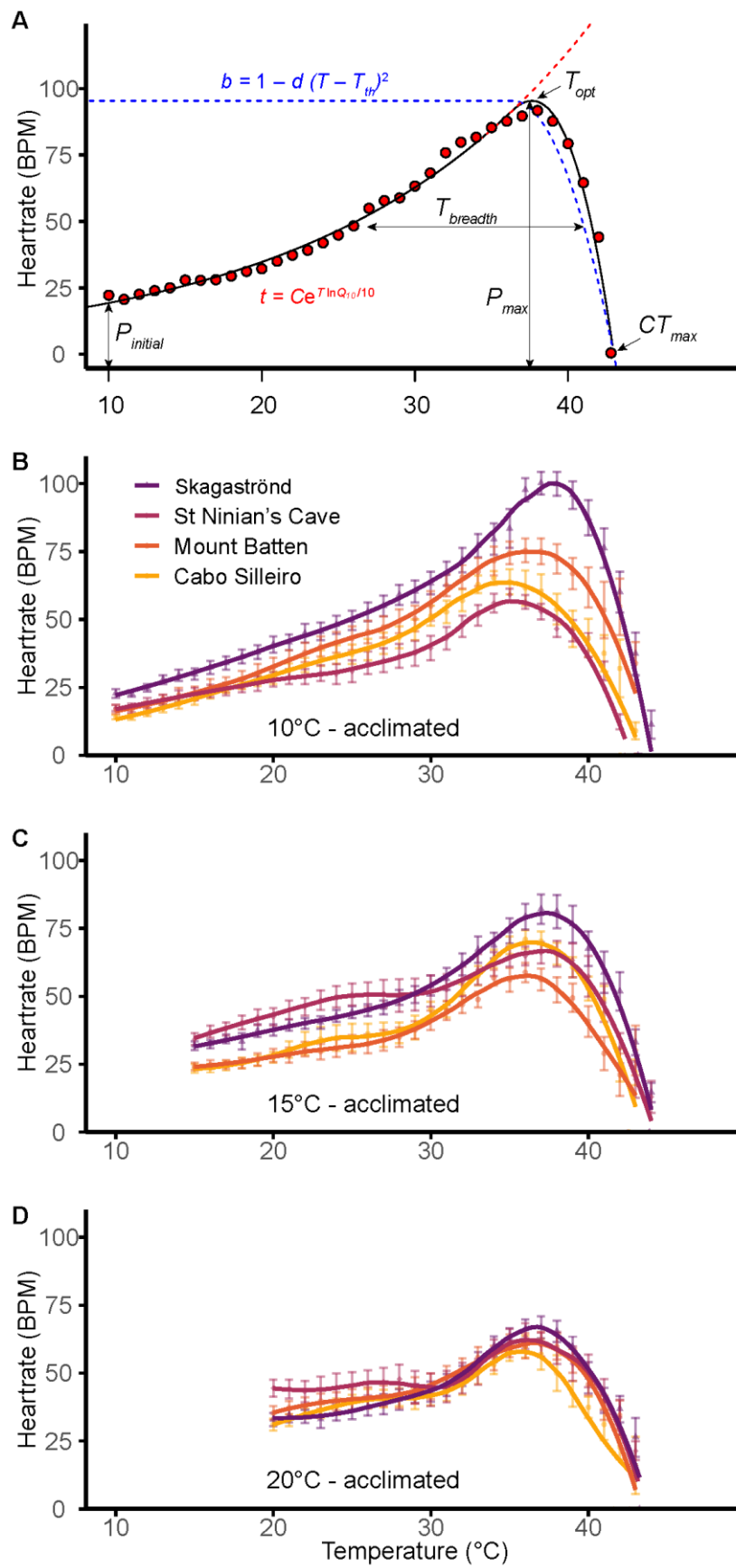


Figure 3.1: Thermal performance curves. A) Graphical representation of the non-linear regression model used in our study, showing a TPC fitted to empirical heartrate data from an individual *Littorina saxatilis* (red circles). Curves are described using the functions $t \times b$, with t describing the rising component of the TPC based on thermodynamic effects, and b representing the falling slope of the TPC due to protein instability. These functions incorporate four TPC parameters (Q_{10} , C , T_{th} , and d , see “Methods”), with T corresponding to ambient body temperature ($^{\circ}\text{C}$). From these curves, five descriptors of thermal performance ($P_{initial}$, P_{max} , $T_{breadth}$, T_{opt} , and CT_{max}) are derived. See Rezende and Bozinovic (2019) for more information. (B-D) Averaged heart rates for each population during acute ramping assays. Heartrates were recorded at 1°C increments throughout ramping assays; error bars denote standard error. Curves were fitted to mean values using the “LOESS” function in the R package ggplot2.

3.2.4 Statistical analysis

Statistical analysis was performed using R (R Core Team 2019). To investigate how widely utilized metrics of thermal tolerance and performance differed in response to latitude and acclimation temperature, as well as to explore the possibility of an interactive effect, General Linear Model (GLM) analysis was performed separately for five standard metrics of thermal performance: CT_{max} , T_{opt} , P_{max} , $P_{initial}$ and Q_{10} . In each model, latitude and acclimation temperature were used as continuous and categorical predictor variables respectively. No significant effect of shell size was observed for any parameter except P_{max} , thus size was not included as a covariate in the final models.

In addition, to determine how different TPC parameters and descriptors generated by the non-linear model interact to contribute to inter-individual variation in the shape of TPCs within the dataset, we conducted a Principal Component Analysis (PCA) incorporating the four parameters Q_{10} , C , T_{th} and log-transformed *decay* (to improve normality), as well as the descriptors T_{opt} , CT_{max} , and P_{max} .

3.3 Results

3.3.1 Thermal performance traits

In contrast to the latitudinal trend in habitat temperature (Fig. A1), critical thermal limits (CT_{max}) displayed a significant positive trend with increasing latitude ($F_{1,122} = 36.953$, $p < 0.001$; Fig. 3.2A), being 1.7°C higher in the northernmost (Skagatrönd) population than in the southernmost (Cabo Silleiro) population when averaged across all three acclimation temperatures. There was neither an effect of acclimation temperature ($F_{2,122} = 0.105$, $p = 0.9$), nor an interactive effect of acclimation

temperature x latitude ($F_{2,122} = 0.027$, $p = 0.974$). Similarly, there was a significant positive effect of latitude on T_{opt} ($F_{1,122} = 5.923$, $p = 0.016$; Fig. 3.2B). While there was no significant interaction between acclimation temperature and latitude on T_{opt} ($F_{2,122} = 1.876$, $p = 0.157$), greater latitudinal differences in mean T_{opt} were observed following 10°C acclimation compared to groups acclimated at 15 or 20°C. For instance, Skagaströnd snails acclimated to 10°C displayed higher thermal optima ($T_{opt} = 38.2^{\circ}\text{C}$) than Cabo Silleiro snails ($T_{opt} = 35.8^{\circ}\text{C}$), while in 15°C - acclimated snails this difference was much less pronounced (0.2°C difference). A similar trend of greater latitudinal differences following cold acclimation was observed for P_{max} (Fig. 3.2C), which in this instance was associated with a significant interaction term ($F_{2,122} = 6.301$, $p = 0.002$). Notably, the Skagaströnd population displayed substantially elevated P_{max} compared to the Cabo Silleiro population following 10°C acclimation (101.6 ± 15.3 vs. 67.2 ± 14.2 BPM, respectively) while at 20°C, P_{max} values were highly consistent across all populations (58.9 ± 13.0 to 64.3 ± 11.5 BPM). In the Skagaströnd population, P_{max} declined significantly with increasing acclimation temperature (One-way ANOVA, $F_{2,30} = 17.14$, $p < 0.001$), while in the St Ninian's Cave and Cabo Silleiro populations P_{max} was not affected by acclimation temperature ($p > 0.05$). In the Mount Batten population, P_{max} was significantly elevated only in the 10°C acclimated group compared to 15 and 20°C (One-way ANOVA with post-hoc Tukey tests, $F_{2,27} = 8.19$, $p = 0.002$). Interestingly, P_{max} and T_{opt} were positively correlated across the entire dataset ($T_{126} = 3.555$, $p < 0.001$, Pearson's correlation coefficient = 0.302), as were P_{max} and CT_{max} ($T_{126} = 4.765$, $p < 0.001$, Pearson's correlation coefficient = 0.391), suggesting an association between maximum performance and upper limits of cardiac activity at the individual level.

Initial heart rates ($P_{initial}$) increased significantly with acclimation temperature ($F_{2, 122} = 5.55, p = 0.005$) and across latitude ($F_{1, 122} = 15.814, p < 0.001$). Although there was no significant interaction ($F_{2, 122} = 1.264, p = 0.286$), a broadly similar pattern was seen to that observed with P_{max} , with the increase in $P_{initial}$ across latitude being more pronounced following 10 and 15°C acclimation compared to 20°C (Fig. 3.2D).

Thermal sensitivity of heart rate, Q_{10} , decreased with latitude ($F_{1, 122} = 5.549, p = 0.02$) and acclimation temperature ($F_{2, 122} = 4.011, p = 0.02$; Fig. 3.2E). Differences in Q_{10} across latitude were more pronounced at lower acclimation temperatures (10 and 15°C), although the interactive effect was not significant ($F_{2, 122} = 2.225, p = 0.112$). Additionally, Q_{10} was elevated following 10°C acclimation compared to 15 and 20°C acclimation in all populations except the Skagaströnd population ($F_{2, 30} = 1.010, p = 0.376$).

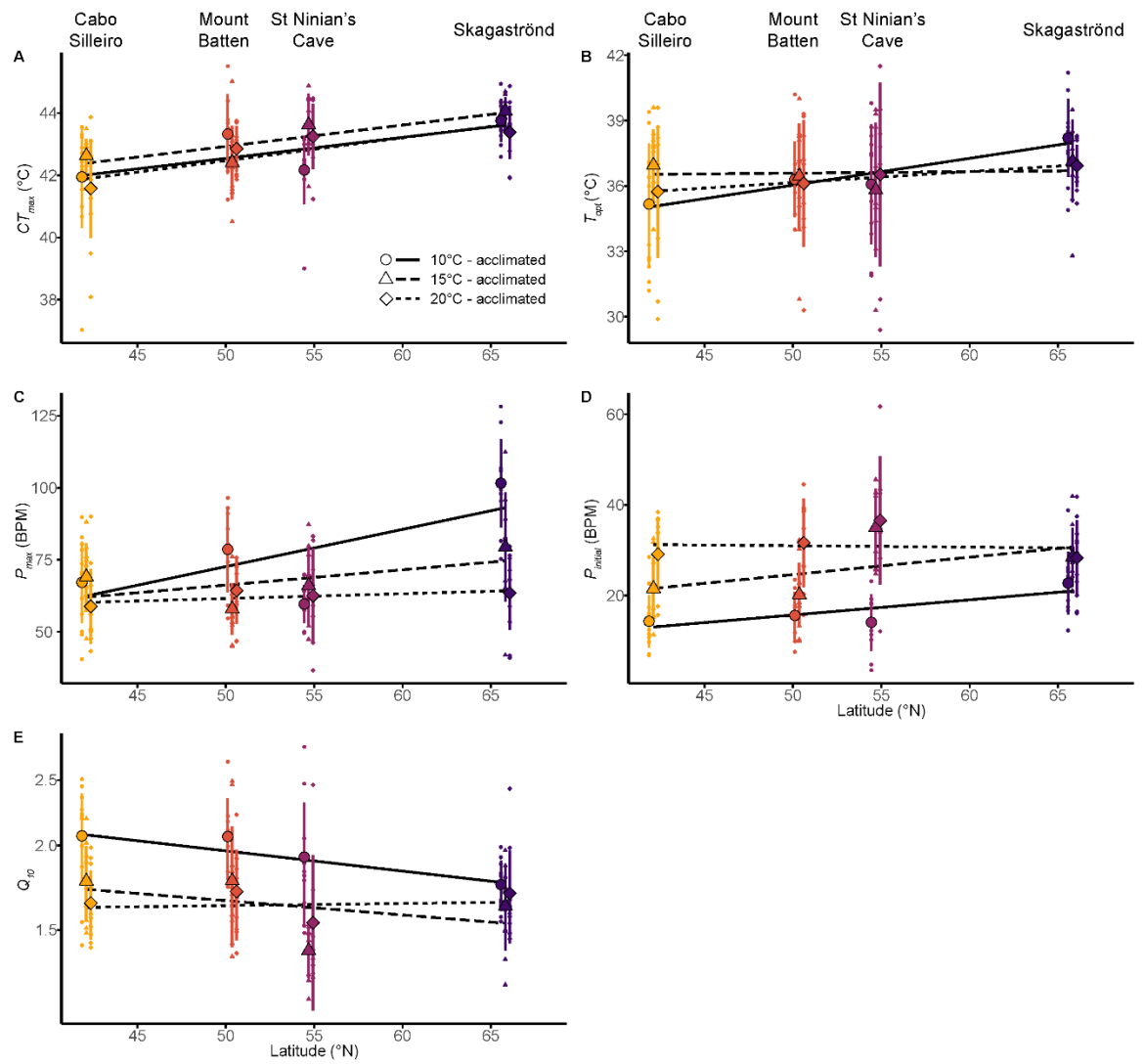


Figure 3.2 Thermal physiological traits derived from TPCs in four populations of *Littorina saxatilis* from across a latitudinal gradient, following acclimation to three different temperatures for a period of at least five weeks. Data points for each acclimation temperature are slightly offset for clarity of presentation. Large and small data points represent mean and individual values, respectively, while whiskers indicate standard deviation.

