Genetics and Thermal Biology of Littorinid Snails of the Genera *Afrolittorina*, *Echinolittorina* and *Littoraria* (Gastropoda: Littorinidae) from Temperate, Subtropical and Tropical Regions

By

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ABSTRACT

With the anticipated effects of climate change due to global warming, there is concern over how animals, especially ectotherms, will respond to or tolerate extreme and fluctuating environmental temperature stress. Littorinid snails are intertidal ectotherms that live high on the shore where they experience both extreme and variable conditions of temperature and desiccation stress, and are believed to live close to their tolerance limits. This study investigated the thermal biology of littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from temperate, subtropical and tropical regions in South Africa and Brunei Darussalam using thermal tolerance, heart function, and proteome approaches. The effects of conditions, such as rate of change in temperature, acclimation, heat shock, season and starvation were also tested. In addition, the evolutionary relationships and genetic diversity between and within the South African *Afrolittorina* spp. were investigated using mitochondrial and nuclear markers.

Genetic results confirmed that these are two distinct species, with the brown to black *A. knysnaensis* predominant in the cool-temperate region of South Africa and the pale blue-grey *A. africana* in the subtropical region. There was low genetic variation and differentiation within each species, suggesting high gene flow among populations as a result of the effects of ocean currents on the dispersal of their planktotrophic larvae.

Tests using exposure to high temperatures revealed differences in the thermal tolerances, heart performance and protein profiles of species from different latitudes, regions and zones on the shore. Thermal tolerance conformed to expectations, with clear, statistically significant trends from high tolerance in subtropical species to lower tolerance in temperate species. However, for *Afrolittorina* spp., there were no significant differences in the thermal tolerances of conspecifics from different regions, though there was a significant difference in thermal tolerance between juveniles and adults. Overall, adults of all species showed higher thermal tolerances than juveniles. Although lethal temperatures for these species were higher in summer than winter, laboratory acclimation had no effect on heat coma temperatures.

All species showed some regulation of heart rate, with a degree of independence of heart rate from temperature across mid-range temperatures. The tropical species showed quick induction and good regulation of heart rate followed by the subtropical and temperate species, which displayed mixed responses including regulation, partial regulation and lack of regulation. Overall, tropical *Echinolittorina* spp. showed good regulation, while the subtropical *E. natalensis* and *Littoraria glabrata* exhibited a mixture of partial regulation and regulation. The subtropical/temperate *Afrolittorina* spp. showed high individual variability, some animals exhibiting regulation, while others did not. These effects seem to be largely phylogenetically determined as there were no differences in the heart rate responses of *Afrolittorina* spp. from different regions.

The temperatures at which heart rate became independent of temperature (thermoneutral zone) were within the range experienced under natural conditions. In addition, there were differences in Arrhenius breakpoint and endpoint temperatures, showing a trend from higher in tropical animals to lower for temperate animals.

Conditions such as acclimation, heat shock and starvation had little or no effect on heart performance. However, a slow increase in temperature induced good regulation of heart rate with noticeable shifts of breakpoints and endpoints for *Afrolittorina* spp.

Lastly, there were differences in the proteome responses between and within *Afrolittorina* spp. as a function of species, size and treatment. Although both large and small *A. knysnaensis* had a greater number of protein spots in their proteome than *A. africana* (though the difference was not significant), the later showed significantly higher differential expression of certain proteins following heat stress. In addition, juveniles of both species displayed greater numbers of protein spots in their proteome than adults.

The results indicate a difference in the physiological and biochemical responses (i.e. adaptations) of these snails to temperature, and this seems to relate to differences in biogeography, phylogeny, species identity and ecology. The ability to regulate heart rate is phylogenetically determined, while thresholds and lethal limits correspond to biogeography and species ecology. The proteome seems to correspond to species ecology. The results also indicate that these littorinids can tolerate high temperature stress and in this respect they are

well suited to life in the intertidal zones or habitats where temperature and other stresses or conditions are extreme and can change abruptly. However, the limited ability of these snails to acclimate to different temperatures suggests that they are already living close to their tolerance limits with small safety margins or narrow thermal windows and so may be vulnerable to small rises in substratum temperature and/or solar radiation.

DEDICATION

This thesis is dedicated to my sister, Mrs **Muhanganei Edith Nesane**, for showing everlasting patience and support throughout my studies from undergraduates to the end of this thesis. Without her, I would have not made it to this far.

DECLARATION AND AUTHOURITY OF ACCESSS

I, **Tshifhiwa Given Matumba**, hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or academic institution, and to the best of my knowledge contains no paraphrase or copy of material previously published or written by other persons, except where due reference is made in the text of the thesis. The views and opinions expressed herein are solely those of the author and do not reflect the views of the university.

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CHAPTER 1: General Introduction

1.1. Organisation

The thesis is divided into six chapters. Chapter One forms a general introduction, and focuses on the study genera and species. Brief descriptions of study areas and conditions are provided here. Chapter Two investigates the phylogenetic relationships of the two closely related *Afrolittorina* spp., *A. africana* and *A. knysnaensis*, and the genetic diversity within these species. Chapter Three investigates the heat tolerance of littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from temperate and subtropical regions. Chapter Four investigates and compares the temperature related heart function of aestivating littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from temperate, subtropical and tropical regions. Chapter Five examines the total protein (proteome) response to heat stress of *Afrolittorina* spp. from a warm temperate region. Chapter Six concludes the thesis with a general discussion and synthesis.

1.2. Temperature and climate change

1.2.1. Environmental temperature and its effects

Temperature is one of the most important environmental factors (e.g. desiccation) that affects the physiology and behaviour, and consequently the distribution and abundance of organisms, including intertidal ectotherms (see Huey and Stevenson, 1979; Huey and Kingsolver, 1989; Huey, 1991; Somero, 1995; 2002; 2005; 2010; Segnini de Bravo *et al.*, 1998; Chan *et al.*, 2006; etc). The effects of temperature on ectotherms have been shown to occur at all levels of organisation from the population to the cellular level (see Sommer *et al.*, 1997; Hickey and Singer, 2004; Allan *et al.*, 2006; Kassahn *et al.*, 2009; Rais *et al.*, 2010). Consequently, temperature affects all aspects of ectotherm biology, from ability to feed and reproduce to the

structural and functional integrity of the biochemical machinery (see Bhaud *et al*., 1995; Burnaford, 2004; Pörtner *et al*., 2006; Silvestre *et al*., 2012). This is because temperature affects biological and physiological processes such as metabolism, growth and reproduction (see Pincebourde *et al*., 2008; Broitman *et al*., 2009; Harley *et al*., 2009; Iftikar *et al*., 2010; Tepler *et al*., 2011), which in turn affect performance and fitness.

Environmental temperature affects performance and fitness through its effect on body temperature (see Cornelius, 1972; Huey and Kingsolver, 1989, 1993; Angilletta Jr. *et al.*, 2002; Feder and Walser, 2005; Martin and Huey, 2008; etc). Essentially, this is because physiological and biochemical performance increases with body temperature until it declines above optimum or near lethal temperatures (see Huey and Berrigan, 2001; Peck *et al*., 2007; Pincebourde *et al*., 2008; Pörtner, 2010; Tattersall *et al*., 2012). Temperature determines physiological processes by limiting reaction (biochemical) rates, which are temperature dependent (see Menge and Sutherland, 1987; Sinclair *et al*., 2006; Kordas *et al*., 2011; Wernberg *et al*., 2011; etc). Since biochemical and physiological processes affect survival, growth and reproduction; environmental temperature determines when and where animals, particularly ectotherms, can survive and thrive (see Menge and Sutherland, 1987; Tomanek and Helmuth, 2002; Helmuth *et al*., 2002; 2010a, b; 2011; Madeira *et al*., 2012b; etc).

As a result, variation in temperature explains much of the spatial and temporal variability in the distributions and abundances of species around the world (see Charles *et al.*, 1992; Warwick and Turk, 2002; Harley and Lopez, 2003; Pincebourde *et al*., 2008; Lima *et al*., 2011; etc). Environmental temperatures are especially important for intertidal animals such as littorinid snails that live under extremely harsh conditions, where they experience both extreme and variable conditions, often living closer to their tolerance limits than species that are confined to purely marine or purely terrestrial environments. In addition, intertidal animals often respond rapidly to environmental changes, and so have been used to study the impact of climate (environmental) change on animals.

Intertidal environments are strongly affected by both atmospheric and oceanic changes, with conditions changing between marine during high tide and terrestrial during low tides, so that

animals face both extreme temperatures and abrupt changes in temperature and desiccation amongst other abiotic stresses that occur during the tidal cycle (see Reese, 1969; Vermeij, 1972; Harley, 2003; Harley and Helmuth, 2003; Jost and Helmuth, 2007; Gracey *et al*., 2008; etc). Although they have evolved from marine ancestors, intertidal animals must regularly contend with terrestrial conditions during low tide (see Hofmann and Somero, 1995; Stillman and Somero, 1996; Tomanek and Helmuth, 2002; Lima *et al*., 2007; Caddy-Retalic *et al.*, 2011). In addition, the timing and duration of exposure at low tide is likely to have a critical effect on temperature and desiccation extremes at a particular site (see Barnes *et al*., 1963; Shick *et al*., 1988; Martin, 1995; Helmuth *et al.*, 2002; 2011; Mislan *et al*., 2009; etc).