3.3.2 Principal Component Analysis

The first three components of our PCA encapsulated 87.8% of total variation across performance metrics derived from the TPC fits (Q_{10} , C , T_{th} , log-transformed $decay$, T_{opt} , CT_{max} , and P_{max}). Plotting of PC1 against PC2 (39.7% and 31.6% of total variance respectively), supported the putative association between upper thermal limits and maximum performance suggested by our univariate analyses (Fig. 3.2A-C), as there was a high positive collinearity between the traits T_{opt} , P_{max} and CT_{max} , as well as the descriptors T_{th} and $decay$ (Fig. 3.3). This cluster of variables was orthogonally separated from a second axis containing traits associated with thermal breadth, namely C , $T_{breadth}$ and the strongly negatively collinear Q_{10} . PC3 (16.5% of the remaining variation) was associated with variation in CT_{max} and P_{max} , which displayed a positive collinearity across our dataset (Pearson's correlation test, $R = 0.47$, $p < 0.001$). In general, TPCs for the different populations were largely displaced based on their thermal breadth, with the two higher latitude populations (St Ninian's Cave and Skagaströnd) displaying a shift towards higher thermal breadth and, in the case of the Skagaströnd population, also towards high T_{opt} (grey arrows and accompanying annotations, Fig. 3.3). Acclimation to 10°C was generally associated with a shift towards lower thermal breadth and reduced T_{opt} , except in the Skagaströnd population, which instead displayed a pronounced shift towards high T_{opt} relative to all other acclimation groups and populations.

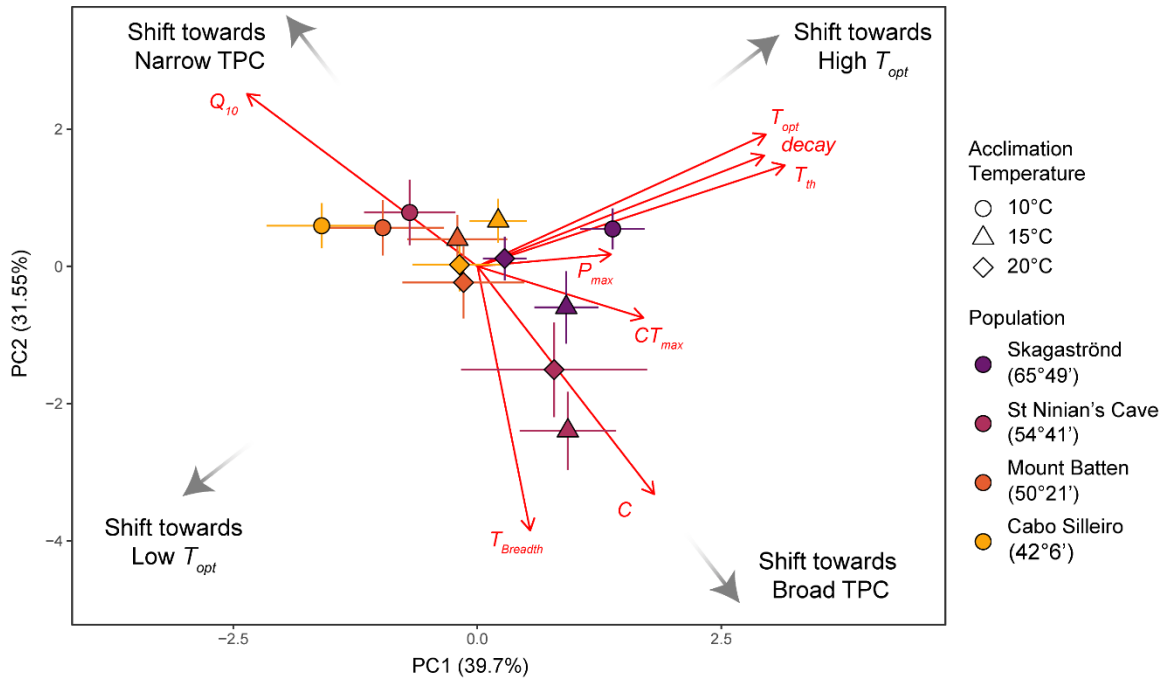


Figure 3.3: Principal Component Analysis incorporating parameters and descriptors used in the nonlinear model for fitting TPCs. The length and direction of red arrows indicate their relative contribution of each variable to the axes. Data points with whiskers indicate mean positions \pm standard error for each population / acclimation temperature combination. Greyed arrows and accompanying text are annotations denoting the theoretical implications of shifts in the position of individual and mean data points in multivariate space, based upon established relationships between TPC parameters and descriptors (Rezende and Bozinovic 2019; Bozinovic et al. 2020).

3.4 Discussion

We assessed local adaptation and acclimation capacity of TPCs for cardiac performance in four latitudinally- separated intertidal populations of *L. saxatilis* from the Northeast Atlantic. Surprisingly, upper thermal limits of cardiac function (T_{opt} and CT_{max}) increased with latitude, being higher in the northernmost population from

Iceland and lowest in the southernmost population from Spain. This response was greatest in 10°C - acclimated individuals, with the northernmost population displaying a shift towards greater T_{opt} relative to other populations at this acclimation temperature. Strikingly, populations from higher latitudes also exhibited elevated heart rates at T_{opt} (P_{max}), as well as high basal heart rates ($P_{initial}$), when acclimated at low temperatures. Because the upper tolerance limits of high latitude populations run counter to environmental temperature variation, we posit that this response is unlikely to have arisen due to thermal adaptation. Instead, the close association between thermal limits and heart rate seen in our results suggests that elevated upper limits in high latitude populations are maladaptive and reflect the consequences of maintaining a high metabolic machinery at low temperatures. This argument is reinforced by the fact that warm-acclimation resulted in an overall reduction in maximum heart rates, this being especially pronounced in cold-adapted populations.

A conceptual framework demonstrating the mechanistic basis for our interpretation is shown in Figure 3.4. Cold-adapted populations (Fig. 3.4A) are subject to thermal selection for elevated metabolic rates at low temperatures in order to compensate for lower thermodynamic reaction rates and reduced growing seasons in cold climates (Addo-Bediako et al. 2002; Clarke 2004; Clarke and Fraser 2004; Seebacher et al. 2010). Due to kinetics, high metabolic rates at low temperatures translate into even higher metabolic rates under elevated temperatures. This elevated performance, in turn, leads to an overall shift in the upper extremes of the TPC (correlated responses, Fig. 3.4A), as higher P_{max} is positively associated with T_{opt} and, ultimately, CT_{max} . An implication of this is that high latitude populations may be highly tolerant of acute thermal stress (Rezende et al. 2014; see Castañeda et al. 2015), although it is unlikely

that this is sustainable over longer exposure periods due to the energetic costs associated with maintaining high metabolic rate. Given the rarity of thermal stress events at high latitudes, such an approach may be sufficient to ensure short term survival, thus the upper portion of the TPC may be under very weak selection pressure in these populations. By contrast, warm-adapted populations (Fig. 3.4B) must contend with higher average temperatures as well as with frequent and extended periods of heat stress during which metabolic costs rise dramatically. Thermal selection therefore acts to reduce the metabolic rate of warm-adapted organisms within their usual thermal range (Fig. 3.4B, greyed area). This has the benefit of reducing metabolic rates at high temperatures, including P_{max} , thus making energy expenditure sustainable under prolonged heat exposure (Marshall and McQuaid 2011), but conversely resulting in lower T_{opt} due to thermodynamic constraints on the shape of TPCs.

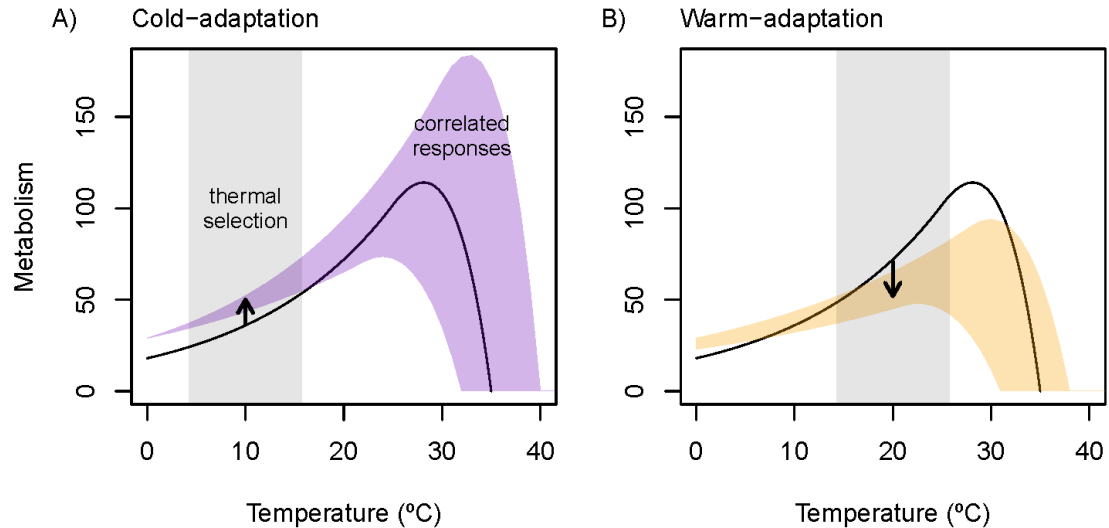


Figure 3.4: Conceptual framework proposed to explain our results, demonstrating how correlated effects on the shape of TPCs may result from thermal selection on metabolism in A) cold-adapted and B) warm adapted populations. In both figures, the black TPC represents thermodynamic effects in a hypothetical ancestral population for the purposes of comparison.

Interestingly, although the differences we observed across latitude closely matched our conceptual model following 10°C acclimation, these differences were reduced following 15°C acclimation, and largely disappeared following 20°C acclimation. This suggests that the correlated effects associated with metabolic cold adaptation in high latitude populations were lost following acclimation to higher temperatures (i.e. a shift from the scenario presented in Fig. 3.4A to 3.4B), and indicates comparatively high metabolic plasticity in high-latitude populations of *L. saxatilis*. In general, species from the upper intertidal tend to display a low capacity for metabolic plasticity in response to acclimation temperature (McMahon et al. 1995; Stillman and Somero 2000; Bozinovic et al. 2011), potentially because temperature fluctuations over tidal cycles are so frequent and dramatic that the temperature conditions experienced at any given time are poor predictors of those likely to occur in the near future (McMahon 1990; Seebacher et al. 2015). However, metabolic plasticity generally increases towards higher latitudes (Stillman and Somero 2000; Compton et al. 2007; Deutsch et al. 2008; Rohr et al. 2018) and accordingly we observed the greatest shifts in TPCs in the Skagaströnd population, potentially suggesting that the greater seasonal variability of higher latitudes may outweigh the low thermal predictability of upper intertidal habitats.

In addition to affecting upper thermal limits and standard metabolic rate, differences in temperature regimes across latitude may also drive shifts in thermal breadth (Spicer and Gaston 1999; Ghalambor 2006; Angilletta 2009). We identified a positive association between thermal optima and performance breadth, both of which increased with latitude. Surprisingly, Q_{10} , which is a major determinant of thermal breadth (Rezende and Bozinovic 2019), was elevated in populations from warmer

environments, but conversely was reduced under warm acclimation, indicating that evolutionary (i.e. interpopulation) and plastic responses of Q_{10} are antagonistic to each other. The fact that Q_{10} is lower under evolutionary cold adaptation seems to contradict the idea that populations from higher latitudes should display a greater temperature sensitivity of metabolism (Clarke 1993; Hochachka and Somero 2002). However, low Q_{10} values would in fact be expected under MCA in cold-origin populations based on first principles, as low Q_{10} is associated with elevated metabolic rate at low temperature, as well as reducing the critical thermal minimum (Rezende and Bozinovic, 2019). Thus, low Q_{10} values may be advantageous in cold-origin populations, and may be especially useful in *Littorina saxatilis* considering that many populations remain active year-round (Reid 1996). Conversely, the reductions in Q_{10} observed under warm acclimation may represent a strategy to reduce energy expenditure under periods of elevated temperatures.

Intertidal organisms have been widely utilized in the study of latitudinal variation in thermal performance, with many studies utilizing CT_{max} as a proxy to assess local adaptation across the entire TPC (Rezende et al. 2011; Rezende and Bozinovic 2019; Dong et al. 2021). However, evidence for broad scale shifts in upper limits across latitude is mixed, with some studies finding no substantial reduction in the upper limits in higher latitude populations (Lee and Boulding 2010; Logan et al. 2012; Gaitán-Espitia et al. 2014). Once we discount the apparent countergradient response of higher T_{opt} and CT_{max} in cold-acclimated northern populations, our results suggest there is remarkably little variation in upper thermal limits of *L. saxatilis* across latitude, although it is unclear whether this pattern would hold under longer periods of exposure to high temperatures. At the same time, the results of this study suggest a

substantial degree of local adaptation of standard metabolic rate across latitude. In conclusion, this study highlights the danger of assuming that variation in any one aspect of the TPC is adaptive without appropriate ecological context and we caution against the use of methods that measure individual TPC characteristics in isolation. Instead, we advocate the use of integrative approaches that enable variation across multiple TPC traits to be explored in tandem, thus allowing for the competing effects of thermodynamic constraints vs. biochemical adaptation to be evaluated.

Chapter 4 Estimates of thermal tolerance variation

across latitude in *Littorina saxatilis* depend on

methodological context

Abstract

Critical thermal limits are widely utilized to assess latitudinal variation in the thermal sensitivity of ectotherms. However, controversy exists over the extent to which methodological differences may affect estimates of critical thermal limits, including the identification of latitudinal trends. In particular, the importance of methodological considerations is poorly understood in intertidal organisms, where thermal tolerance is measured using a variety of protocols. In this study, I assessed the effects of different methodological approaches on thermal tolerance estimates in the intertidal gastropod *Littorina saxatilis*, using four populations from across a latitudinal gradient. Using dynamic ramping, I considered the effects of changes in ramping medium (air vs. water) and ramping rate ($6\text{ }^{\circ}\text{C h}^{-1}$ vs. $12\text{ }^{\circ}\text{C h}^{-1}$) on the thermal limits of cardiac activity. Then, I compared how predictions based on dynamic assays differ from those generated using the static Thermal Death Time (TDT) approach. The use of different ramping media had a substantial impact on thermal tolerance estimates, with cardiac breakpoints occurring at lower temperatures in water than in air. Importantly, a countergradient latitudinal trend in cardiac flatline temperatures observed under ramping in air was not detected under ramping in water. The use of the different ramping rates had only a subtle effect on thermal tolerance limits, although maximum

cardiac activity was elevated under faster ramping, potentially indicating greater acute metabolic stress. Lastly, the TDT approach revealed a contrasting pattern of variation in thermal limits across latitude to those identified using ramping approaches, potentially demonstrating its increased sensitivity in detecting differences across environmental gradients. This study demonstrates that the use of different methodologies may have important repercussions for the assessment of thermal tolerance variation across latitude.

4.1 Introduction

Critical thermal limits represent the maximum habitat temperatures that organisms can withstand (Cowles and Bogert 1944; Lutterschmidt and Hutchison 1997) and are important in defining an organism's thermal niche (Somero 2002). As such, they are widely utilized to quantify differences in thermal sensitivity across latitude (Bozinovic et al. 2011), for which a range of methodological approaches exist. A debate on how methodological considerations may impact upon tolerance estimates is ongoing (Lutterschmidt and Hutchison 1997; Terblanche et al. 2007; Cooper et al. 2008; Rezende et al. 2011; Santos et al. 2011; Rezende and Santos 2012; Bjelde and Todgham 2013). Of particular concern is the potential for experimental conditions to obfuscate underlying trends across environmental gradients (Sgrò et al. 2010; Santos et al. 2011; Rezende et al. 2014), yet several broad-scale macrophysiological studies incorporate a mixture of different methodologies into their analyses (Addo-Bediako et al. 2000; Sunday et al. 2011; Hoffmann et al. 2013). There is therefore a pressing need to assess how methodological differences may impact our ability to assess how

thermal tolerance varies across latitude (Rezende et al. 2011; Santos et al. 2011; Rezende and Santos 2012).

Critical thermal limits are typically measured using one of two approaches. In static assays, organisms are held acutely at constant stressful temperatures and the time taken to reach a physiological endpoint is measured (Cowles and Bogert 1944; Lutterschmidt and Hutchison 1997). In dynamic ramping assays, organisms are instead exposed to a benign temperature, which is then increased or decreased at a constant rate until a physiological endpoint is reached. The temperature at which this occurs is referred to as CT_{max} (Cowles and Bogert 1944; Lutterschmidt and Hutchison 1997). The dynamic approach has historically enjoyed greater popularity (Kingsolver and Umbanhowar 2018), and has seen widespread use in studies comparing variation in thermal tolerance across environmental gradients (Stillman and Somero 2000; Stenseng et al. 2005; Deutsch et al. 2008; Sunday et al. 2011; Hoffmann et al. 2013; Buckley and Huey 2016; Dong et al. 2017, 2021). However, differences in ramping rates used in dynamic assays may have a substantial effect on estimates of thermal tolerance limits. At slow ramping rates, increased exposure times may either lead to reduced CT_{max} due to greater injury accumulation and depletion of energy reserves (Terblanche et al. 2007; Sgrò et al. 2010; Rezende et al. 2011), or to increased CT_{max} via heat hardening or acclimatory responses (Lutterschmidt and Hutchison 1997; Angilletta 2009; Terblanche et al. 2011). There is also evidence that the use of dynamic assays may obscure or misrepresent the extent of thermal adaptation across latitude (Santos et al. 2011). This is because organisms adapted to different climates may differ in their ability to tolerate different combinations of intensity and duration of thermal stress, which cannot be disentangled under dynamic assays. In contrast, Thermal

Death Time (TDT) curves, based upon the static approach, have the ability to distinguish the effects of duration vs. intensity of heat exposure on critical limits, for which they have gained renewed attention (Rezende et al. 2014; Jørgensen et al. 2019). The TDT approach has recently been used to identify previously unnoticed latitudinal trends in thermal tolerance (Rezende et al. 2014; Castañeda et al. 2015; but see Jørgensen et al. 2019; Alruiz et al. 2021), as well as trends across body size (Peralta-Maraver and Rezende 2021), life-history stages (Truebano et al. 2018), and shore height (Dwane et al. 2021). Disadvantages of the TDT method include the requirement for large numbers of individuals, an inability to assess variation at the inter-individual level, and an inability to incorporate thermal performance prior to collapse (Kingsolver and Umbanhowar 2018). In addition, the heightened ecological relevance of ramping assays in highly variable thermal environments has led to the continued prevalence of the dynamic approach in study systems such as the intertidal (Stenseng et al. 2005; Moyon et al. 2019; Dong et al. 2021). However, the effects of different rates of change on estimates of CT_{max} , and the extent to which these estimates can be reconciled with data collected using static approaches (Jørgensen et al. 2019; Rezende et al. 2020) are still poorly understood in intertidal taxa (Moyon et al. 2019; Dong et al. 2021).

Another factor which may influence estimates of thermal tolerance limits is the choice of medium in which animals are held during the trials. In semi-aquatic organisms, exposure to high temperatures in water vs. air during the assays pose substantially different challenges, as the oxygen solubility of water decreases dramatically at high temperatures (Abele et al. 2007). The oxygen limitation hypothesis (Pörtner 2001, 2010; Verberk et al. 2011; Pörtner et al. 2017) proposes that constraints on oxygen

supply may be the main mechanism limiting thermal tolerance in ectotherms, as limited oxygen supply reduces the ability of an organism's respiratory system to meet spiralling metabolic demands at high temperatures, leading to impaired performance and ultimately death. Based on this, it has been suggested that biochemical, and morphological adaptations to cope with oxygen limitation at high temperatures may comprise a crucial component of thermal adaptation of ectotherms across latitude, particularly in aquatic environments (Pörtner 2001, 2002; Verberk et al. 2016a). However, the upper thermal limits of air-breathing species do not appear to be constrained by oxygen limitation, at least in arthropods (Verberk et al. 2016b), suggesting that it is not a universal mechanism (Verberk et al. 2016a; Bowler 2018). In semi-aquatic intertidal taxa, the choice of medium may substantially affect estimates of upper tolerance limits (Marshall and McQuaid 1992; Bjelde and Todgham 2013; Bjelde et al. 2015), although the nature of differences may be highly species-specific (Kankondi et al. 2018) and dependent on respiratory mode (Koopman et al. 2016). While intertidal taxa are widely used in studies comparing thermal tolerance across latitude, under both emersion (Logan et al. 2012; Dong et al. 2015, 2017) and submersion (Clarke et al. 2000; Stillman and Somero 2000; Compton et al. 2007; Gaitán-Espitia et al. 2014), few studies to date have attempted to compare between the two (Bjelde and Todgham 2013; Kankondi et al. 2018). Therefore, little is known about whether the use of different ramping media may affect our ability to identify latitudinal trends in the upper limits of these organisms.

The aim of this study was to examine the effect of different methodological approaches on estimates of upper thermal tolerance limits, with a focus on how any potential differences in these estimates may affect estimates of thermal tolerance

differences across latitude. To do this, I compared upper thermal limits of four populations of the intertidal gastropod *Littorina saxatilis* from along a latitudinal gradient spanning 23° across the east Atlantic coastline.

- First, I examined how upper thresholds of cardiac activity varied in response to ramping under different media (air vs. water). Given that aerobic performance becomes limited at lower temperatures under immersion in this species (Sokolova and Pörtner 2003), I predicted that both Arrhenius breakpoint temperature (ABT) and cardiac flatline (CT_{maxFL}) would be reached at lower temperatures in water compared to air, and that this would potentially have implications for latitudinal trends in both traits.
- Second, I explored the effect of differences in heating rate on upper thermal limits under dynamic ramping. I predicted that upper limits would increase under faster ramping (Terblanche et al. 2007; Rezende et al. 2014), and that this may affect latitudinal trends, given that differences in sensitivity to heating rate are known to exist across shore height (Moyen et al. 2019).
- Lastly, I generated thermal tolerance landscapes for each population using a static TDT approach for Littorinids (Truebano et al. 2018; Dwane et al. 2021) and compared this with data generated from dynamic assays to explore how latitudinal trends in CT_{max} vary between the two approaches. Given that static approaches have been shown to be superior in detecting underlying latitudinal trends in thermal sensitivity compared to dynamic approaches (Rezende et al. 2014; Castañeda et al. 2015), I predicted that latitudinal trends in thermal tolerance would be more pronounced under the TDT approach, and potentially be associated with a trade-off between chronic and acute thermal tolerance at different

exposure temperatures and durations, as has been observed in terrestrial insects (Castañeda et al. 2015; Alruiz et al. 2021).

4.2 Methods

4.2.1 Animal collection and husbandry

Adult populations of *Littorina saxatilis* were obtained from four sites across a latitudinal gradient spanning 23° along the northeast Atlantic coastline, from Galicia in Northwest Spain to the north coast of Iceland, which experience contrasting temperature regimes (Fig. A1). Details of study sites are given in Chapter 3, Table 3.1. Collection dates for all populations and individuals used in this chapter are the same as those given in Chapter 3: Table 3.1, unless otherwise noted below. Following collection, snails were placed in plastic containers with dampened cardboard and transported by car, or express airmail, to the University of Plymouth. Upon arrival, snails were distributed randomly between 5 L aquaria containing aerated seawater (salinity = 35 ± 1) with a stocking density of ~ 10 snails L^{-1} . All populations were initially held in a 15°C controlled temperature room for > 1 week to recover from handling stress, except for the Skagaströnd population, which were initially held at 10°C on account of the lower sea temperatures at the locality at the time of collection. Following this period, all populations were held at 15°C for at least five weeks, which is a period sufficient to limit differences in metabolism arising from prior acclimation in the field (Newell 1979; Hawkins 1995; Sokolova and Pörtner 2001, 2003). Water changes were conducted weekly, and snails were fed ad libitum on *Ulva lactuca*. Mortality during the acclimation period was low (< 10 %) for the majority of

populations and acclimation temperatures but was observed to be higher in St Ninian's Cave snails (~ 20 %).

4.2.2 *Comparison of ramping in air vs. water*

To compare upper tolerance limits of snails under different media (in water vs. in air), heart rates of snails from each population were recorded under an acute thermal ramp of 6°C h^{-1} using infrared photoplethysmography (Burnett et al. 2013; Dong et al. 2017). This was done using an infrared monitoring setup and method (Chapter 3), with several adjustments to facilitate ramping in water. Briefly, after being fitted with sensors, snails were transferred individually to either: (for ramping in air) unsealed dry plastic envelope bags, or (for ramping in water) 150 mL glass beakers (Pyrex, UK) containing filtered seawater (salinity = 35 ± 1), which were gently aerated during the trials to maintain oxygen saturation. In both cases, holding vessels were placed inside a Grant R5 water bath (Grant Instruments, UK) preheated to 15°C . Following addition of the snails, the setup was left for 15-20 min to allow snail body temperatures to equilibrate with water bath temperatures, and to allow recovery from handling stress. Temperature during the trials was monitored using k-type thermocouples, which were either (for ramping in air) inserted inside an empty snail shell filled with blu-tak (Bostic, UK), or (for ramping in water) placed directly into beakers containing the snails to monitor ambient water temperature. Temperature was then steadily increased at a rate of 6°C h^{-1} and cardiac activity was continuously recorded until a temperature was reached beyond which all snails had reached cardiac flatline (at around $47\text{-}50^{\circ}\text{C}$), at which point the experiment was terminated. Separate trials were run for snails ramped in air, vs in water, with five snails being run simultaneously in each trial for ramping in

air and four snails per trial in water. To ensure sufficient replication, a minimum of three trials per population were run using snails ramped in air, and four trials per population for snails ramped in water. Snails displaying faint or inconsistent heartbeat traces were discarded from further analysis. It should be noted that raw heartbeat data for snails ramped at 6°C h^{-1} in air in this chapter is the same data used for the 15°C acclimation group in Chapter 3. Raw signals were converted into heartbeat interval data using the MATLAB-based software package ARTiiFACT (Kaufmann et al. 2011). Heartbeats were counted for 1 min periods at 1°C ($\pm 0.1^{\circ}\text{C}$) intervals through the course of the temperature ramp and used to calculate heartrate in beats per minute (BPM). However, snails ramped in water sometimes displayed extremely low heartrates (< 3 BPM) at temperatures beyond 30°C . In these instances, heartrates were instead counted over 2-3 min windows and averaged to provide more reliable estimates of heartrate in BPM.