Intertidal environments are often characterized by high variability of physical factors such as temperature, desiccation, salinity, oxygen, solar radiation, wind and wave action amongst others (see Barnes *et al*., 1963; Vernberg, 1969; McMahon and Wilson, 1981; Shumway, 1983; Martin, 1995; Denny and Wethey, 2001; Tepler *et al*., 2011; etc). There are also regular cycles of tides, seasonal patterns of heat and cold, environmental changes due to storms, and extreme weather conditions such as heavy rains (see Vernberg, 1969; Underwood and McFadyen, 1983; Mouritsen and Poulin, 2002; Morritt *et al.*, 2007; Wethey *et al.*, 2011). Particularly critical are short-term (i.e. tidal or daily) and long-term (i.e. seasonal) changes in temperature (see Vernberg, 1969; Spaargaren and Achituv, 1977; Clarke and Crame, 1992; Hofmann and Somero, 1995; Stillman and Tagmount, 2009; Rais *et al.*, 2010). For example, temperature in the intertidal may change between 10-20°C within minutes or hours as the tide fluctuates (see Todd and Dehnel, 1960; Burggren and McMahon, 1981; Wilbur and Hilbish, 1989; Muñoz *et al.*, 2005; Finke *et al.*, 2009). In addition, there are also stresses caused by interactions, including predation and competition, with other animals (see Wethey, 1983; 1984; 2002; Hall *et al*., 1992; Chapman, 2000; Harley and Lopez, 2003; Rochette *et al.*, 2003) which can be modified by physical factors (see Vermeij, 1972; Menge and Olson, 1990; Dahlhoff *et al*., 2001; Yamane and Gilman, 2009; Kordas *et al*., 2011; etc).

Fluctuations and extremes of temperature are critical to intertidal ectotherms whose body temperatures are in equilibrium with those of the environment (see Sommer *et al.*, 1997; Boutilier, 2001; Mora and Ospina, 2001; Mislan *et al*., 2009; Helmuth *et al*., 2010a; 2011). This is particularly problematic during low tides on hot summer days when air temperatures

can reach as high as 50-55°C in the tropics (Lewis, 1963; Garrity, 1984; Williams and Morritt, 1995; Marshall and McQuaid, 2010; Cartwright and Williams, 2012) and 33-45°C in subtropical and temperate regions (pers. obs.; Morley *et al*., 2009); but see Whiteley *et al*. (1997) for temperatures as high as 50°C in the temperate regions. In addition, microhabitats within a shore may differ in thermal stress over small scales (see Helmuth, 1998; 1999; Sinclair *et al*., 2006; Mislan *et al*., 2009; Judge *et al*., 2011). As a result, the body temperatures of intertidal animals such as littorinid snails can be 10-20°C above that of sea surface temperature during low tide periods on hot summer days (see Garrity, 1984; Tomanek and Somero, 1999; Dahlhoff *et al*., 2001; Rais *et al.*, 2010; Caddy-Retalic *et al.*, 2011). For example, Judge *et al*. (2011) found the body temperature of the supralittoral snail *Cenchritis muricatus* exhibited daily fluctuations of more than 20°C and regularly exceeded 46°C.

Also important is the fact that body temperatures of animals or individuals from different regions, shore levels, microhabitats, etc. vary during the tidal cycle (see Helmuth, 1998; 2002; Helmuth *et al*., 2002; 2006a, b; Gilman *et al*., 2006; Broitman *et al*., 2009; Szathmary *et al*., 2009; Chapperon and Seuront, 2011a, b; etc). For example, Dahlhoff *et al*. (2001) found that the body temperature (which mirrored that of air) of the whelk *Nucella ostrina* from a Strawberry Hill population in Oregon was higher than for a Boiler Bay population, kilometres away. In addition, individuals from wave-protected shores had higher body temperatures than those from wave-exposed shores. Fitzhenry *et al*. (2004) found higher body temperatures for mussels of *Mytilus californianus* from wave-protected shore than for wave-exposed shore, which they interpreted as a result of the cooling effect of wave splash on wave exposed shore.

On the other hand, Helmuth and Hofmann (2001) found that individuals of *M. californianus* on horizontal, upward-facing substrata experienced temperatures 10°C higher than those on vertical, north-facing slopes located a few centimetres away. Seabra *et al*. (2011) found that sun-exposed robolimpets of the genus *Patella* routinely reached higher temperatures than their counterparts attached to north-facing shaded surfaces during low tide. In addition, the differences between sunny and shaded robolimpets were consistently larger than the variability associated with season and shore level. Sokolova *et al.* (2000c) found the body temperature of *Littorina saxatilis* individuals from mid and low shores differed by 10°C during low tide.

In addition, animals living in close proximity may experience and/or display different body temperatures due to differences in body size and morphology as well as behaviour (see Helmuth, 1998; 2002; Fitzhenry *et al*., 2004; Jost and Helmuth, 2007; Denny *et al*., 2011; etc). In summary, during aerial exposure, body temperature of intertidal animals is driven by multiple, interacting climatic factors such as substratum and air temperature, wind speed, cloud cover, solar radiation, relative humidity as well as physical factors such as substratum slope, orientation, type, colour and size, and is further affected by organism size, shape, mass and colour as well as behaviour (see Vermeij, 1971; Etter, 1988; Helmuth, 1998; Pincebourde *et al*., 2008; Finke *et al*., 2009; Gedan *et al*., 2011; Miller and Denny, 2011; etc).

1.2.2. Climate change and its effects

With the anticipated effects of climate change due to global warming (whether caused by natural variability or anthropogenically induced), there is concern over how ectotherms that have limited independence from environmental temperatures will respond to or tolerate extreme and fluctuating environmental conditions (see Sommer *et al.*, 1997; Fitzhenry *et al*., 2004; Harley *et al*., 2005; Parmesan, 2007; Helmuth *et al*., 2010a, b; 2011; etc). This is because climate and weather (which are frequently modified by multiple nonclimatic factors such as tidal cycle) directly control the distribution and other aspects of species, populations, community and ecosystems (see Wethey, 2002; Leemans and Eickhout, 2004; Poulin and Mouritsen, 2006; Jentsch *et al*., 2007; Terblanche *et al*., 2007; Mislan *et al*., 2009).

Although climate change is an old phenomenon (see Clarke and Crame, 1992; Crowley and Kim, 1999; Crowley, 2000; Shindell *et al*., 2001; Brierley and Kingsford, 2009), human activities such as burning of fossil fuels as well as urbanisation and land use activities such as deforestation and desertification are causing a rise in atmospheric greenhouse gases (e.g. carbon dioxide, methane, nitrous oxide, ozone, chlorofluorocarbons, etc) resulting in global climate change, often called "global warming" (see Partridge, 1993; Vitousek, 1994; Feely *et al*., 2001; Thuiller, 2007; Tambrian, 2012). The build-up of concentrations of greenhouse gases in the atmosphere (which leads to the „Enhanced Greenhouse Effect") affects the heat- or energy-exchange balance between the Earth"s systems (continents, oceans, atmosphere,

cryosphere and space), thereby inducing global warming (see Trenberth and Solomon, 1994; Cox *et al*., 2000; Karl and Trenberth, 2003; Miller, 2006). In addition, some of the excess greenhouse gases (e.g. CO_2) are absorbed by the oceans, with the result of decrease in ocean pH, also known as "ocean acidification" (see Billings *et al*., 1982; Feely *et al*., 2004; 2009; Barnett *et al*., 2005; Caldeira and Wickett, 2005; Doney *et al*., 2009; etc).

The mean global air temperature has increased by 0.2 to 1.0°C over the last one hundred years and is expected to increase by 1.5 to 7.0°C in the next fifty to one hundred years (see Levitus *et al*., 2000; 2001; McCarty, 2001; Angilletta Jr., 2009; Caddy-Retalic *et al.*, 2011). On the other hand, the mean global sea surface temperature (SST) has also increased by 0.57°C (Hulme and Jenkins, 1998; Levitus *et al*., 2000; 2001; 2005; Brierley and Kingsford, 2009; Caddy-Retalic *et al.*, 2011) and will continue to increase by between 1.4 and 3.9°C. The magnitude of global warming is predicted to vary among regions as a result of differences in ocean circulation patterns and other processes which contribute to regional and temporal changes in climate (see Partridge, 1993; Leemans and Eickhout, 2004; Barnett *et al*., 2005; Jentsch *et al*., 2007; Heller and Zavaleta, 2009; Xie *et al*., 2010). Most temperate regions and higher latitudes are expected to experience a greater magnitude of warming than the tropics and lower latitudes (see Oviatt, 2004; Williams *et al*., 2008; Helmuth *et al*., 2010a; Kordas *et al*., 2011; Wernberg *et al*., 2011). However, regions nearer to the equator and the poles experience enhanced and/or faster warming compared to those in the subtropics and temperate regions (Liu *et al*., 2005; Xie *et al*., 2010; Nguyen *et al*., 2011). In addition, aquatic systems such as coastal waters, estuaries and internal seas are areas that are expected to experience the strongest impacts of global warming (see Thompson *et al*., 2002; Lozano *et al*., 2004; Thuiller, 2007; Provan and Maggs, 2012).