All statistical analysis in this study was performed in R (R Core Team 2019). Cardiac performance of *L. saxatilis* under ramping in air can be encapsulated using thermal performance curves (TPCs; see Chapter 3). However, preliminary data indicated that snails ramped in water did not display a typical TPC response, but instead displayed an abrupt transition towards bradycardia at relatively low temperatures which was then maintained until flatline (CT_{maxFL}) was reached. For this reason, I instead utilized Arrhenius breakpoint Temperatures (ABTs), an appropriate approach for the comparison of thermal tolerance limits in animals held in air vs. in water (Bejde and Todgeham, 2013). As described previously (Stillman and Somero 1996; Stenseng et al. 2005; Kankondi et al. 2018), the natural logarithm of heart rate was plotted over temperature (in $1000/\text{K}$), after which piecewise linear regression lines were fitted

using the R package “segmented” (Muggeo 2008). ABT was determined using the point on the x axis at which the line describing the initial increase in heartrate intersected with a line describing the subsequent collapse of heart rate and was converted back into °C. Maximum performance (P_{\max}) was recorded as the point on the y-axis at which the intersection describing ABT occurred. Thermal sensitivity of heartrate (Q_{10}) during the initial increasing slope was calculated using the equation:

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{\frac{10}{T_2 - T_1}}$$

With R_2 being P_{\max} , T_2 being ABT, R_1 being the heartrate at 15°C predicted by the regression line describing the initial increase in heartrate, and T_1 being the temperature at the start of the ramp (15°C). In addition, the critical limit of cardiac flatline ($CT_{\max FL}$) was recorded as the temperature ($\pm 0.05^\circ\text{C}$) at which cardiac activity was observed to cease in the raw heartbeat signals.

To assess how upper thermal limits and performance differed across ramping media, and whether this affected our interpretation of trends across latitude, I performed General Linear Model (GLM) analyses separately for $CT_{\max FL}$, ABT, P_{\max} and Q_{10} , with ramping medium and latitude as categorical and continuous predictor variables respectively, as well as an interaction term (ramping medium x latitude). Q_{10} and P_{\max} required log-transformation to meet model expectations of normality of residuals; however, as results did not differ qualitatively from those using transformed values; statistical tests for untransformed values are reported below for simplicity.

4.2.3 Comparison of ramping rates

To compare the effect of ramping rates on upper tolerance limits across latitude, cardiac activity was recorded from snails subjected to increasing temperature in air at one of two different ramping rates (6°C h^{-1} or $12^{\circ}\text{C h}^{-1}$). This was done following the methodology described above. Raw signals from snails subjected to either ramping rate were then converted into heartbeat interval data in the same manner. Heartbeat interval data was assessed over 1 min periods (every 1°C increase) across both ramping rates for consistency, thus the faster increase in temperature at the $12^{\circ}\text{C h}^{-1}$ ramping rate resulted in a wider temperature interval being captured within each 1-minute window ($\pm 0.2^{\circ}\text{C}$). These heartbeat interval data were then converted into heartrate (in BPM), which was then used to fit TPCs using either the “fit.thermal.curve” or “boot.thermal.curve” functions (Rezende and Bozinovic 2019; Bozinovic et al. 2020; Padfield et al. 2021), following the protocol described in Chapter 3. TPCs were used to generate four standard metrics of thermal performance: CT_{max} , T_{opt} (the temperature at which peak cardiac performance occurred), P_{max} (heartrate at T_{opt}) and Q_{10} of the rising (exponential) phase of the TPC.

To assess how thermal performance and tolerance differed in response to ramping rate, and whether this affected latitudinal trends, I performed General Linear Model (GLM) analysis separately for four standard metrics of thermal performance derived from the TPC fits: CT_{max} , T_{opt} (the temperature at which peak cardiac performance occurred), P_{max} (heartrate at T_{opt}) and Q_{10} of the rising (exponential) phase of the TPC. In each model, latitude and ramping rate (6°C h^{-1} or $12^{\circ}\text{C h}^{-1}$) were included as continuous and categorical predictor variables, respectively, as well as an interaction term (ramping rate x latitude).

4.2.4 *Comparison of static and dynamic approaches*

To determine tolerance times under immersion in static TDT trials, I utilized sublethal heat coma. This approach is identical to that described in Chapter 2 and Dwane et al. (2021) and is an adaptation of the approach used by Truebano et al. (2018). Briefly, snails were fitted with a small piece of thread to the operculum using superglue (Loctite). Following an overnight recovery period, trials were run by holding snails individually inside 350 mL unsealed glass jars, which were filled with seawater preheated to one of six test temperatures (35°C, 36°C, 37°C, 38°C, 39°C, 40°C). Time taken to enter heat coma was assessed by gently prodding the foot tissue at regular intervals, with the thread being gently pulled to re-extrude the foot if the snails retracted into their shells and sealed their operculum. Heat coma time was recorded when no reaction to the stimulus was noted. The Skagaströnd population was tested across five temperatures (35°C, 36°C, 37°C, 38°C, 39°C), the St Ninian's Cave population at four temperatures (36°C, 37°C, 38°C, 39°C), and the Mount Batten population at five temperatures (36°C, 37°C, 38°C, 39°C and 40°C). Data for the Cabo Silleiro population was taken from the RB ecotype used in Chapter 2 (Dwane et al. 2021) and assessed at five temperatures (36°C, 37°C, 38°C, 39°C and 40°C). Only male individuals were used in static trials.

To generate TDT curves for each population, I regressed log-transformed heat coma times against treatment temperature (Rezende et al. 2014, 2020). GLM was used to test for differences in the regression slopes of TDT curves across latitude, with temperature and latitude as continuous predictor variables and log-transformed heat coma time as a response variable. In addition, $CT_{\max HC}$ (representing the extrapolated

temperature at which heat coma would be reached after 1 min of exposure) and z were calculated for each population using the equations $CT_{max} = - \text{intercept/slope}$ and $z = 1/\text{slope}$. To model how estimates of thermal tolerance generated using the static approach would have differed had the same assay methodology been adopted under a dynamic approach, I additionally utilized the function “dynamic.landscape” (Rezende et al. 2020) to estimate heat coma times under a simulated 6°C h^{-1} dynamic ramp starting at 15°C (i.e. the same methodology used in comparisons of the dynamic approach in air and water). CT_{maxHC} was recorded as the 0.1°C temperature increment at which 50 % of individuals would be estimated to reach heat coma under the simulated ramp in each population.

4.3 Results:

4.3.1 Comparison of ramping in air vs. water

Thermal performance of cardiac activity displayed strikingly different patterns under ramping in air vs. water across all four populations (Fig. 4.1A). Overall, the medium under which estimates were made had a significant effect on CT_{maxFL} ($F_{1,84} = 13.36$, $p < 0.001$), and crucially, also affected our ability to detect latitudinal differences in thermal tolerance, indicated by a significant interaction between latitude and ramping medium ($F_{1,84} = 14.21$, $p < 0.001$; Fig. 4.1B). While CT_{maxFL} increased significantly with latitude under ramping in air (one-way ANOVA, $F_{1, 41} = 17.99$, $p < 0.001$), this latitudinal pattern was not present under ramping in water ($F_{1,43} = 0.47$, $p = 0.5$).

Ramping medium had an even more pronounced effect on ABT, which occurred at much lower temperatures under ramping in water compared to air ($F_{1,84} = 29.55$, $p <$

0.001; Fig. 4.1C). However, there was no corresponding effect on patterns across latitude, indicated by a non-significant interaction between ramping medium and latitude on ABT ($F_{1,84} = 0.75$, $p = 0.39$), and a similar (non-significant; $F_{1,84} = 3.89$, $p = 0.052$) latitudinal trend across both ramping in air and water.

Surprisingly, despite this earlier collapse in heartrate, there was no significant effect of ramping medium on P_{\max} ($F_{1,84} = 0.43$, $p = 0.5$; Fig. 4.1D). However, when latitude as a continuous variable was substituted for populations as discrete factors, a significant interaction between population and ramping medium was revealed ($F_{1,83} = 9.65$, $p < 0.001$). This was likely due to the Plymouth population displaying high P_{\max} in water compared to the other three populations (Fig. 4.1A). By contrast, Q_{10} values of heartrate leading up to ABT displayed a highly consistent pattern across latitude between individuals ramped in air and water (Fig. 4.1E). There was no significant interaction between latitude and ramping medium on Q_{10} ($F_{1,84} = 0.01$, $p = 0.9$). However, once this was dropped from the model there was a significant effect of latitude ($F_{1,85} = 6.47$, $p = 0.01$) and of ramping medium ($F_{1,84} = 7.4$, $p = 0.007$) on Q_{10} , indicating that while Q_{10} was elevated under ramping in water across all populations, the negative trend in Q_{10} with increasing latitude was preserved across both ramping media.

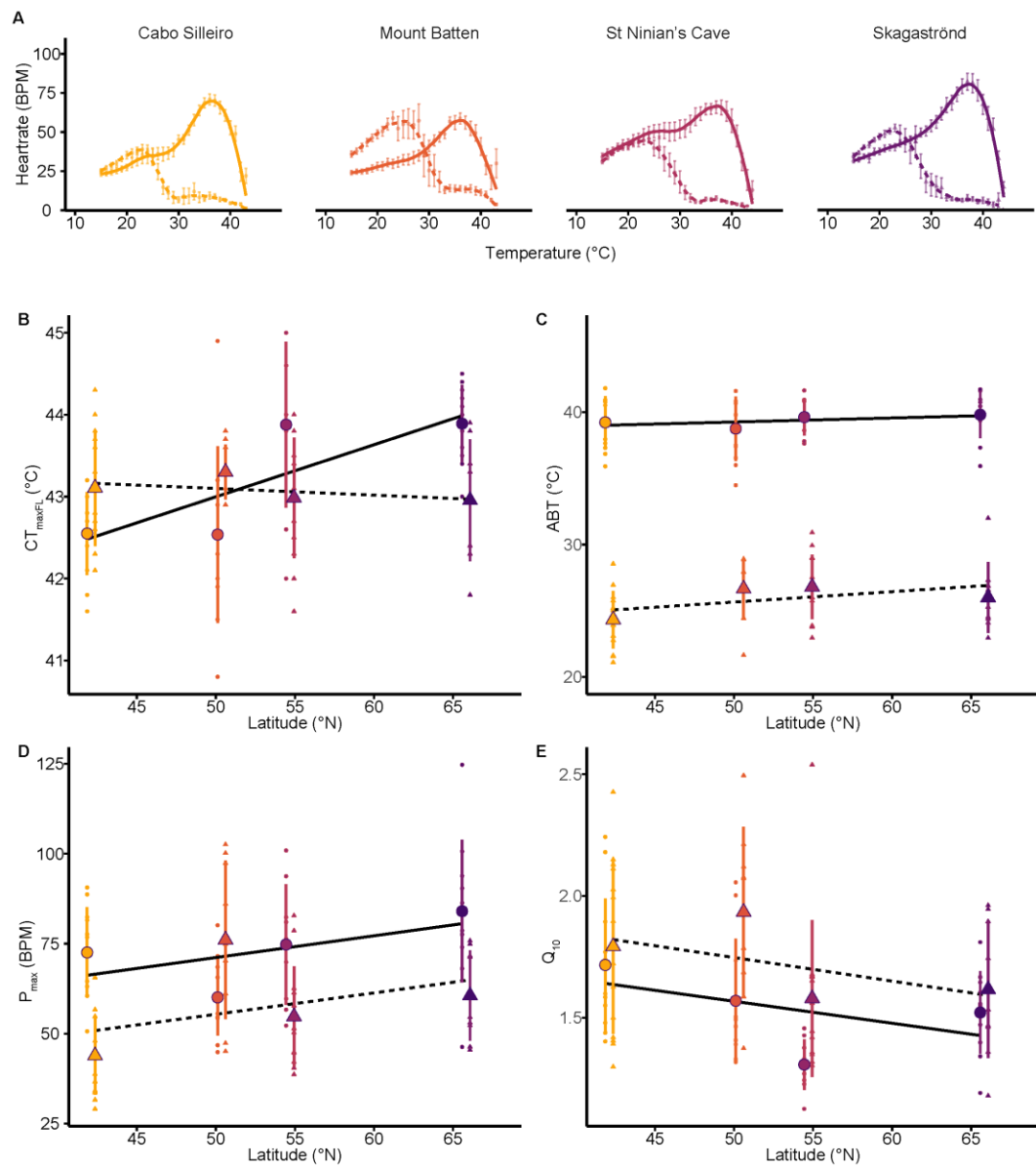


Figure 4.1: Effects of ramping medium (air vs. water) on cardiac performance in four populations of *Littorina saxatilis* from across a latitudinal gradient. A) Averaged heart rates for each population under ramping in air (solid line) and ramping in water (dashed line). Error bars denote standard error at each 1°C increment at which heartrates were recorded during the trials. Curves were fitted to mean values using the “loess” function in the R package ggplot2. B-E) Variation in four cardiac performance traits across ramping media and latitude. Data points for each group are slightly offset for clarity of presentation. Large and small data points represent mean and individual values, respectively, while whiskers indicate standard deviation.

4.3.2 Comparison of ramping rates

Ramping rate did not have a significant effect on CT_{maxFL} ($F_{1,81} = 0.09$, $p = 0.76$). In addition, our ability to predict CT_{max} across latitude was not affected by the ramping rate used in the assays, as indicated by the lack of a significant interaction between ramprate and latitude ($F_{1,81} = 0.02$, $p = 0.9$), meaning that a significant decline in CT_{maxFL} across latitude ($F_{1,81} = 38.84$, $p < 0.001$) was preserved across both ramprates.

For T_{opt} , there was likewise no significant ramprate x latitude interaction ($F_{1,81} = 1.67$, $p = 0.2$), and no main effect of ramprate on T_{opt} even once this interaction term was dropped ($F_{1,82} = 0.01$, $p = 0.12$). However, there did appear to be a (non-significant) trend towards greater latitudinal differences in T_{opt} in $12^{\circ}C\ h^{-1}$ as opposed to $6^{\circ}C\ h^{-1}$ – ramped snails (Fig. 4.2B).

In spite of the minimal differences in upper tolerance limits, there were marked differences in the thermal performance of cardiac activity between snails ramped at $6^{\circ}C\ h^{-1}$ vs. $12^{\circ}C\ h^{-1}$. There was a near significant ramp rate x latitude interaction on P_{max} ($F_{1,81} = 3.77$, $p = 0.055$; Fig. 4.2C), and once the interaction term was dropped there was a significant main effect of ramping rate ($F_{1,82} = 12.93$, $p < 0.001$) and latitude ($F_{1,82} = 22.21$, $p < 0.001$) on P_{max} , indicating that P_{max} was generally elevated across all four populations under the faster ramping rate. This appeared to be most pronounced in the northernmost Skagaströnd population (100.12 ± 15.18 BPM vs. 79.43 ± 18.90 BPM, respectively), and least pronounced in the southernmost Cabo Silleiro population (72.02 ± 13.69 BPM vs. 69.08 ± 11.71). It should be noted that this difference in P_{max} was not driven by a difference in initial heart rates ($P_{initial}$) at the start of the trials, as there was no significant ramp rate x latitude interaction on $P_{initial}$ ($F_{1,81} = 0.36$, $p = 0.55$), nor a significant main effect of ramp rate ($F_{1,81} = 0.56$, $p = 0.46$). Instead, higher P_{max}

under fast ramping appeared to be associated with elevated Q_{10} values; once a non-significant interaction term was dropped ($F_{1,81} = 0.04$, $p = 0.84$;). There were significant main effects of ramprate ($F_{1,82} = 11.78$, $p < 0.001$) and latitude ($F_{1,82} = 7.42$, $p = 0.007$) on Q_{10} , with values being consistently elevated under $12^{\circ}\text{C h}^{-1}$ ramping compared to 6°C h^{-1} (Fig. 4.2D). In summary, while exposure to different ramping rates did not significantly affect upper tolerance limits, it did influence the thermal sensitivity of cardiac activity during the ramp, which in turn appeared to be linked to higher maximal performance at faster ramping rates.

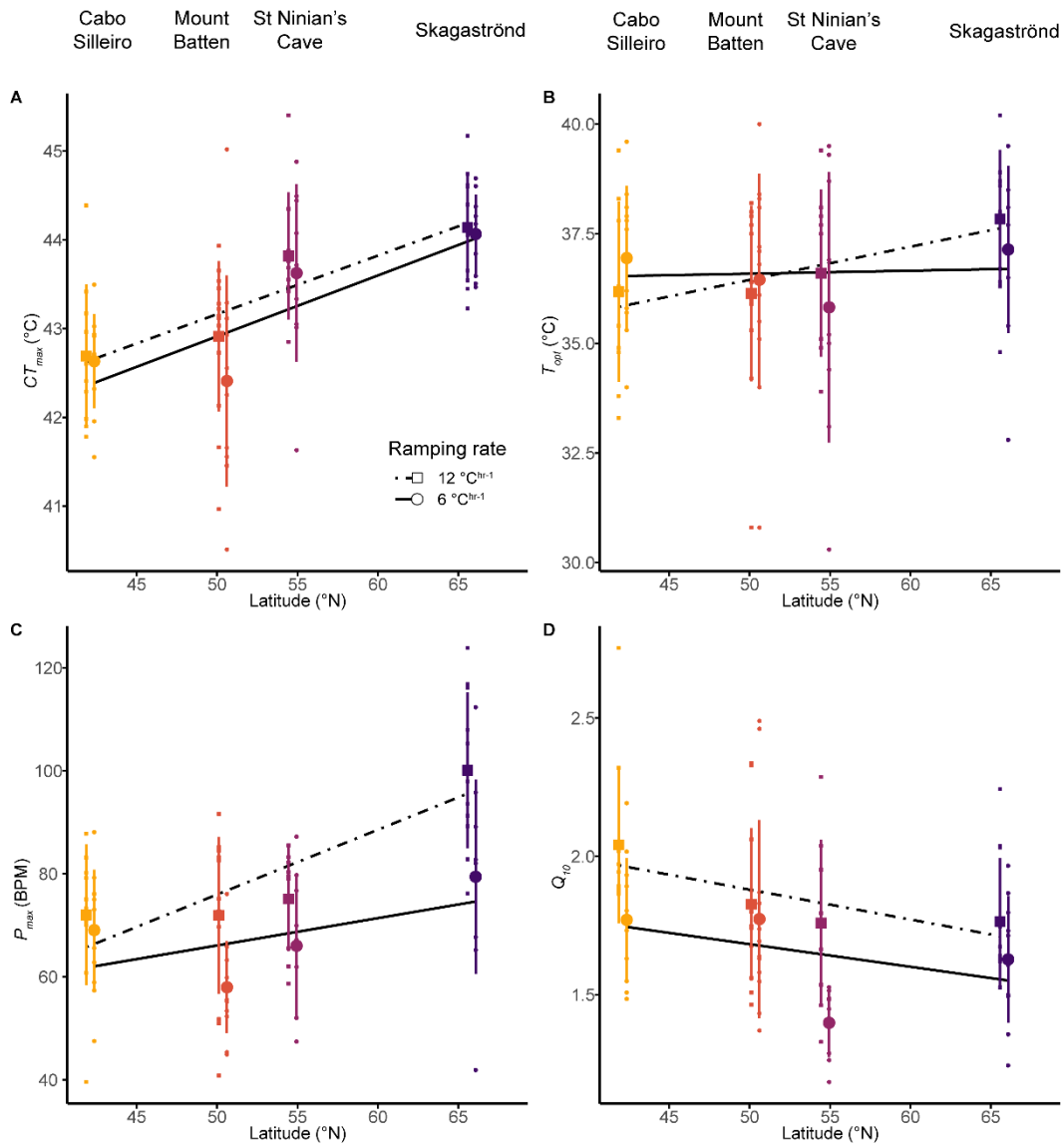


Figure 4.2 Effects of ramping rate on cardiac performance traits in four populations of *L. saxatilis* from across a latitudinal gradient. Data points for each ramping rate are slightly offset for clarity of presentation. Large and small data points represent mean and individual values, respectively, while whiskers indicate standard deviation.

4.3.3 Comparison of static and dynamic approaches

In contrast to data derived from dynamic assays (Figs. 1-2), TDT curves derived from static assay data displayed a trend of declining thermal tolerance with increasing latitude (Figure 3A), matching clinal variation in temperature across the sites of origin for each population. This was indicated by a significant effect of both latitude ($F_{1,273} = 327.4$, $p < 0.05$) and temperature ($F_{1,273} = 4225.1$, $p < 0.05$) on log-transformed heat coma time (Fig. 4.3A). However, there was no significant latitude x temperature interaction ($F_{1,272} = 0.01$, $p = 0.9$; dropped from the linear model prior to the calculation of main effects). This indicates that, unlike in other species (Rezende et al. 2014; Castañeda et al. 2015; Alruiz et al. 2021), there is no trade-off across latitude in the ability of *L. saxatilis* to tolerate very high temperatures for short durations vs. less extreme temperatures for longer durations. $CT_{\max HC}$ values extrapolated from static TDT curves displayed a strong negative correlation with latitude (Pearson's correlation coefficient, $R = -0.986$, $p = 0.014$; Fig. 4.3B). Similarly, estimated $CT_{\max HC}$ values for heat coma under a simulated 6°C h^{-1} ramp also displayed a negative trend with latitude (Pearson's correlation coefficient, $R = -0.935$), although this was not significant ($p = 0.065$; Fig. 4.3B). Differences in $CT_{\max HC}$ between the southernmost and northernmost populations under the simulated ramp (39.3°C vs 38.3°C in the Cabo Silleiro and Skagaströnd) were not as extreme as those based on the static trials (41.07°C vs 39.78°C), likely due to the higher z value seen in the Cabo Silleiro population ($z = 1.90$; Fig. 4.3C) compared to the other populations ($z = 1.71 \sim 1.74$), which would result in a reduction in endurance times under longer exposure periods.

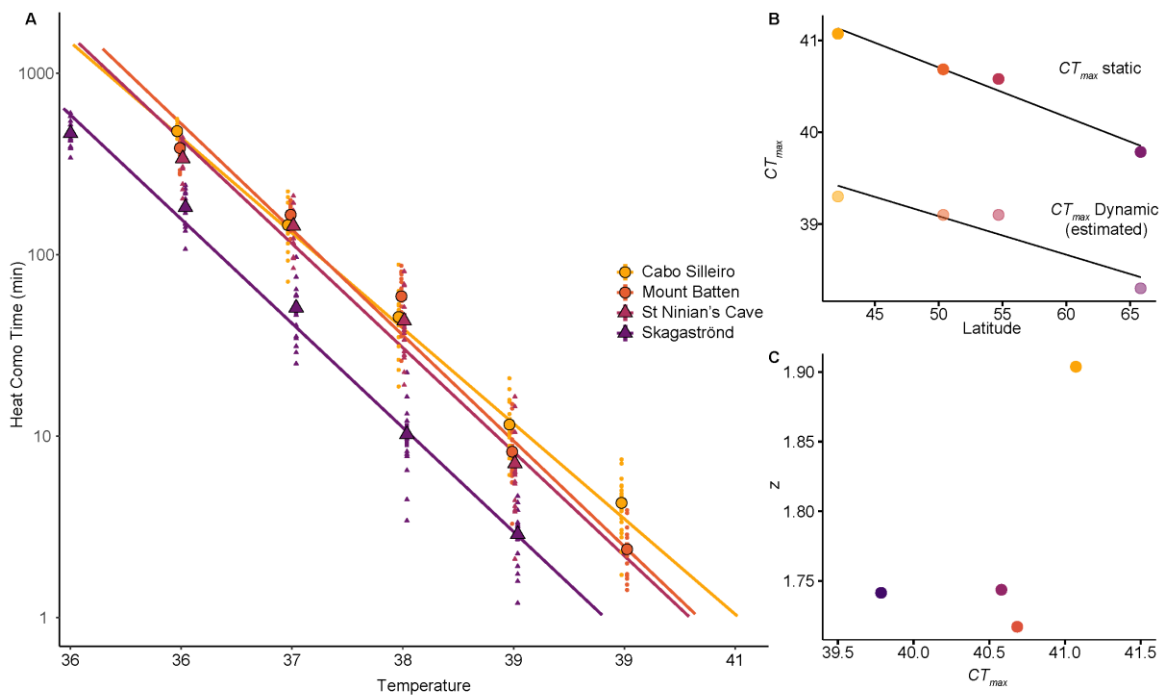


Figure 4.3: Data generated using static TDT assays in water for four populations of *L. saxatilis* from across a latitudinal gradient. A) TDT curves for each population, with the y-axis plotted in log₁₀ scale, demonstrating the semilogarithmic relationship between exposure time and temperature tolerance associated with the TDT method. Regression lines displayed high R^2 for all populations (Cabo Silleiro = 0.96, Mount Batten = 0.95, St Ninian's Cave = 0.91, Skagaströnd = 0.96). B) The relationship across latitude of CT_{max} estimates derived directly from the static TDT approach (thus representing the temperature at which tolerance would cease after 1 min), and those estimated under a simulated 6° h⁻¹ dynamic ramp conducted using the function “dynamic.landscape” (Rezende et al. 2020). C) Plot of CT_{max} and z values for each population, illustrating the lack of a general relationship between CT_{max} and z across latitude.

Comparison of the latitudinal trends in CT_{max} generated by the three methods used in this study (dynamic ramping in air, dynamic ramping in water, and static assays in water; Fig. 4.4) revealed dramatically different predictions of changes in CT_{max} across latitude. Namely, the positive latitudinal trend observed in CT_{max} under dynamic ramping in air disappeared under ramping in air and was reversed under static assays in water resulting in a negative latitudinal trend.

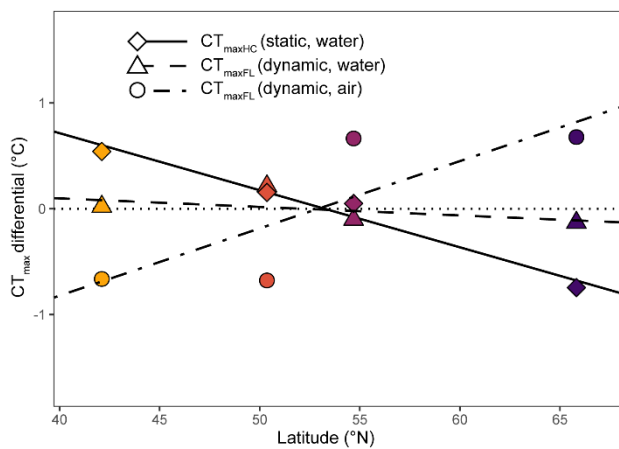


Figure 4.4: Comparison of latitudinal trends in CT_{max} revealed by different static and ramping assay techniques in four populations of *L. saxatilis* from across a latitudinal gradient. To facilitate the visual comparison of slopes, CT_{max} values measured using the different techniques were standardized so that mean CT_{max} = zero across latitude.

4.4 Discussion

In this study, I assessed how estimates of upper thermal tolerance limits, as well as our ability to detect differences between populations across latitude, may be affected by differences in methodological approaches, namely: the effect of different ramping

media, changes in ramping rate, and differences in assay technique. Broadly, the use of different ramping media had a large influence on upper tolerance limits in *Littorina saxatilis*, as well as knock-on effects on our ability to identify trends across latitude. By contrast, the use of different ramping rates did not significantly affect upper tolerance limits, although an effect on the thermal performance of cardiac function was observed. Under the static TDT approach, CT_{max} estimates were lower compared to those obtained during ramping assays, likely due to the use of a different endpoint. Importantly however, the TDT approach revealed a contrasting pattern of thermal tolerance differences across latitude compared to that found using the dynamic approach, potentially reflecting its ability to detect underlying (cogradient) interpopulation differences which were otherwise masked by thermodynamic effects under the dynamic approach.