These temperature increases will be paralleled by a rise in the frequency and magnitude of thermal fluctuations and extreme (hot and cold) events (Sommer *et al.*, 1997; Stenseth *et al*., 2002; Lima *et al.*, 2006; Tebaldi *et al*., 2006; Lannig *et al*., 2010; Wethey *et al.*, 2011; etc), more frequent and/or intense storms and coastal upwellings (Bakun, 1990; Lozano *et al*., 2004; Harley *et al*., 2006; McGregor *et al*., 2007), changes in precipitation patterns (Karl and Trenberth, 2003; Poulin and Mouritsen, 2006; Thuiller, 2007), changes in ocean circulation patterns and/or the distribution of water masses (Trenberth *et al*., 1994; Rahmstorf, 2002;

Macdonald *et al*., 2005; Böning *et al*., 2008), change in ocean water oxygen and salinity levels (Macdonald *et al*., 2005; Harley *et al*., 2006; Piñeiro *et al*., 2010), sea level rise and coastal flooding (Karl and Trenberth, 2003; Riegl, 2003; Omann *et al*., 2009), ultraviolet (UV) or solar radiation rise (Lean *et al*., 1995; Coelho *et al*., 2000; Richier *et al*., 2008), shifts in climate zones (Thompson *et al*., 2002; Brierley and Kingsford, 2009; Omann *et al*., 2009) and ocean acidification.

The frequency and intensity of extreme events will have greater impacts, and so may be more threatening than the rise in mean temperatures (see Clarke, 1993a; Bijlsma and Loeschcke, 2005; Williams *et al*., 2008; Stillman and Tagmount, 2009; Morley *et al*., 2009; Lagos *et al*., 2011). This is because unusually high temperatures occurring during the daytime in summer months are/or have been associated with mass mortalities in marine animals and plants (see Tsuchiya, 1983; Williams and Morritt, 1995; Helmuth, 2002; Riegl, 2003; Chan *et al*., 2006; Harley, 2008; Bergmann *et al*., 2010; etc). Therefore, animals must not only adapt to changing mean temperature ranges, but also to extreme events as well as other factors or conditions, and their interactions (see Stenseth *et al*., 2002; Jentsch *et al*., 2007; Menge *et al*., 2008; Mislan *et al*., 2009; Wethey *et al*., 2011).

Increasing temperatures and extreme events will have different (positive and negative) effects, altering species distributions, abundances and community composition, and this may be especially problematic for intertidal organisms as they live in harsh, fluctuating environments, and temperature gradients generally correlate with species distributions and abundances (Charles *et al.*, 1992; Warwick and Turk, 2002; Brierley and Kingsford, 2009; Miller and Denney, 2011; Nguyen *et al*., 2011). There are already signs of the effects of climate change on polar, temperate and tropical species with shifts (contraction or expansion) of distribution ranges as well as local extinctions (Barry *et al*., 1992; Southward *et al*., 1995; McCarty, 2001; Hawkins *et al*., 2003; Parmesan and Yohe, 2003; Perry *et al*., 2005; Lima *et al*., 2006; 2007; Menge *et al*., 2008; Heller and Zavaleta, 2009; Hoegh-Guldberg and Bruno, 2010; etc). Densities of ectotherm populations are predicted to decrease exponentially with increasing body temperature due to thermal constraints (see Hall *et al*., 1992; Sebens, 2002; Pardo and Johnson, 2005; Chan *et al*., 2006; Muñoz *et al.*, 2008; Helmuth *et al*., 2010a). In addition, species invasions are expected to increase (Stachowicz *et al*., 2002; Thompson *et*

al., 2002; Occhipinti-Ambrogi, 2007; Thuiller, 2007; Sorte *et al*., 2010), and this will further threaten global biodiversity (see Bax *et al*., 2003; Occhipinti-Ambrogi and Savini, 2003; Molnar *et al*., 2008; Cheung *et al*., 2009; Teske *et al*., 2011b).

Increased temperatures will also have impacts on processes such as growth, reproduction and metabolism as well as species interactions and dispersal, which are strongly influenced by environmental temperature (see Bertness *et al*., 1999; Thomas *et al*., 2000; Stenseth *et al*., 2002; Brooker *et al*., 2007; Piñeiro *et al*., 2010; etc). Thus, the nature of physiological responses or adaptations to environmental temperatures will determine the biological fitness of individuals in a population, and in turn define its distribution (see Tomanek and Helmuth, 2002; Leemans and Eickhout, 2004; Helmuth *et al*., 2010a; 2011; Kordas *et al*., 2011; Wernberg *et al*., 2011).

Changes in air and sea surface temperatures as well as other climate change related scenarios, also called "secondary factors", and their effects on animals have been experienced and predicted for southern Africa including South Africa (see Shannon *et al*., 1992; 1998; Lutjeharms and Ruijter, 1994; Scott *et al*., 1995; Hulme *et al*., 2001; Roy *et al*., 2001; Reason *et al*., 2006; Shillington *et al*., 2006; Crawford *et al*., 2008; Rouault *et al*., 2009; etc). However, it must be noted that the effect of increasing temperatures or climate change on species range shifts and/or biodiversity will also depend on the impacts of biotic interactions (e.g. competition), dispersal and the rate of climate change as well as their interactions (see Warwick and Turk, 2002; Pearson and Dawson, 2003; Brooker *et al*., 2007; Menge *et al*., 2008; Pincebourde *et al*., 2008; 2012; Wernberg *et al*., 2011, etc).

1.3. Study Animals

This study explicitly investigates and compares the thermal biology as well as the genetics of temperate, subtropical and tropical littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* in order to understand how littorinids and other ectotherms will respond to global warming and climate change related scenarios.

1.3.1. Introduction to the Littorinidae family

Littorinids are marine gastropod snails (Phylum: Mollusca, Class: Gastropoda, Subclass: Prosobranchia, Order: Neotaenioglossa, Infraorder: Discopoda, Family: Littorinidae) (see Reid, 1989; 1996b; 2002; Bieler, 1992; Winnepenninckx *et al*., 1998a, b; Colgan *et al*., 2000; 2007; Backeljau *et al*., 2001). Together with limpets (Family: Patellidae, Siphonariidae, Acmaeidae, etc) and slugs (Family: Opisthobranchia), littorinids (Family: Littorinidae) are the most abundant group of molluscs (constituting 80% all together), followed by bivalves which constitute 15%, while the other five classes constitute only 5% all together (see Bieler, 1992; Winnepenninckx *et al*., 1998b; Colgan *et al*., 2000; 2007).

The family Littorinidae (Anon., 1834) consists of approximately 200 living species that are commonly found on the mangrove and rocky shores of polar, temperate and tropical regions where they occupy the littoral (shallow and high intertidal) zones (see Reid, 1989; 1990; 1996a, b; 2002; McQuaid, 1996a, b; Libertini *et al*., 2004; Reid and Williams, 2004; Sanpanich *et al*., 2004; etc). Consequently, littorinids are important grazers on the littoral zones feeding on a wide range of food (e.g. diatoms, bacteria, fungi, algae, lichens, etc), and as such important in structuring the littoral ecosystems (Norton *et al*., 1990; Williams, 1990; 1994; McQuaid, 1996a, b; Christensen, 1998; Saier, 2000; Kaehler and Froneman, 2002; etc).

Members of the family Littorinidae sometimes also called Littorinacae fall into three subfamilies namely: the Laevilitorininae, Lacuninae and Littorininae (Reid, 1986; 1989; 1996; 2002; McQuaid, 1996a; Reid and Williams, 2004; Reid *et al*., 2012). The first two subfamilies are found in temperate and polar regions where they occupy the low eulittoral zone and continental shelf, while members of the subfamily Littorininae are found in the high eulittoral zone and the eulittoral fringe in tropical, subtropical and temperate regions (see Reid, 1989; 1996a, b; 2002; 2007; McQuaid, 1996a; Williams *et al*. 2003; Reid and Williams, 2004; Williams and Reid, 2004; Reid *et al*., 2010; 2012). Snails of the family Littorinidae are also referred to as “periwinkles” or “winkles”; and the latter is more specific to the snails in the subfamily Littorininae (Reid, 1989; 1996a, b; 2002; Reid *et al*., 2012).

The subfamily Littorininae has approximately 152 species in several genera, *viz*. *Littorina*, *Littoraria*, *Austrolittorina*, *Afrolittorina*, *Nodilittorina*, *Echnolittorina*, *Tectarius*, *Mainwaringia*, etc. (see below; Reid, 1990; 1996a, b; 2002; Reid and Geller, 1997). Only five genera, *viz*. *Littorina*, *Littoraria*, *Afrolittorina*, *Nodilittorina* and *Echnolittorina*, inhabit the coastline of southern Africa (McQuaid and Scherman, 1988; McQuaid, 1992; Sinclair *et al*., 2004; d'Errico *et al*., 2008). The genus *Afrolittorina* includes four species (see Williams *et al*. 2003; Reid and Williams, 2004; Reid *et al*., 2012); while the genera *Echinolittorina* and *Littoraria* comprise approximately 50 and 39 species respectively (see Reid, 1986; 1989; 2007; Reid and Mark, 1999; Inness-Campbell *et al*., 2003; Williams and Reid, 2004; Torres *et al*., 2008; Reid *et al*., 2010), though not all occur in southern Africa.