4.4.1 *Comparison of ramping in air vs. water*

As predicted based upon previous work in intertidal gastropods (Bjelde and Todgham 2013; Kankondi et al. 2018), thermal tolerance estimates differed substantially between *L. saxatilis* individuals ramped in air and water. These differences were mechanistic in nature, as indicated by dramatic differences in cardiac activity at high temperatures. Notably, breakpoints in cardiac performance (ABT) occurred at substantially lower (> 10°C) temperatures in water compared to air, a response likely to be associated with oxygen-limitation (Sokolova and Pörtner 2003; Bjelde and Todgham 2013; Bjelde et al. 2015; Kankondi et al. 2018). Indeed, the temperatures at which cardiac breakpoints occurred in water closely matches the temperature at which the onset of anaerobiosis has previously been reported to occur in *L. saxatilis*

(Sokolova and Pörtner 2003). This indicates that the suppression of heart rate observed in the present study was likely associated with a switch to anaerobic metabolism. Suppression or “quiescence” of metabolism, during which metabolic rate becomes temperature- independent, is a well-described response of intertidal gastropods to periods of hypoxia (Cossins and Bowler 1987; McMahon 1990; McMahon et al. 1995; Marshall and McQuaid 2011). On the other hand, the fact that CT_{maxFL} did not collapse substantially earlier in water than in air is consistent with its status as a near lethal endpoint, and with the cardiac muscle being regarded as the last trait to fail prior to death (Stenseng et al. 2005; Dong et al. 2017; Moyen et al. 2019). Indeed, this result is consistent with the suggestion that suppression of heart rate under hypoxia may serve a protective role in preserving low levels of heart function until ultimate failure occurs (Bjelde et al. 2015).

Interestingly, although CT_{maxFL} did not occur substantially earlier in water, a positive trend of increasing CT_{maxFL} across latitude that was apparent under ramping in air was not present in water, demonstrating that differences in ramping media may affect the estimation of latitudinal trends in critical limits. In previous work (Chapter 3), I identified that a counter-gradient trend in upper limits under ramping in air appeared to be driven by thermodynamic effects of high maximum cardiac performance in high-latitude populations, and that this, in turn, appeared to be driven by metabolic cold adaptation across latitude. In the present study, the fact that maximal cardiac performance, Q_{10} and ABT all displayed consistent latitudinal trends across both air and water suggests that, despite the much earlier collapse of heart rate in water, the thermodynamic effects responsible for the latitudinal trend in ABT in air have a corresponding effect in water. By contrast, CT_{maxFL} in water did not display the same

counter-gradient latitudinal trend as seen in air, potentially suggesting that critical limits measured in water are less susceptible to the thermodynamic effects of physiological compensation on performance, and therefore may be more appropriate at detecting underlying differences in upper limits across latitude. Such a conclusion is controversial given that ramping in air is generally seen as more ecologically realistic (Sokolova and Pörtner 2003; Bjelde and Todgham 2013). However, although intertidal gastropods typically only experience extreme temperatures at low tide, a common response to extended periods of emersion is for snails to withdraw into the shell and to occlude the aperture with the operculum to avoid desiccation (McMahon 1990; Marshall and McQuaid 2011). In these instances, oxygen availability may be limited and metabolic depression may occur (Newell et al. 1971; Marshall and McQuaid 2011), and it is possible that latitudinal differences in CT_{max} under such conditions would therefore mirror those experienced under ramping in water. Future studies comparing thermal limits in air under different levels of hypoxia (Bjelde et al. 2015), within a latitudinal context, may assist in shedding further light on whether latitudinal differences in upper tolerance limits are influenced by thermodynamic effects on performance when oxygen availability is unconstrained.

4.4.2 Comparison of ramping rates

I predicted that snails subjected to an elevated ramping rate ($12^{\circ}\text{C h}^{-1}$) would display increased upper tolerance limits compared to those under slower ramping (6°C h^{-1}), as snails under faster ramping would experience less accumulation of heat damage (Rezende et al. 2014). In general, CT_{maxFL} values appeared to be slightly elevated under fast ramping ($12^{\circ}\text{C h}^{-1}$), although this difference was small ($\sim 0.34^{\circ}\text{C}$) and not

statistically significant. The fact that this difference was so small may indicate that a change in ramping rate from $12^{\circ}\text{C h}^{-1}$ to 6°C h^{-1} is not great enough to result in substantial differences in CT_{maxFL} . For comparison, Allen et al. (2016) reported the effect on CT_{max} of a tenfold change in ramping rate ($0.05 - 0.5^{\circ}\text{C min}^{-1}$) to be between $0.1 - 2.4^{\circ}\text{C}$ (average 1.1°C) across six species of springtail, suggesting that the effect of ramping rate may be quite small in absolute terms, even across a wider range of ramping rates than were used in the present study. This, coupled with high inter-individual variability in responses, may account for the lack of substantial differences in CT_{maxFL} observed herein. Alternatively, the lack of differences may indicate that CT_{maxFL} is a relatively fixed trait and unaffected by changes in ramping rates. Moyen et al. (2019) found that, in *Mytilus edulis*, flatline temperatures were highly consistent across different ramping rates, while cardiac breakpoints displayed greater variability. In the present study, a non-significant trend towards greater latitudinal variability in T_{opt} under the faster ramping rate of $12^{\circ}\text{C h}^{-1}$ may indicate that it is more responsive to differences in ramping rate than CT_{maxFL} . Unfortunately, firm conclusions cannot be drawn due to high inter-individual variability in responses. The strength of such a conclusion could be enhanced in follow-up work through the use of additional ramping rates, especially as this would facilitate the estimation of z values and the direct comparison with thermal sensitivity estimates generated using the static approach (an approach not adopted here, because estimates of z based on only two ramping rates are likely to be unreliable; Jørgensen et al. 2021).

By contrast, both Q_{10} and maximum cardiac performance were significantly elevated under faster ramping across all populations. This suggests that changes in ramping rate may have a substantial impact on the thermal sensitivity of cardiac performance and

the shape of TPCs. The effects of ramping rates on thermal performance metrics have not been assessed to the same extent as critical limits, likely because the majority of studies assessing the effect of ramping rate have utilized single endpoints, such as knockdown time (Rezende et al. 2014; Castañeda et al. 2015; Jørgensen et al. 2019) or heat coma (Lah et al. 2017), and so have not incorporated TPCs. One study which did explore this (Moyen et al. 2019), found no effect of ramping rate on Q_{10} in the mussel *Mytilus edulis*, although an increase in maximum cardiac performance was observed in high shore mussels under a faster ($8.0^{\circ}\text{C h}^{-1}$) compared to a slower (3°C h^{-1}) rate of ramping. These results suggest that populations may differ in their ability to control their cardiac activity in response to different rates of temperature increase. Given that heating rates experienced at low tide may vary substantially across shore height (Miller and Dowd 2017), and presumably across latitude, this is clearly an aspect of the thermal physiology of intertidal ectotherms which warrants further investigation in future studies.

4.4.3 *Comparison of static and dynamic approaches*

Recent studies have demonstrated that use of the static TDT approach can reveal patterns of variation in thermal sensitivity and tolerance across latitude which differ substantially from those previously reported using ramping assays (Rezende et al. 2014; Castañeda et al. 2015). The findings of this study provided strong support for this. In contrast to estimates of variation in CT_{maxFL} based on dynamic assays of heart rate, which displayed either an increase (under ramping in air) or no difference across latitude (under ramping in water), CT_{maxHC} values generated using the static approach displayed a negative latitudinal trend. Such a trend matches (in directionality) the

decline in environmental temperatures with increasing latitude experienced across the four populations (Fig. A1). Several previous studies have indicated an apparent trade-off between CT_{max} and z value across latitude, with high latitude populations displaying high acute temperature tolerance (in the form of high CT_{max}) at the expense of reduced chronic tolerance at lower temperatures (due to high z values) (Rezende et al. 2014; Castañeda et al. 2015; Alruiz et al. 2021). However, this was not the case here, as the latitudinal trend in CT_{maxHC} was not associated with a substantial shift in z values across latitude, indicating that responses were largely consistent across different exposure time and temperature combinations. The only exception was the Cabo Silleiro population, which displayed a higher z value and reduced tolerance times relative to the other populations at lower test temperatures. In addition, the fact that the latitudinal trend was preserved (although weakened) under a simulated 6°C h^{-1} ramp, suggests that the observed differences in CT_{maxHC} would have consequences under a dynamic ramp and are not the result of over-extrapolation due to use of CT_{max} values based upon 1 min exposure times (Jørgensen et al. 2019).

The latitudinal trend in CT_{maxHC} observed under the TDT approach appears to better reflect broad-scale differences in environmental temperatures experienced by the populations used in this study (Fig. A1). This potentially suggests that the TDT approach is more sensitive at detecting underlying adaptive differences in the stress responses of *L. saxatilis* across latitude. Because static thermal tolerance is time-limited and determined by the ability to resist injury accumulation and the depletion of energy reserves (Rezende et al. 2014; Jørgensen et al. 2019), variation in tolerance times between populations is likely to be influenced by the presence of mechanisms which mitigate these effects, such as the expression of heat shock proteins and

reliance upon anaerobic metabolic pathways (Burggren and McMahon 1981; Jørgensen et al. 2017; Monaco et al. 2017; Pörtner et al. 2017). Passive stress responses such as these comprise a major component of physiological variation between populations of *L. saxatilis* (Grahame et al. 1995; Sokolova and Pörtner 2001; Maltseva et al. 2016, 2021). Therefore, it is possible that the latitudinal trend identified using the static approach better reflects underlying adaptation in these traits across the latitudinal temperature gradient, in contrast to the dynamic assessment of cardiac activity which is potentially less sensitive to these differences.

4.5 Conclusions

In this study, I found evidence that differences in methodological approaches not only substantially affect estimates of upper thermal tolerance limits in absolute terms, but also alter estimates of thermal tolerance differences across latitude. This study also suggests that thermal limits measured in water, particularly using the static TDT approach, provide estimates of thermal tolerance that more closely match the latitudinal cline in temperature across these populations. Importantly, this study has highlighted that the use of different methodologies may reveal contrasting patterns of thermal tolerance across latitude that are dependent on different underlying mechanisms, demonstrating that datasets collected using different assay techniques must be handled with caution when incorporated into a comparative framework.

Chapter 5 General Discussion

5.1 Thesis recap and introduction

The central aim of my thesis was to characterise intraspecific variation in thermal tolerance in the intertidal periwinkle *Littorina saxatilis*, to understand the processes involved in local adaptation across environmental gradients. In Chapter 2, I focused upon variation across intertidal gradients, characterising thermal tolerance differences between two shore height-segregated ecotypes. In Chapter 3, I focused upon variation across latitudinal gradients, comparing thermal performance and tolerance between four different upper shore populations across a wide range of the species' latitudinal distribution. In Chapter 4, I used *L. saxatilis* as a model to compare different widely used methodological approaches to assess thermal tolerance, and whether the use of different methods affects our ability to detect differences across gradients. In this final chapter, I aim to synthesise the main findings of my thesis and provide recommendations for future work. The below discussion is structured into three sections. In the first two (5.2-5.3), I discuss the contribution of my thesis to our knowledge of local adaptation in *L. saxatilis* and comparable intertidal organisms. In the third section (5.4), I critically appraise the different methodological approaches used in this thesis and evaluate how each has contributed to my findings.

5.2 Thermal adaptation across shore height in *L. saxatilis*

Littorina saxatilis is well-suited to the intraspecific comparison of populations living at different shore heights, possessing low dispersal ability with direct development, conditions generally thought to favour local genetic change over plasticity (Hollander

2008; Sanford and Kelly 2011). In addition, populations may be found living at multiple levels on the shore, often (but not always; Maltseva et al. 2016; 2021; Sokolova et al. 2003) accompanied by morphological differentiation in the form of vertically segregated ecotypes (Johannesson 2016). Such populations represent a suitable but under-utilised model for the study of intraspecific thermal adaptation across shore height. For my comparison across shore height in Chapter 2, I utilised two ecotypes of *L. saxatilis* from Northwest Spain, RB and SU, chosen as they represent perhaps the best studied and most morphologically and genetically divergent ecotype pair known (Butlin et al. 2014) and are subject to substantial differences in heat exposure. I identified that differences in thermal tolerance exist between adults of the two ecotypes in line with their vertical position on the shore. My study confirmed predictions in the literature that the RB ecotype should be more resistant to thermal stress (Rolán-Alvarez et al. 1997; Rolán-Alvarez 2007; Keller and Seehausen 2012), filling an important gap in our knowledge of this particular study system. Significantly, I also demonstrated that the two ecotypes differ in their sensitivity to temperature change (z values). While differences in z values have previously been demonstrated across latitude in terrestrial and marine ectotherms, and across life history stages in an intertidal gastropod (Truebano et al. 2018), my study is the first to demonstrate that these differences may also exist across intertidal gradients. Future work could confirm the generality of these findings through interspecific comparisons of related species from different shore heights. Littorinids offer a promising model for intraspecific studies using the TDT approach, given that closely related species within the *Neritrema* subgenus of *Littorina* often exhibit vertical segregation on the same shores (Reid 1996), share a close genetic affinity, but also display substantial metabolomic differences (Maltseva et al. 2016). Intriguingly, certain locations are known where

height-segregated populations of *L. saxatilis* coexist with the two closely related congeners *L. arcana* (on the upper shore) and *L. compressa* (on the lower shore) (Maltseva et al. 2021). This would potentially allow for an experimental design in which the extent of intra- vs, interspecific differences in CT_{max} and z across shore height can be directly compared. Maltseva et al. (2021) adopted such a design to assess metabolomic differences in *Littorina*, finding that the extent of proteomic divergence between upper and lower shore populations of *L. saxatilis* closely mirrored interspecific differences between two sympatric congeners. A comparable study utilising the TDT approach could provide insights into the extent to which intraspecific divergence in thermal sensitivity may reflect interspecific differences across shore height.