1.3.2. Study species

Six littorinid species (see Fig. 1.1) of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* were used, namely: *A. knysnaensis* (Philippi, 1847), *A. africana* (Philippi, 1847), *E. natalensis* (Krauss in Philippi, 1847), *E. malaccana* (Philippi, 1847), *E. vidua* (Philippi, 1847), and *L. glabrata* (Philippi, 1847). These species show different distribution ranges and display clear patterns of vertical zonation as well as microhabitat use and aestivation (a mechanism which extends the time an animal can survive on stored energy) periods, which was expected to determine their adaptations or acclimation to different thermal regimes.

A. africana is found in the Southwest Indian Ocean (SIO) from near Cape Town to Natal in South Africa, southern Mozambique and southeastern Madagascar, whereas *A. knysnaensis* occurs from Walvis Bay in Namibia to the vicinity of Durban in South Africa (Hartnoll, 1976; Reid, 1996; Reid and Williams, 2004; d'Errico *et al*., 2008). *L. glabrata* occurs in the subtropics and tropics of the Indo West Pacific (IWP) including the eastern coasts of South Africa (Torres *et al*., 2008; Reid *et al.*, 2010); while *E. natalensis* is found in the SIO from the eastern coast of South Africa to Kenya, Madagascar and the Seychelles (Hartnoll, 1976; Williams and Reid, 2004; Reid, 2007).

E. malaccana occurs in the IWP including India, mainland coasts of Southeast Asia, Southern China, Taiwan, Philippines, Borneo and Sulawesi; while *E. vidua* has a wide distribution in the Central IWP, including Pakistan, India, Southeast Asia, Indonesia, tropical Australia, New Guinea and southern Japan (Williams and Reid, 2004; Reid, 2007).

In addition, these species display clear patterns of vertical zonation which were expected to determine their exposure and aestivation periods. *E. malaccana* inhabits the eulittoral fringe to the upper eulittoral zone (Williams and Reid, 2004; Reid, 2007), where it can aestivate for more than 60 days (see Marshall and McQuaid, 2010); this is also true for *E. vidua*, which extends from the lowermost eulittoral fringe into the upper eulittoral zone (Williams and Reid, 2004; Reid, 2007). *E. natalensis* is abundant in the eulittoral fringe and extends to the eulittoral zone (Williams and Reid, 2004; Reid, 2007), whereas *L. glabrata* inhabits the uppermost eulittoral fringe to the eulittoral zone (pers. obs.; Silva *et al*., 2013). However, while all these species tend to live very high on shore (littoral fringe to upper eulittoral zone), they can show differences in microhabitat use (pers. obs.). All species can be found on open rock or in more shaded spots including crevices, pits etc., but this is especially true for *L. glabrata*, which prefers shaded and humid microhabitats (pers. obs.).

The two *Afrolittorina* species dominate the upper shore, ranging from the upper-mid eulittoral zone to the lower eulittoral fringe and show broad overlap where both species co-occur, except on the southeast coast of South Africa where *A. africana* has a higher vertical limit (McQuaid and Scherman, 1988; McQuaid, 1992; Reid and Williams, 2004; d'Errico *et al*., 2008). Both species occur on exposed rock, but frequently group together in clusters during low tide and are often found at very high densities around the margins of shallow and temporary pools (pers. obs.). Although there is no information on the aestivation periods of the South African species, they can be expected to aestivate for 14 days or more during neap tides (see McQuaid and Scherman, 1988; Sinclair *et al*., 2004).

Figure 1.1. Study species. (A) *Afrolittorina africana*, (B) *A. knysnaensis*, (C) *Littoraria glabrata*, (D) *Echinolittorina natalensis*, (E) *E. vidua* (picture downloaded from the web: www.roboastra.com/brunsmoll1/brpr213.html) and (F) *E. malaccana* (picture courtesy of Gray Williams and David Marshall).

1.4. Description of study Areas

This study explicitly compares the thermal biology of temperate, subtropical and tropical species as well as the genetics of subtropical and temperate species (see above); and the study areas chosen are found in Brunei Darussalam and South Africa (see Fig. 1.2).

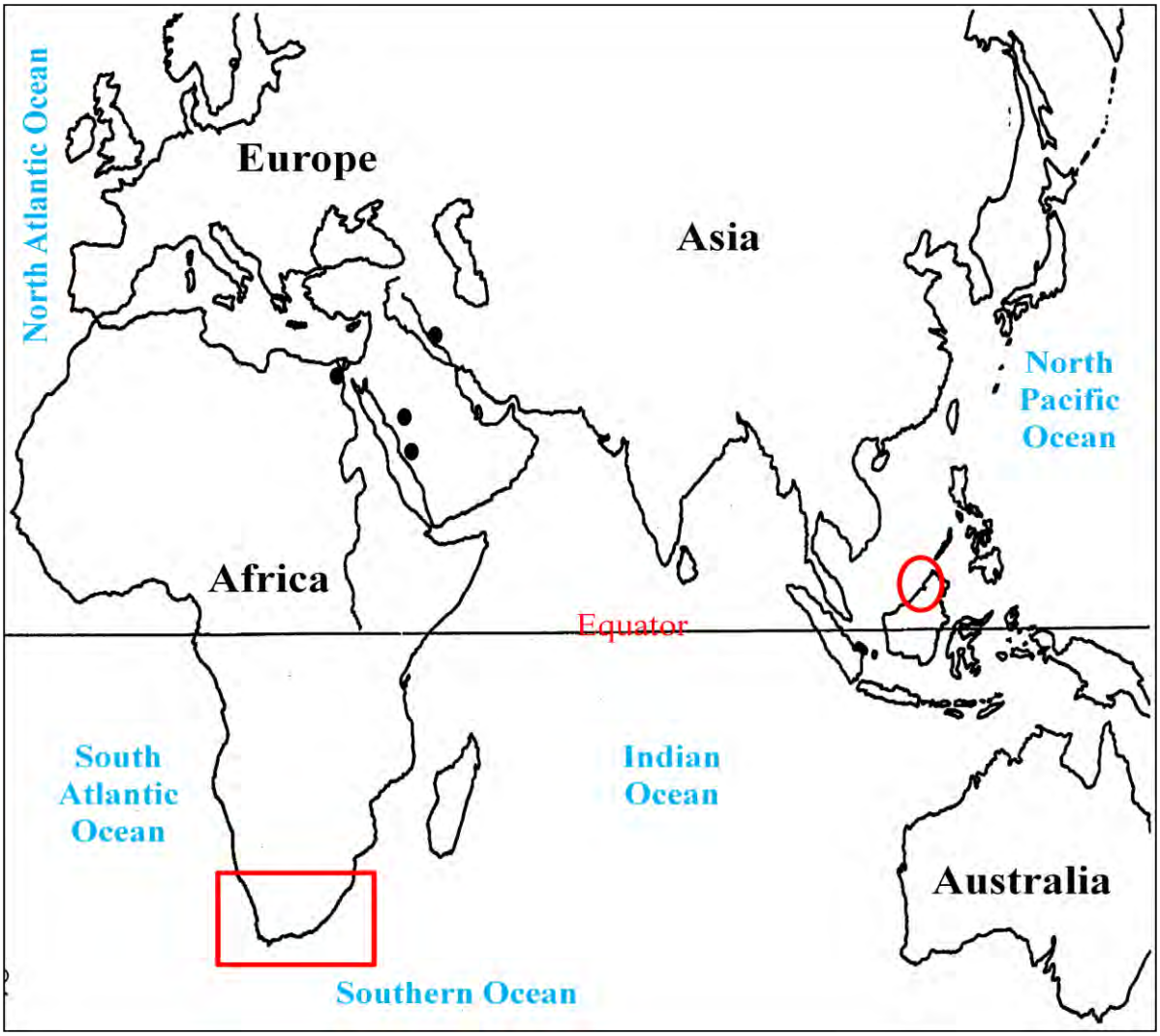

Figure 1.2. Map showing study areas. Red block and circle indicates South Africa and Brunei Darussalam respectively. Picture downloaded from the web: www.mapofworld.com.

1.4.1. Brunei Darussalam

One of the study areas is found in Brunei, Negara Brunei Darussalam, which is located between 4°N and 5.8°N and 114.6°E and 115.4°E on the north coast of the island of Borneo in Southeast Asia (see Fig. 1.2). Brunei Darussalam occupies a northern portion of the island of Borneo, with Malaysia and Indonesia on the southern parts of the island (see Curiale *et al*., 2000; Hiscott, 2001; Malik, 2011). As a result of its position, Brunei has a consistently warm-humid tropical climate (see Malik and Abdullah, 1996; Hiscott, 2001; Malik, 2011); and is influenced by two seasons, the northeast and southwest monsoon. The northeast monsoon (winter) is characterised by stronger and relatively constant dry winds, while the southwest monsoon (summer) is rain bearing (see Malik and Abdullah, 1996; Morton and Blackmore, 2001; Malik, 2011). Air temperatures are lowest during the winter (average maximum of 29-30°C), and are highest in the transition period before the onset of summer (average maximum of 33-35°C) (see Malik *et al*., 2011; Malik, 2011).