Furthermore, I demonstrated that subtle differences also exist in the thermal tolerance times of embryos of the two ecotypes. Although these differences are less pronounced than those of adults, this finding is nonetheless significant. It suggests there is an underlying developmental basis to differences between adults and embryos, and that these differences could be heritable and not solely due to physiological plasticity. Several previous studies have demonstrated metabolomic and physiological differences between *L. saxatilis* adults living at different shore heights, indicating upper shore populations display substantial biochemical adjustments to cope with greater physiological stress (Sokolova and Pörtner 2001, 2003; Maltseva et al. 2021). Notably, using dynamic assays with a ramping rate of $12^{\circ}\text{C h}^{-1}$, Sokolova et al. (2000) found a similar magnitude of difference ($\sim 1^{\circ}\text{C}$) in upper lethal limits to that observed in my thesis between upper and lower shore populations of *L. saxatilis* from the White Sea and North Sea. However, a limitation of these studies is that they utilised

populations where vertically-segregated ecotypes are not present and the level of genetic intermixing across shore height is unknown, making it difficult to exclude the effect of physiological plasticity (but see Grahame et al. 1995 for a counter-example). A promising avenue for future work is to identify differences in thermal tolerance between adult individuals across different shore heights (including both populations where ecotypes are present and those where they are not), and then to track differences in their offspring. This would enable us to better understand the heritability and genetic basis of such differences, as well as whether differences are dependent upon the presence of ecotypes.

5.3 Thermal adaptation across latitude in *L. saxatilis*

In Chapter 3 and 4 I compared thermal performance and upper tolerance limits using four upper-shore populations of *L. saxatilis* from across a wide range of thermal environments spanning 23° of latitude. These ranged from the southernmost population from Cabo Silleiro, Galicia (the same population as the RB ecotype used in Chapter 2), two populations from the UK (St Ninian's cave and Mount Batten) and a northern population from just below the Arctic Circle (Skagaströnd, Iceland). Although genetic data was not collected during my thesis, phylogeographic studies suggest that genetic differences between the four populations roughly mirror their degree of geographic separation, with the Iceland and Galician populations both being particularly distinct and the two UK populations sharing greater similarity to each other (Doellman et al. 2011; Panova et al. 2011). Comparisons across latitude in intertidal organisms are often complicated by site-specific differences in temperature regimes, which may lead to "mosaic" patterns of local adaptation and obscure

latitudinal trends (Helmuth and Hofmann 2001; Helmuth et al. 2006; Kuo and Sanford 2009). Unfortunately, *in-situ* temperature data was not available from each site used in my thesis. However, temperature data extracted from climate databases (Fig. A1) suggests that the four sites experience substantial differences in climatic regimes, although it should be noted that these data are likely to underestimate maximum *in-situ* habitat temperatures (Seabra et al. 2015). Based upon these genetic and climatic differences, I had predicted that the four populations of *L. saxatilis*, would exhibit substantial differences in thermal tolerance limits. Surprisingly, my results across both chapters indicate that the degree of latitudinal variation in upper tolerance limits between the four populations studied is, at best, *modest*, although, as explored in Chapter 4, the exact interpretation of such a trend is highly dependent on methodology. In Chapter 3 I found that both CT_{max} (cardiac flatline) and T_{opt} increased with latitude under dynamic ramping in air, a counter-gradient trend driven by higher cardiac activity in the northern populations due to metabolic cold adaptation. Under ramping in water (Chapter 4), this counter-gradient trend disappeared, at least for CT_{max}. By contrast, use of the static TDT method in Chapter 4 indicated a negative latitudinal trend in CT_{max} for heat coma, with the Cabo Silleiro population possessing a CT_{max} value 1.3°C higher than the Skagaströnd population (reducing to 1°C higher under a simulated dynamic ramp).

My findings are broadly in line with those of previous studies which have assessed the thermal tolerance of *Littorina saxatilis* across latitude, in general also finding latitudinal differences in upper thermal limits to be slight or non-existent. Sokolova et al. (2000) reported comparable differences in thermal tolerance to those of my study between two populations of *L. saxatilis* from the North Sea and White Sea (12° latitudinal

difference), finding that upper lethal limits differed by approximately 1°C between the two populations. By contrast, Sorte et al. (2011) compared the survival of three *L. saxatilis* populations from across a latitudinal gradient of ~2.4° along the Northwest Atlantic coast in response to 1 h of exposure at 40°C, finding that it was actually highest in the northernmost (Northern Maine) population, although this difference disappeared following three weeks of acclimation at 17°C. Clarke et al. (2000) used ramping methodology to compare the heat coma temperatures of 21 populations of *L. saxatilis*, along with several congeners, across 6° of latitude within the British Isles. They found that while interpopulation differences were substantial, there was no systematic variation across latitude, suggesting that differences were largely driven by site-specific effects.

Although upper thermal limits provided little evidence of differences in thermal physiology across latitude, substantial differences were observed in the thermal performance of cardiac activity, as revealed under dynamic ramping (Chapters 3 and 4), revealing important insights into physiological adaptation in *Littorina saxatilis*. Increased cardiac activity at higher latitudes was observed in snails acclimated to 10 and 15°C, consistent with metabolic cold adaptation and physiological compensation across latitude in northern populations of this species. This increased cardiac activity was, in turn, linked to greater cardiac performance throughout the dynamic ramping experiments including P_{\max} . In Chapter 3, I argued that this represents a maladaptive response to increasing temperatures, as elevated heart rates are energetically costly to maintain and are often seen in individuals which have failed to acclimate to high temperatures (Havird et al. 2020). My argument that this response is indicative of a lack of physiological “preparedness” for exposure to high temperature in cold-

acclimated northern populations is reinforced by data from Chapter 4, where I compared the effect of different ramp rates on cardiac activity in 15°C acclimated individuals across latitude. I found that the maximum heart rate reached during warming was higher at the faster rate of change (12°C h⁻¹) compared to the slower rate (6°C h⁻¹). This occurred to a disproportionate extent in the northernmost Skagaströnd population, with the increased ramping rate having much the same effect as acclimation to 10°C compared to 15°C in Chapter 3. The fact that these responses were similar indicates that the Skagaströnd population possesses greater sensitivity of cardiac activity to changes in temperature. Interestingly, following acclimation to 20°C the Skagaströnd population displayed similar cardiac responses to the other populations. This indicates that metabolic cold adaptation can be reversed following acclimation to warmer temperatures and demonstrates substantial physiological plasticity in this population. In addition, T_{breadth} increased with latitude, consistent with the Climate Variability Hypothesis, and was linked to a trend of decreasing Q_{10} of heart rate across latitude. Indeed, the latitudinal trend of Q_{10} was remarkably consistent across both ramping media and changes in ramping rate (Chapter 4). This may suggest that Q_{10} is under strong selection across latitude, while the fact that the latitudinal trend disappeared following acclimation at 20°C suggests that it is also associated with metabolic cold adaptation (Hochachka and Somero 2002; Angilletta 2009).

Drawing together these findings, a broad picture emerges of the nature of thermal adaptation across latitude in *L. saxatilis*. Namely, it appears that the species displays little variation in upper tolerance limits, instead relying on extensive physiological compensation, as well as elevated physiological plasticity in northern populations, to cope with differences in temperature regimes across latitude. This is consistent with

research indicating that upper tolerance limits evolve slowly relative to other aspects of physiology (Kellermann et al. 2012; Hoffmann et al. 2013) and are evolutionarily conserved at lower taxonomic levels (Calosi et al. 2010; Faria et al. 2017). As noted in my introduction, there is limited evidence for latitudinal differences in upper thermal limits from within-species comparisons, particularly in intertidal species (Lee and Boulding 2010; Logan et al. 2012; Gaitán-Espitia et al. 2017). Although much of this evidence has previously come from species with planktonic larval dispersal, the fact that I observed the same pattern in a direct-developing species with limited dispersal may suggest that the extent for intraspecific adaptation of upper thermal limits across latitude is generally low, regardless of developmental mode.

The comparatively limited intraspecific variation in upper tolerance limits in *L. saxatilis* may also be closely linked to its broad distribution and eurythermal nature. Based upon the Climate Variability hypothesis, highly variable temperate environments favour species with broad tolerance windows (Deutsch et al. 2008; Tewksbury et al. 2008; Clusella-Trullas et al. 2011; Verberk et al. 2016a). This appears to be particularly true in terrestrial (Deutsch et al. 2008; Tewksbury et al. 2008; Sunday et al. 2011) and by extension upper intertidal habitats (Stillman and Somero 2000; Compton et al. 2007; Dong et al. 2021), where upper thermal limits vary relatively little across latitude due to high seasonal temperature variability at high latitudes. In addition, because physiological flexibility facilitates survival in a wider diversity of thermal environments, widely distributed species tend to possess wider thermal tolerance windows and higher upper limits than those of narrowly distributed species (Spicer and Gaston 1999; Calosi et al. 2008, 2010; Bozinovic et al. 2011). For example, Tepolt and Somero (2014), found that the widely distributed crab species *Carcinus maenas* displayed

higher thermal tolerance limits than related species with smaller overlapping ranges, as well as a high degree of acclimatory capacity. These conditions likely facilitated its recent invasive range expansion onto the North American coastline. Given its similarly wide range across the temperate north Atlantic and excellent ability to colonise new habitats (Reid 1996), the physiological characteristics of *L. saxatilis* I observe in this thesis appear consistent with those of a eurythermal species (Verberk et al. 2016a; Maltseva et al. 2021). In other words, *L. saxatilis* possesses broad tolerance windows across its entire range, but also the capacity for metabolic adaptation in response to climatic variation.

The fact that I found limited evidence of adjustment of upper thermal limits across latitude may also indicate a substantial role of behavioural thermoregulation, especially in southern populations. The role of behaviour as a means for ectotherms to regulate body temperature in lieu of biochemical adaptation has long been established in the literature. Indeed, in 1944 Cowles and Bogert observed that many species of desert lizard are only able to survive midday temperatures in their native habitat through burrowing, leading to the phenomenon of behavioural mitigation in lieu of physiological adaptation being known as the “Bogert effect” among some authors (Sunday et al. 2014; Gunderson and Stillman 2015). Given that many terrestrial organisms already experience habitat temperatures exceeding their thermal safety margins, behavioural temperature regulation is likely to be a necessity in many ectotherms (Sunday et al. 2014), including intertidal gastropods (Dong et al. 2017). Behavioural mitigation of temperature stress in intertidal gastropods is well described and widespread (McMahon 1990; Miller and Denny 2011; Chapperon et al. 2017; Monaco et al. 2017; Dong et al. 2021). For instance, shell standing behaviour may

reduce heat absorption from the rock face (Chapperon et al. 2017; Miller and Dowd 2017; Seuront et al. 2018), and has been shown variously to reduce body temperature by 1-2°C (Chapperon et al. 2017) or as much as 2-4°C (Miller and Denny 2011) relative to the substrate. Similarly, evaporative cooling may be used to mitigate against short-term thermal stress, although potentially risky over longer time periods due to the risk of desiccation (McMahon 1990). Indeed, water retention ability appears greater in low-latitude and high shore *L. saxatilis* (Sokolova et al. 2000), hinting that these populations may opt for reduced exploratory behaviour in order to retain water during prolonged heat stress. The existence of behavioural differences between the populations used in my thesis is tentatively supported by substantial differences in cardiac performance across latitude, observed across Chapters 3 and 4. These differences could potentially suggest that higher latitude populations remain more active and potentially continue feeding even at low tide, while lower latitude populations may reduce activity levels in order to mitigate against high temperatures (Sokolova et al. 2000). Miller and Denny (2011) modelled how the effects of thermoregulatory behaviour in Littorinids may mitigate against thermal stress at different locations along the Californian coastline. They reported that, while at more northern sites shell standing behaviour is unnecessary to prevent snails from exceeding their upper critical limits, at warmer locations such behaviour may be critical in ensuring survival. Given that behavioural thermoregulation may circumvent the need for other forms of thermal adaptation across latitude (Gunderson and Stillman 2015), understanding how behaviour differs between populations across latitude may be a crucial “missing piece of the puzzle” in our understanding of thermal adaptation in intertidal species. To the best of my knowledge, no study has explicitly tested whether the extent of thermoregulatory behaviour varies between populations of the same

species living at different latitudes, at least in intertidal species, and it is critical that research is undertaken to fill this gap in our knowledge.