The Brunei coastline is linear with very few bays; and it stretches for about 161 km from Muara (5.8°N; 115.4°E) near Brunei Bay on the north to the vicinity of Kuala Belait (4N°; 114.6°E) on the south (see Fig. 1.3). It is dominated by a high profile sandy beach aligned in a southwest direction, and a complex estuarine, mangrove and mudflat zone within Brunei Bay in the northeast (see Marshall *et al*., 2010; Malik, 2011). Most of the rocky shore habitats of the coastline comprise artificial seawalls with few patches of natural rocky shores. In addition, the whole coastline is dominated by the South China Sea, which is the largest semi (marginal) closed sea in the western tropical Pacific Ocean, the circulation of which is driven by monsoonal winds resulting in variation (seasonal) in surface currents and SST (see Shaw and Chao, 1994; Wu *et al*., 1998; Kuo *et al*., 2000; Wang *et al*., 2006; 2008).

The surface (warm) currents move to the southwest (i.e. cyclonic) in winter and the northeast (i.e. anticyclonic) in summer with stable eddies (see Shaw and Chao, 1994; Hu *et al*., 2000; Liu *et al*., 2001; Morton and Blackmore, 2001; Shi *et al*., 2002). Sea surface temperatures in the South China Sea vary with relative lows (approximately 18-29°C) in March and December and highs (approximately 27-37°C) in June and September (see Chou, 1994; Chu

et al., 1997a, b; 1998a,b; Qu, 2001; Wang *et al*., 2008). The tides are generally less than 2 m except for about 2.3 m (reaching a maximum of 2.7m in Brunei Bay) in spring tides, with wider ranges occurring during storms (see Hiscott, 2001). In addition, wave heights can reach as high as 3 m during the northeast monsoon (see Chou, 1994; Hiscott, 2001).

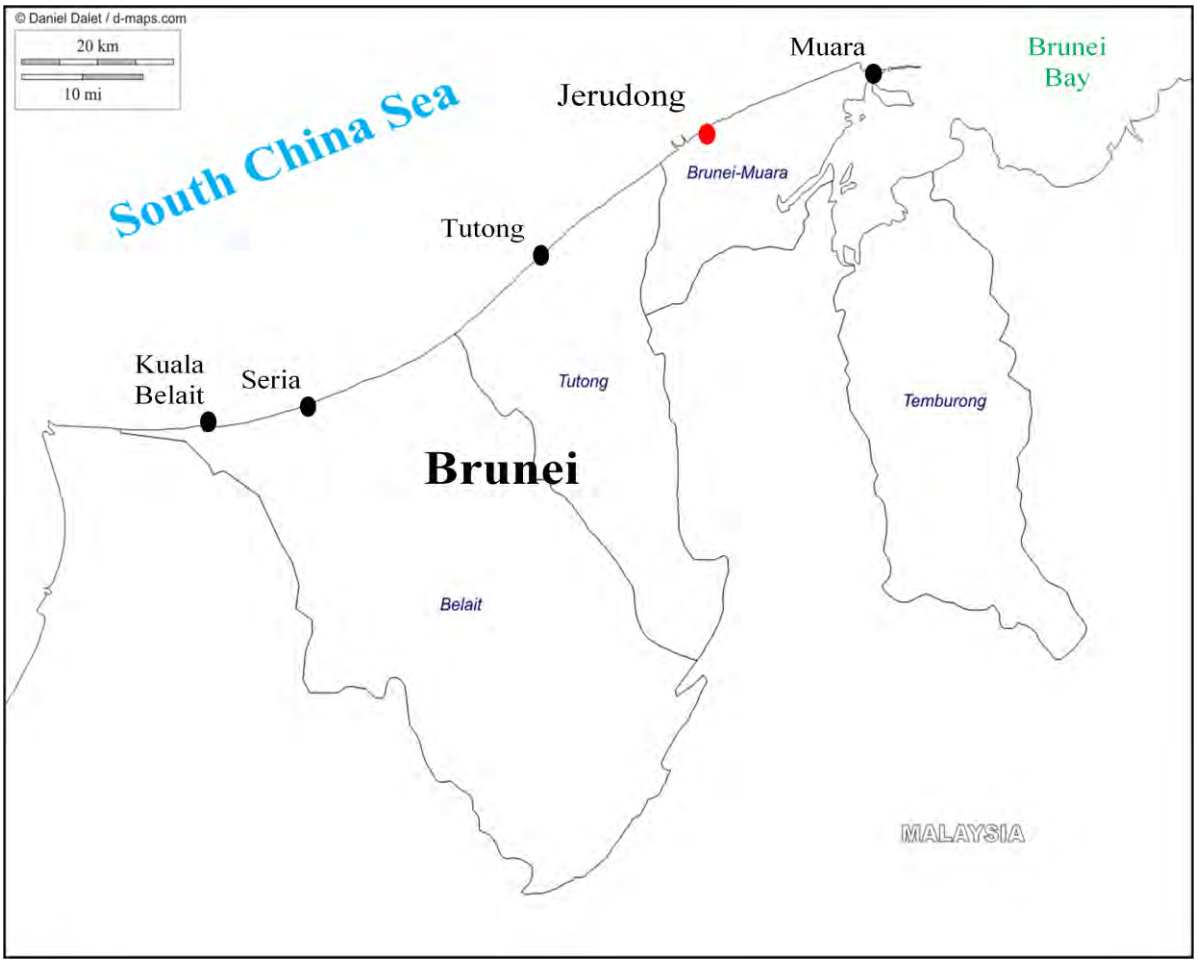

Figure 1.3. Map showing the Brunei coastline with study area, Jerudong, indicated by red dot. Picture downloaded from the web: wwww.mapofworld.com.

1.4.2. South Africa

The other study areas were found in the Republic of South Africa, which is located between 26.5°S and 28.4°S and 33.5°E and 16.3°W on the southern portion of Africa (see Fig. 1.2). South Africa (SA) is divided into four regions, namely; the east, south, southwest and west,

each of which experience different conditions, especially weather and climate as well as rainfall (see below). The west coast has a hyper-arid tropical climate with winter rainfall; the southwest has a Mediterranean climate with winter rainfall; the south coast has a warm climate and experiences varying rainfall regimes with some areas experiencing year round rainfall while the east coast has a moist warm-tropical climate characterised by summer rainfall (see Lutjeharms and Ruijter, 1994; 1996; Cowling *et al*., 1999; Cooper, 2001; Reason *et al*., 2002; Peter *et al*., 2003; Calf and Underhill, 2005). Air temperatures are diurnally and seasonally variable reaching 30-35°C in summer and falling to 3°C in the subtropical and to 0°C along the west and south coast during winter (see Kruger and Shongwe, 2004 and reference herein; Sinclair *et al*., 2004).

The South African coastline stretches for approximately 3000 km from Kosi Bay (26°54'S; 33°48'E) near the Moçambique border on the east coast to Alexander Bay (28°38'S; 16°27'W) at the Namibian border on the west coast (see Fig. 1.4). The coastline is almost linear with very few significant bays or inlets, and is dominated by long stretches of sandy beaches and sand dunes (constitute 1700 km) with some patches of rocky shores (constitute 1300 km) (see Marshall and McQuaid, 1993b; Ramsay, 1996; Hutchings *et al*., 2002; Ramsay and Cooper, 2002; Peter *et al*., 2003). In addition, the SA coastline is divided into three primary biogeographic provinces or regions: the cool temperate, warm temperate and subtropical regions (see Fig. 1.4; Emanuel *et al*., 1992; Pether, 1994; Bustamante and Branch, 1996; Turpie *et al.*, 2000; Maree *et al*., 2000; Harrison, 2002; 2004; etc). Each of these biogeographic provinces is characterised by its own unique environmental and oceanographic conditions as well as the topographic features (see below). Of more interest is the difference in environmental and oceanographic conditions (which also show variability) between and within the regions.

South Africa‟s marine environment is unique in that it is surrounded by three major oceans, the cool South Atlantic Ocean to the west, the very cold Southern Ocean to the south and the warm Indian Ocean to the east (see Fig. 1.5; Pether, 1994; Bustamante *et al*., 1995; Lutjeharms *et al*., 2001; Lucas and Griffiths, 2012). As a consequence, SA‟s marine environment is influenced by two current systems; the strong and intense southwards fast (~2 ms^{-1}) flowing warm (23-26°C) Agulhas current on the east and south coasts and the upwelled

northwards slow (0.25-0.50 ms^{-1}) flowing cold (~ 10°C) Benguela current on the west coast (see Fig. 1.5), which show variability (Darbyshire, 1963; 1964; Hutson, 1980; Lutjeharms and de Ruijter, 1996; Hutchings *et al*., 2002; Bryden *et al*., 2005; etc).

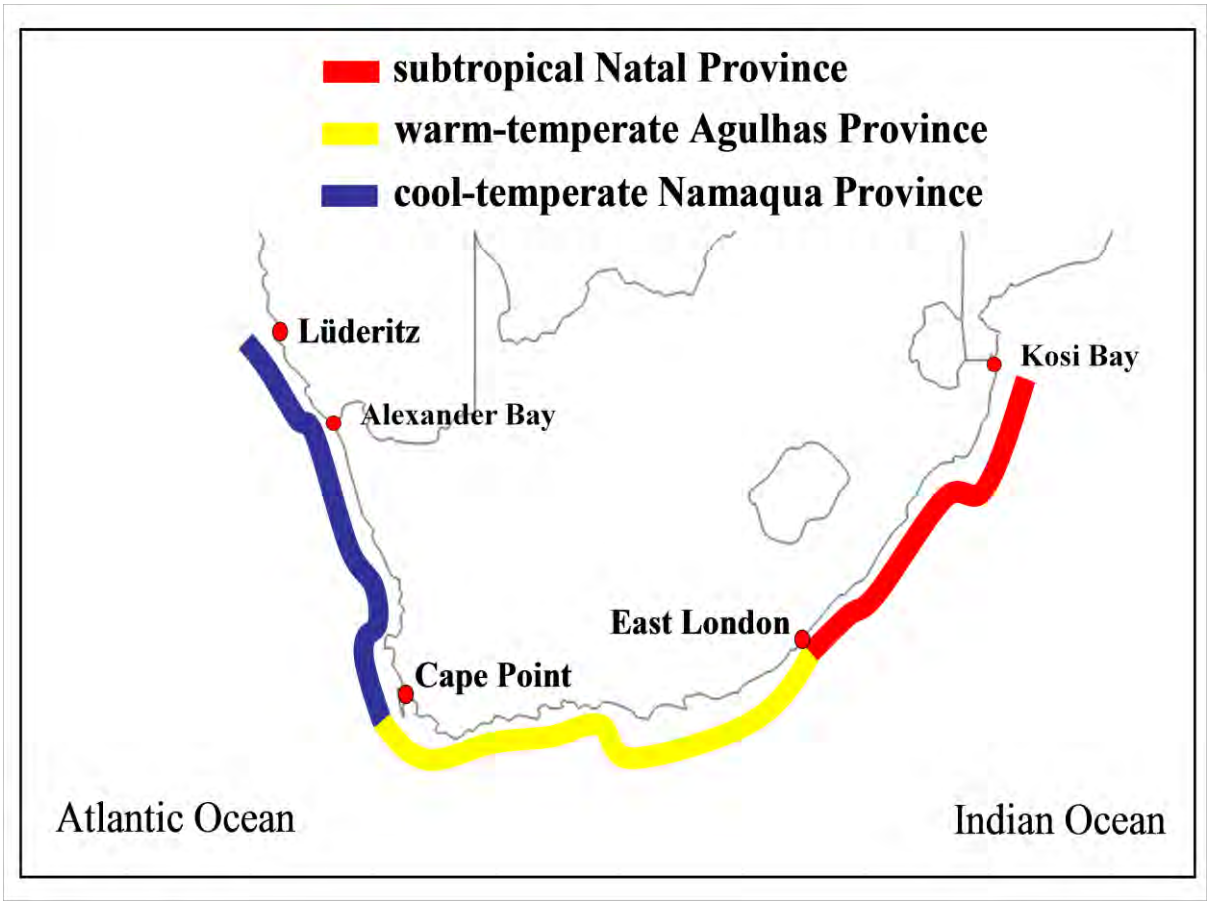

Figure 1.4. Map showing the South African coastline and zoo- or biogeographic provinces indicated by different colours. Picture courtesy of Christopher D McQuaid.

The Agulhas current carries warm waters from the tropics, resulting in SST of 20 to 22°C in winter and 22 to 27°C in summer in the subtropics (see Isaac, 1937; Darbyshire, 1964; Hutson, 1980; Harris and Cyrus, 1996; Harrison, 2004). On the south coast (the warm temperate region) where the Agulhas current diverges away from the coastline, SST ranges from 15 to 20°C in winter and 15 to 22°C in summer (see Isaac, 1937; McQuaid and Branch, 1984; Flores *et al*., 1999; Demarcq *et al*., 2003; Laudien *et al*., 2003; Harrison, 2004). On the other hand, the Benguela current brings cold waters from the Atlantic/Southern Oceans, resulting in SST of 10 to 15°C in winter and 10 to 19°C in summer in the cool temperate

region (see Isaac, 1937; Darbyshire, 1963; Roy *et al*., 2001; Laudien *et al*., 2003; Harrison, 2004). There are counter-currents, also called "occasional currents" which flow close inshore in the opposite direction to the main currents (see Isaac, 1937; Bustamante and Branch, 1996; Weeks *et al*., 1998; Thibault-Botha *et al*,. 2004; Luschi *et al*., 2006), which together with coastal upwellings result in variability in currents flow patterns and SST (see Pether, 1994; Lutjeharms *et al*., 2000; 2001).

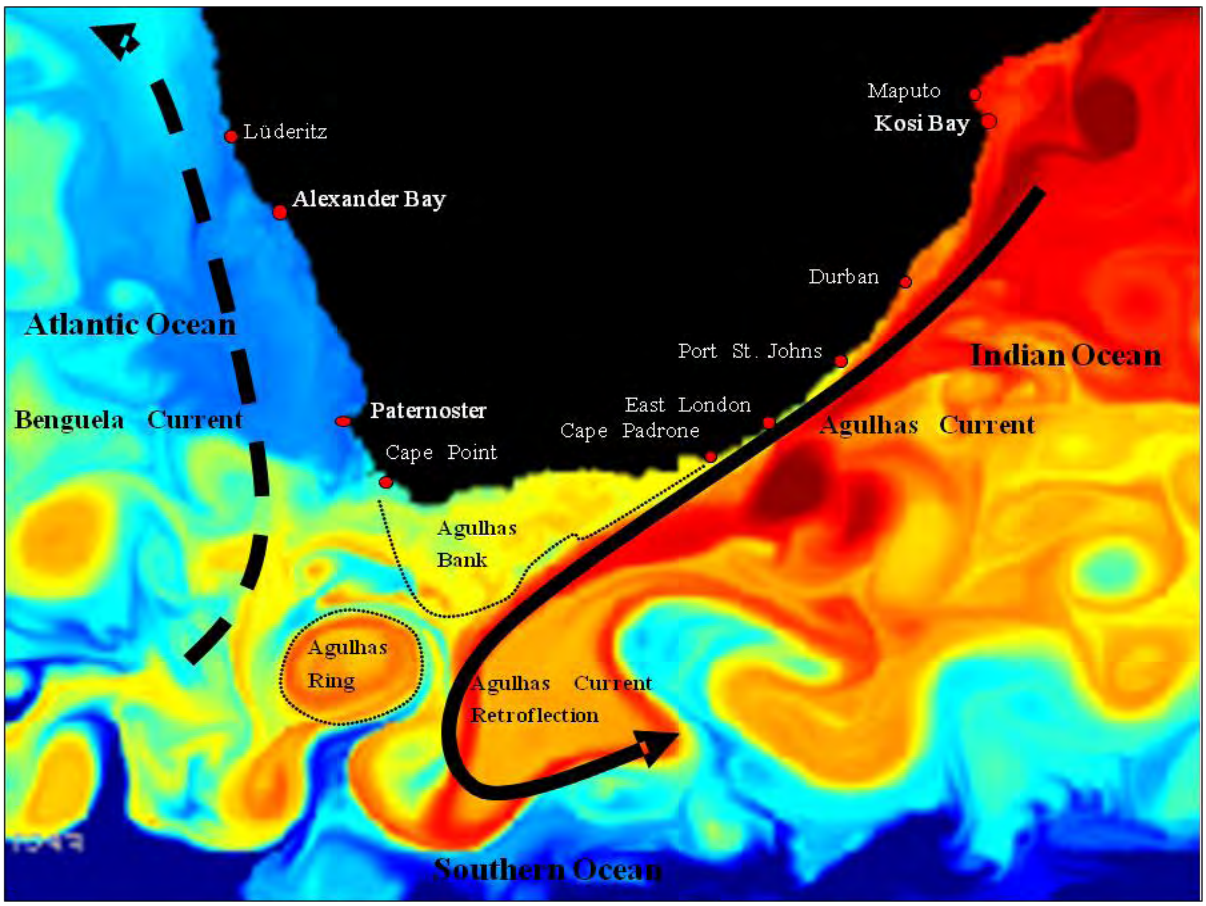

Figure 1.5. Satellite image of the South African coastline, sea surface temperature, two currents, Agulhas and Benguela currents and oceans around the South African coastline. Picture courtesy of Christopher D McQuaid.

In addition, SST and salinity (also oxygen and nutrients) show differences between and within regions, with a slight and/or irregular decrease from east to west along the coastline (see Isaac, 1937; Darbyshire, 1963; 1964; Hutson, 1980; Shannon *et al*., 1990; Harrison, 2004; Roberts, 2005). More important is the marked daily and seasonal variation in SST, with

greater variability in summer than winter (see Beckley, 1983; Shannon *et al*., 1988; Cohen *et al*., 1992; Schumann *et al*., 1995; Harrison and Whitfield, 2006; Reason *et al*., 2006). Tidal range is relatively small and varies little around the coast, with most areas experiencing a neap tidal range between 0.56 to 0.80 m (average 1 m) and a spring tidal range of 1.59 to 2.5 m (average 1.4 m) (see Cooper, 2001; Calf and Underhill, 2005; Ramsay and Cooper, 2002; Laudien *et al*., 2003). In addition, extreme wave heights (as high as 3.5-5.5 m) occur during unusual events such as strong stormy weather (see Cooper, 2001; Ramsay and Cooper, 2002; Calf and Underhill, 2005).