5.4 Evaluation of methodological approaches used in the study

An important aspect of my thesis was the use of two novel methodological approaches which have previously been used to only a limited extent in comparative studies. The use of different methodologies in my first two data chapters is justified by their differing aims. For my comparison across shore height in Chapter 2, I adopted the TDT approach to explore whether there was a trade-off across shore height in the ability to withstand thermal stress intensity vs. duration. Trials in both adults and embryos were conducted in water, to facilitate a valid comparison across life-history stages (Truebano et al. 2018), as responses in embryos needed to be measured in an aqueous medium to avoid desiccation. In Chapter 3, I was interested in exploring how thermal tolerance varied across latitude, and how this related to metabolic performance and acclimation ability. I therefore utilised a TPC-based approach (Rezende and Bozinovic 2019; Bozinovic et al. 2020), allowing me to explore how different characteristics of thermal performance varied in tandem across latitude and acclimation temperatures. I combined this with dynamic ramping, which is a widely utilised and ecologically relevant technique in intertidal organisms (Dong et al. 2021), and conducted trials in air as this represents the conditions under which thermal stress is generally experienced. In Chapter 4 I compared between the two approaches, as well as the effect of measuring thermal tolerance in different media and at different ramping rates, to examine how methodological considerations may affect our ability to measure thermal tolerance across latitude. In the following section, I offer an appraisal

of the relative advantages and disadvantages of the two approaches and offer recommendations as to their future application in comparative studies.

5.4.1 Static TDT approach

My thesis represents the first experimental application of the TDT approach to compare across both latitude and shore height in an intertidal species, revealing important insights into the nature of thermal adaptation across both gradients. In Chapter 2, the approach was crucial in revealing differences in thermal tolerance and thermal sensitivity between upper and lower shore ecotypes of *L. saxatilis*, as well as building upon the previous work of Truebano et al (2018) and enabling me to explore how the magnitude of these differences changed during ontogeny. Similarly, the employment of the TDT approach in the latitudinal comparison presented in Chapter 4 revealed an underlying latitudinal trend of declining thermal tolerance with increasing latitude, which was obscured under dynamic ramping. This added weight to my conclusion that upper tolerance limits under dynamic ramping were chiefly the result of thermal effects, and highlights the utility of the static method in removing the influence of confounding rate effects associated with dynamic ramping (Rezende et al. 2014). Interestingly, while substantial differences in z values were observed across adults from different shore heights in Chapter 2, differences in z across latitude were less substantial. This may suggest that the trade-off between duration and intensity of heat exposure is a greater selective factor across intertidal gradients than across latitude in intertidal species. Indeed, such a conclusion seems intuitive, considering that the duration of thermal stress experienced at low tide increase predictably with

tidal height, but is more consistent across comparable shore heights at different latitudes.

At the same time, the lack of differences in z values indicated by my latitudinal comparison in Chapter 4 does not appear to match up with findings of dynamic ramps in Chapters 3 and 4, which conversely revealed substantial differences in the thermal sensitivity of cardiac activity across latitude. This discrepancy highlights a potential shortcoming of the TDT approach, namely that it can only assess differences in thermal sensitivity at extreme temperatures at which tolerance becomes time-limited, and therefore may be limited in its ability to detect differences in thermal sensitivity at lower temperatures. Admittedly, it is possible that differences in z values were reduced due to my use of only a single acclimation temperature in my TDT comparison, as a previous study has demonstrated that z values may be modified by acclimation to different temperatures (Castañeda et al. 2015). It is therefore possible that different patterns of variation in z across latitude would have become apparent had acclimation to higher or lower temperatures been used, as was the case with the dynamic approach in Chapter 3. Unfortunately, it was not possible to test this due to logistical constraints, namely the large number of individuals required to run static assays, but the potential for thermal acclimation to alter patterns of thermal sensitivity under the TDT approach needs to be considered in future studies.

The choice of endpoints used created complications when comparing the results of the TDT experiments with the other approaches I adopted. In adults, I utilised heat coma, which is considered a suitable indicator for when gastropods become physiologically compromised and thus unable to escape adverse conditions (Sandison 1967; Lutterschmidt and Hutchison 1997). However, I found this endpoint to be sublethal in

L. saxatilis. In addition to making it difficult to compare to tolerance limits measured in embryos in Chapter 2, it also makes CT_{max} estimates from the TDT approach difficult to compare with cardiac activity used elsewhere in the study. Although both cardiac failure and heat coma are ultimately linked to neuromuscular failure, cardiac activity is typically one of the last activities to cease under thermal stress and so is regarded as a good proxy for lethality (Dong et al. 2021). Indeed, CT_{max} values for cardiac flatline (at $\sim 42-44^{\circ}C$) were considerably higher than CT_{max} values based on heat coma (at $\sim 39-41^{\circ}C$), with the former closely matching lethal temperatures reported by Sokolova et al. (2000) of $\sim 45^{\circ}C$ both in air and water. Currently, knowledge of how discrepancies between different endpoints may affect the interpretation of thermal tolerance estimates generated using the TDT approach is lacking, limiting our ability to compare across datasets collected using different methodologies. In addition, it is currently unclear to what extent z-values, or indeed the relationship between CT_{max} and z, might be affected using different endpoints, as different levels of biological organisation may be compromised at different temperatures (Rezende et al. 2014; Rezende and Bozinovic 2019). Future work should therefore focus on assessing multiple endpoints using the static method and assessing how they vary systematically. Given the logistical challenges associated with assessing multiple endpoints within a single animal using the static approach (Rezende et al. 2014; Jørgensen et al. 2017, 2020), study species and endpoints would need to be chosen with care.

5.4.2 *Dynamic TPC approach*

In spite of recent critiques (Rezende et al. 2011, 2014; Castañeda et al. 2015), dynamic assays remain widely utilised in comparative studies using intertidal species (Moyen et

al. 2019; Dong et al. 2021). In particular, the assessment of cardiac breakpoints under dynamic ramping represents a widely used technique to assess thermal tolerance in molluscs (Dong et al. 2021). However, while I adopted cardiac breakpoints to compare thermal tolerance under ramping in air vs water in Chapter 4, in other parts of Chapter 4 and in Chapter 3 I instead used a TPC approach developed by Rezende and Bozinovic (2019) to assess cardiac activity during the thermal ramp. The approach I adopted is unusual, as TPCs are conventionally constructed by holding individuals at multiple different temperatures and measuring performance at each temperature (Schulte et al. 2011), as opposed to under a continuous ramp. However, this approach was crucial for my ability to interpret my results in Chapter 3. Using it, I revealed that an apparent counter-gradient variation in T_{opt} and CT_{max} was associated with metabolic cold adaptation via thermal effects on the overall shape of TPCs. This is a finding which would not have been identified using more conventional breakpoint-based approaches (e.g. Stenseng et al. 2005), highlighting the utility of approaches able to assess variation in thermal performance metrics in tandem (Rezende and Bozinovic 2019; Bozinovic et al. 2020).

At the same time, my use of dynamic ramping to explore TPC traits likely had a direct knock-on effect on the measured traits themselves. This was hinted at by my comparison across ramping rates in Chapter 4, in which both increased Q_{10} and an “overshoot” of maximum cardiac activity was observed under the faster ramping rate. Importantly, the “overshoot” was much greater in the high-latitude Skagströnd population and may, as noted earlier, be indicative of heightened metabolic stress under rapid heating in these animals. This, in turn appeared to influence T_{opt} , (Fig. 4.2) although admittedly, differences were non-significant. Although it is therefore difficult

to draw firm conclusions, the potential for dynamic ramping to have knock-on consequences for upper performance limits could hold important implications for the comparison of thermal tolerance in intertidal species. Crucially, this appears to arise as a result of “carryover” thermal effects on performance experienced as a consequence of rapidly increasing temperature (Cossins and Bowler 1987; Schulte et al. 2011), and so is a distinct phenomenon from the effect of ramping rate on critical thermal limits (Santos et al. 2011; Rezende et al. 2014), which by contrast are caused by differences in injury acclimation at different ramping rates (Jørgensen et al. 2019). Surprisingly, although numerous studies have tested the effect of ramping rate on CT_{max} (Terblanche et al. 2007; Santos et al. 2011; Allen et al. 2016), remarkably few have rigorously tested the effect of ramping rate on thermal performance thresholds (Dong et al. 2021). One notable exception, Moyen et al. (2019), found that faster ramping rates significantly increased upper limits of cardiac function (analogous to ABT), as well as maximum heart rate, in mussels from the upper shore, but not in those from the lower shore. The comparison of ramping rates in Chapter 4 indicated that similar differences might also exist between populations at different latitudes. Given that there is substantial variation in heating rates across both shore height and latitude (Helmuth and Hofmann 2001; Seabra et al. 2015; Miller and Dowd 2017), it seems likely that differences in the sensitivity of performance to ramping rate may be of adaptive significance (Moyen et al. 2019). Given the widespread use of acute ramping methodologies in intertidal ectotherm research (Dong et al. 2021), understanding variation in the thermal sensitivity of physiological performance traits to different heating rates, in addition to critical limits, clearly comprises a significant gap in our knowledge of the physiology of intertidal organisms which requires urgent investigation in future work.

5.5 Conclusion

As highlighted in my introduction, the discipline of comparative physiology saw a transition in the late 20th century away from being a largely descriptive field, towards one which aims to understand the general principles underpinning physiological adaptation. This trend is only likely to continue, thanks to the ongoing profusion of novel techniques and meta-analyses which are revealing broad-scale patterns in the evolution of thermal sensitivity across environmental gradients. At the same time, comparisons using individual taxa still serve a vital function in allowing us to test these predictions by applying them to species with unique characteristics. I believe that my thesis has amply demonstrated the continued utility of such an approach. In particular, I hope that my thesis has highlighted the value of *L. saxatilis* as an ideal vehicle for further enquiry, at the intraspecific level, into many of the adaptive patterns that have been well described at the interspecific level. In doing so, this will help to answer broader questions regarding local thermal adaptation in intertidal organisms. Importantly, my work has highlighted that studying how intertidal organisms cope with variation in both intensity and duration of thermal stress, and different rates of temperature change, is crucial to understanding patterns of thermal adaptation in the intertidal. Achieving this knowledge will provide us with a much greater ability to predict how these organisms are likely to be affected by the challenges of the future.

Appendix

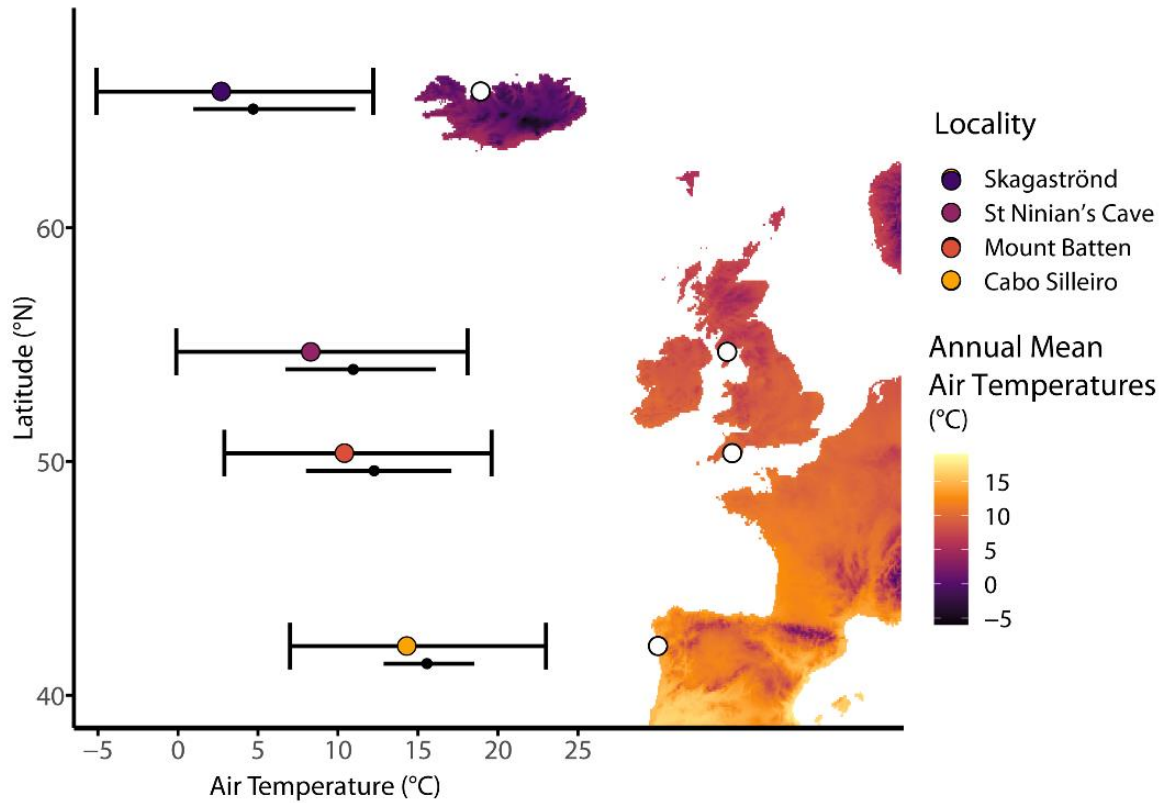


Figure A1: Air and sea surface temperatures (SST) for the four *Littorina saxatilis* localities used in latitudinal comparisons in Chapters 3-4. Coloured data points represent annual mean air temperatures for each locality, with bars indicating the temperature range bounded by the average maximum daily air temperatures for the warmest month of the year, and average minimum daily air temperatures for the coldest month of the year. Smaller points and bars represent the respective values for SSTs at each locality. White circles represent the geographic location of each locality, the map is coloured based on annual mean air temperatures. Note that the colour coding used to distinguish each locality does not exactly correspond to temperature/hue used in the key in the lower right. Air temperatures were obtained

from the WorldClim dataset spanning from 1970-2000

(<https://www.worldclim.org/>), while SSTs were derived from the Bio- ORACLE dataset spanning from 2002-2009 (Tyberghein et al. 2012).

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