1.5. Background to the study

On rocky shores, there is a general perception that species‟ upper limits are set by abiotic factors and their lower limits by biotic interactions (see Wethey, 1984; Britton, 1992; Yamada and Boulding, 1996; Duncan *et al*., 1998; Harley and Helmuth, 2003; Judge *et al*., 2009; Miller *et al*., 2009; etc). The study species live on the upper shore (i.e. eulittoral fringes and zones), often where there are no other animals. Therefore, interspecific competition (e.g. for food and/or space) is unlikely to be important while there is little evidence of high levels of predation (see McQuaid, 1981a, b; 1985; 1992; Mak and Williams, 1999; Lee *et al*., 2009). But intraspecific competition might determine the distribution of these species (see McQuaid, 1981b; Mak and Williams, 1999; Lee and Kim, 2009; Lee *et al*., 2009; Stafford *et al*., 2012). Physiological (e.g. temperature and desiccation) stress however, is likely to be important in setting the distribution of these species on the shore (see McQuaid and Scherman, 1988; McQuaid, 1992; Sinclair *et al*., 2004; Lee and Kim, 2009; Marshall *et al*., 2010; 2011; etc). Of course, studies in the intertidal tend to emphasize a major role of physiological adaptations to temperature and desiccation stress (McMahon, 1990; Sokolova and Pörtner, 2001b; Horowitz, 2001; 2002; Tomanek and Helmuth, 2002; Miller and Denny, 2011).

To date, few studies have investigated how heat affects the physiology of South African littorinid snails (see Marshall unpub. data; McQuaid and Scherman, 1988; McQuaid, 1992). The present study investigates how heat stress affects the physiology of South African

littorinids, with the aim of drawing conclusions about the effects of temperature on other littorinids and other ectotherms. This study further investigates whether the two *Afrolittorina* spp. are distinct species as suggested by previous studies. Thus, the phylogeography of *Afrolittorina* spp. was determined. Measurement of phylogeography throughout the South Africa coastline would provide insight to the possible role of bioregions in phylogeography of other marine invertebrates, assuming that the phylogeography of *Afrolittorina* spp. will be similar to that of invertebrates with planktonic larvae.

1.6. Thesis overview

The main focus of this thesis was to investigate the phylogeography and thermal biology of the two closely related littorinid snails of the genus *Afrolittorina*, *A. africana* and *A. knysnaensis*, from subtropical and temperate regions of South Africa. However, littorinid snails of the genera *Echinolittorina* and *Littoraria* from subtropical (South Africa) and tropical (Brunei Darussalam) regions were included for other reasons. *E. natalensis* and *L. glabrata* were included because they occur together with *A. africana* in the subtropical region where they occupy higher levels on the shore (see Hartnoll, 1976; McQuaid, 1992; Sinclair *et al*., 2004; Sink *et al*., 2005; d'Errico *et al*., 2008). The tropical species *E. malaccana* and *E. vidua* were included because they are in the same genus as the subtropical *E. natalensis* (see Williams and Reid, 2004; Reid, 2007; Reid *et al*., 2012) offering the opportunity to compare the heat stress response of temperate *Afrolittorina* spp. to that of other littorinid snails from the same or different regions and shore heights.

It is assumed that there would be differences in temperature responses between species and sizes as different species and sizes are found at different latitudes, regions, shore levels and microhabitats. Thus, one might predict that *Afrolittorina* spp. would show similar responses to temperature that are different from those of subtropical and tropical species.

1.6.1. Aims and Objectives

The objectives of this study were as follows: First, to investigate the phylogenetic relationships between *A. africana* and *A. knysnaensis* and the diversity within members of these species using DNA sequence data. These data also tested if there is a genetic basis for the range of colour morphs found within the distributional ranges of the two species. Second, to investigate the tolerance of high temperatures of snails in the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from the temperate and subtropical regions of South Africa using heat coma (temperature at which cessation of activity occurs) and lethal limits (temperature at which 50% mortality of the population occurs). Third, to investigate the temperature-heart rate relationship of the two co-existing *Afrolittorina* spp., comparing this to that of the subtropical *E. natalensis* and *L. glabrata*, and the tropical *E. malaccana* and *E. vidua*. Fourth, a proteomic approach was used to analyse the protein profiles of *Afrolittorina* spp. from the warm temperate region of South Africa in order to compare their whole protein responses to temperature stress.

CHAPTER 2: Phylogeography of the two closely related *Afrolittorina* species, *Afrolittorina africana* and *A. knysnaensis*, from South Africa

2.1. Introduction

The term "phylogeography" was introduced by Avise *et al*. (1987) to explain the striking phylogenetic patterns observed after analysis of mitochondrial DNA (mtDNA), usually below "species" level. Thus, molecular analysis of mtDNA from coastal marine taxa had revealed intraspecific genealogies that were geographically coincident with each other and with biogeographic patterns (Avise *et al*., 1987; Avise, 1992, 1998; Hewitt, 2004). These commonalities suggested that intraspecific mtDNA phylogenies or phylogenetic groupings were shaped by common biogeographic barriers to gene flow associated with physical and biological factors, and possibly other factors (Avise, 1992, 1998; 2004; Burton, 1998).

Although mtDNA led the way into phylogeography and is the most commonly used marker in phylogeographic studies, nuclear DNA markers such as microsatellites and other markers are also used (see below; Féral, 2002; Hewitt, 2004; Panova *et al*., 2008; Pleines *et al*., 2009). The properties of mtDNA, including its non-recombining characteristics, maternal inheritance, reduced effective population size and rapid rate of evolution, make it one of the best makers to study phylogenetic relationships (Avise *et al*., 1987; Moritz *et al*., 1987; Hwang and Kim, 1999; Galtier *et al*., 2009, etc). However, a combination of mitochondrial and nuclear sequence data is emerging as an optional strategy for phylogeographic analysis, as the two genomes have the potential to validate historical inferences (see Bowen and Grant, 1997; Bermingham and Moritz, 1998; Hare, 2001; Williams *et al*., 2002; Kuo and Avise, 2005; Rubinoff and Holland, 2005; Beheregaray, 2008; Teske *et al*., 2009).

The field of phylogeography is concerned with the principles and/or processes that govern the geographical patterns of genetic or evolutionary lineages within and among closely related species or taxa (Avise *et al.*, 1987; Avise, 1992; 1998; 2004; Beheregaray, 2008; Teske *et al*.,

2009; 2011; Hickerson *et al*., 2010; etc). Thus, phylogeography deals with the spatial distribution of genetic lineages (i.e. genealogies), and processes that have shaped such genealogies or partitions. The spatial distribution of genetic lineages is under the control of historical (e.g. vicariance and dispersal) and contemporary (e.g. variation in climate and hydrological conditions) processes (Bernardi *et al*., 2003; Templeton, 2003; Rocha *et al*., 2007; Arbogast and Kenagy, 2008; Waters, 2008a, b; Pleines *et al*., 2009; etc). In summary, phylogeography can be regarded as a subdiscipline of biogeography that applies phylogenetic techniques to achieve a comprehensive understanding of how physical and biological factors have shaped the distribution of genetic lineages.

In addition, the field of phylogeography provides a powerful tool for identifying cryptic species and/or hybridizations that are difficult or sometimes impossible to distinguish or identify morphologically (Templeton, 2003; Beheregaray and Caccone, 2007; Teske and Beheregaray, 2009; Teske *et al.*, 2009; 2011c; Azuma *et al*., 2011). Phylogeography is also useful in management or conservation studies since it can be used to estimate population connectivity as well as levels and patterns of genetic diversity between and within populations (Féral, 2002; Hellberg *et al*., 2002; Palumbi, 2003; 2004; Rocha *et al*., 2007; von der Heyden, 2008; 2009; Ni *et al*., 2012; Provan and Maggs, 2012). Thus, the results of phylogeographic studies can be applied not only to answer questions of evolutionary significance, but can also have management and conservation applications.

Dispersal and vicariance are the two historical processes that are invoked to account for the origins of spatially disjunct genetic lineages (see Reid, 1990; Bowen and Grant, 1997; Bernardi *et al*., 2003; Waters and Roy, 2004a; Bilodeau *et al*., 2005; Waters, 2008a, b). Under a dispersal scenario, lineages come to occupy their present ranges through active or passive dispersal from one or more ancestral centres of origins (see Nelson, 1974; McDowall, 1978). On the other hand, under vicariance scenarios, lineages become separated when more or less continuous ranges of ancestral forms are split apart by natural events such as plate tectonics or the formation of land bridges (see Nelson, 1974; Rosen, 1978). However, dispersal is regarded as a central process affecting the distribution of genetic lineages or species (see Pole, 1994; Bernardi *et al*., 2003; Waters and Roy, 2004a; Waters, 2008a; Teske *et al*., 2009).

Recently, studies show that contemporary processes such as variation in oceanographic (e.g. currents and upwellings) and environmental (e.g. temperature and salinity gradients) conditions as well as topographical features (e.g. bays and habitats) can also account for the origins of spatially disjunct genetic lineages (Banks *et al*., 2007; Rocha *et al*., 2007; Waters, 2008b; Pelc *et al*., 2009; Teske *et al*., 2009; 2010; 2011a; Zardi *et al*., 2007; 2011; Dong *et al*., 2012). This shows that contemporary processes should be considered in the maintenance of genetic structure (i.e. diversity), perhaps more than historical processes from which diversity originated (Teske *et al*., 2011a). This means that the effects of historical and contemporary factors acting on or interacting with an organism‟s life history (see below), determine the phylogeographic patterns seen in marine organisms. Phylogeographic patterns in marine organisms can also result from anthropogenic activities, for example human-mediated translocation of fouling organisms (Waters and Roy, 2004a; Cunningham, 2008; Zhan *et al*., 2009; Panova *et al*., 2011; Teske *et al*., 2011a; Ni *et al*., 2012).

Several studies have investigated the phylogeographic patterns and/or genetic structure (i.e. variation) of marine animals, including coastal and estuarine invertebrates and fishes (see below). Generally, species with planktonic larvae are thought to show little or no evidence of genetic structure along their distribution ranges, while those with non-planktonic larva or direct developers show high genetic structure (Johannesson, 1988; Palumbi, 1994; 2003; Chambers *et al*., 1996; 1998; Bohonak, 1999; Dawson, 2001; Bernardi *et al*., 2003; Bowen *et al*., 2006; Rocha *et al*., 2007; Bell, 2008; Pelc *et al*., 2009; etc). This shows that the presence or absence of genetic variation is due to differences in dispersal potential of that particular species. Thus, the magnitude of genetic structure appears partially related to the life history (type and duration of larval development) pattern and dispersal capability of a particular species (Johannesson, 1988; Avise, 1992, Kyle and Boulding, 2000; Bowen *et al*., 2006; Rocha *et al*., 2007; Teske *et al*., 2007b; 2011a). As a result, the duration of the larval (pelagic) stage is often regarded as an indication of dispersal potential and related to population or genetic structure.

However, there is growing evidence that species with planktonic larva can show as much genetic structure as direct developers. This suggests that dispersal potential on its own does not determine genetic variation within marine species, and other factors also play a role. For

example, local oceanographic processes, active behaviour of larvae and spawning events that coincide with certain tides and current regimes are among the biological and physical factors that may influence larval dispersal and thus genetic structure (Johannesson, 1988; Palumbi, 2003; Waters and Roy, 2004b; Rivadeneira and Fernández, 2005; Rocha *et al*., 2007; Zardi *et al*., 2007b, e; Sherman *et al*., 2008; Ayre *et al*., 2009; Zhan *et al*., 2009; Cheang *et al*., 2012; Díaz- Ferguson *et al*., 2012; von der Heyden *et al*., 2013). For example, larval retention and self-recruitment may be higher than previously expected in marine animals with planktonic larva (Johannesson, 1988; Palumbi, 2003; Bowen *et al*., 2006; Andrade and Solferini, 2007; Small and Wares, 2010; Teske *et al*., 2007b; 2008; 2012).

For those species which show genetic structure, phylogeographic breaks often coincide with known biogeographic boundaries or limits, but this is not the case for all marine species studied to date since other species shows patterns which do not conform to known biogeographic barriers or bioregions (Burton, 1998; Irwin, 2002; Kou and Avise 2004; Bilodeau *et al*., 2005; York *et al*., 2008; Pelc *et al*., 2009). Studies on littorinids from different regions have also found phylogeographic structures and/or breaks that coincide with known or unknown boundaries (Knight and Ward, 1991; Gosling *et al*., 1998; Wilson and Gosling, 1998; Andrade *et al*., 2003; Reid *et al*., 2006; Andrade and Solferini, 2007; Waters *et al*., 2005; 2006; Van den Broeck *et al*. 2008; Lee and Boulding, 2007; 2009; Doellman *et al*., 2011; Panova *et al*., 2011; Díaz- Ferguson *et al*., 2012; etc).

The most recent phylogeographic studies along the South African coastline support this phenomenon. It is well known that there is genetic variation in marine species, including invertebrates, that seems to be influenced by life history (i.e. mode of dispersal) and the effect of oceanographic (e.g. currents and coastal upwelling) and environmental (e.g. temperature and salinity) conditions as well as topographical features (e.g. sand dunes and beaches). Thus, together with topographic features, environmental and oceanographic conditions have been found to have an influence on the genetic structure of various taxa found within the South African coastline (Teske *et al.*, 2006; 2007a, b; c; e; 2008; 2009; 2010; 2011a, c; Zardi *et al.*, 2007; 2011; Nicastro *et al.*, 2008; von der Heyden, 2007; 2008; 2009; 2010; etc). As such, the South African coastline offers an interesting area to study evolutionary relationships between and within closely related species.

In addition, most of such studies have shown that phylogeographic breaks (including limits) within and among taxa often coincide with known biogeographic boundaries (Ridgway *et al*., 1998; Evans *et al*., 2004; Teske *et al.*, 2006; 2007a, b, e; 2008; 2009; Zardi *et al.*, 2007), although there are some exceptions (Tolley *et al*., 2005; Matthee *et al*., 2007; Teske *et al*., 2007b; 2011a; Neethling *et al*., 2008; Mmonwa, 2009; 2013 unpub. data). This shows that the prevailing biogeographic boundaries do not affect the phylogeography of all marine invertebrates in a similar way (Teske *et al*., 2011a, b). For example, the deflection (which also show seasonal variability) of the Agulhas current as it approaches the Agulhas Bank creates a semi or incomplete permeable barrier (by deflecting the dispersing larvae offshore) to gene flow of some animals but not others (Teske *et al*., 2006; 2008; Zardi *et al*., 2007; Mmonwa, 2009 unpub. data). Biogeographic boundaries also act to maintain genetic breaks evoked by ancient climate changes (Pelc *et al*., 2009; Teske *et al*., 2011a). Thus, species which are found in more than one region show, or are expected to show, phylogeographic breaks (including limits) that coincide with known biogeographic boundaries.

Afrolittorina africana and *A. knysnaensis* are two closely related southern African species belonging to the new genus *Afrolittorina* within the family Littorinidae (Williams *et al.*, 2003; Reid and Williams, 2004; Reid *et al*., 2012). They are the most widespread, conspicuous and abundant littorinids on rocky shores along the southern African coast (see McQuaid, 1992). In South Africa, the pale blue-grey *Afrolittorina africana* is abundant in Kwazulu-Natal, in the eastern part of the country, while the brown to black *A. knysnaensis* ranges from Namibia to southern Kwazulu-Natal (Hughes, 1979; Grant and Lang, 1991; Reid and Williams, 2004; d'Errico *et al*., 2008). Thus, *A. africana* is predominant in the subtropical east coast region, while *A. knysnaensis* is abundant in the cool temperate west coast region.

These species, as well as individuals that are morphological intermediates (in colour pattern) occur together in the warm temperate south coast region (Hughes, 1979; McQuaid and Scherman, 1988; Reid and Williams, 2004; Sinclair *et al.*, 2004) where they even occupy the same microhabitats from the upper-mid eulittoral zone to the lower eulittoral fringe (pers. obs.; McQuaid, 1992; d'Errico *et al*., 2008). Although these species occur along the South Africa coastline, differences in colour pattern have been previously observed, and this has led

Hughes (1979) to suggest that they form a single species which differs in its morphological appearance along its distribution range. For example, certain specimens of *A. africana* had dark brown dashes or streaks (form Natal region) and pale brown flecks or spots (from Transkei region) superimposed on the typical pale blue-grey or bluish-white background (see Hughes, 1979; Reid and Williams, 2004). On the other hand, for *A. knysnaensis*, a specimen from Lüideritz had cream dashes, with the predominance of individuals throughout its range bearing pale blue upper margins to the whorls on a uniform dark brown to black background (see Hughes, 1979; Reid and Williams, 2004).

Of particular importance is the reproduction and development of these two species, which are expected to determine their geographic distribution and/or phylogeography. Both these species are predicted to have pelagic spawning and planktotrophic development based on: 1) the dimension of protoconch and large capsule gland of both species (see Reid, 1989; Reid and Williams, 2004), 2) the small (87 µm) egg size of *A. knysnaensis* (McQuaid, 1981) and 3) low genetic variation on a geographical scale in *A. knysnaensis* (Grant and Lang, 1991). In addition, these species seem to breed throughout the year with continuous recruitment followed by settlement of juveniles high on the shore (McQuaid, 1981). However, recruitment is erratic or inconsistent and depends on the currents.

This means that like most littorinid snails in the subfamily Littorininae (Reid, 1990; Kyle and Boulding, 2000; Kim *et al*., 2003; Williams *et al*., 2003; Reid *et al*., 2006; 2012; Lee and Boulding, 2007; 2009), these species are pelagic spawners with planktotrophic veliger larvae which could disperse widely with the help of ocean currents, resulting in high gene flow and preventing genetic discontinuity. Thus, one would expect little, if any, genetic variation among populations of *Afrolittorina* spp. provided that external forces act in the same way across their distribution ranges. In fact, several studies have shown that marine invertebrates with highly dispersive stages unexpectedly displayed high genetic variation which was linked to abiotic factors such as ancient oceanography and habitat availability (Teske *et al*., 2006, 2007b; 2011a; Zardi *et al*., 2007; Mmonwa, 2009 unpub. data). But, using life history of the organism to predict its genetic structure can be problematic, especially in a dynamic marine realm like the southern African coastline. This means that when conditions vary across the area in question, genetic variation may evolve as suggested, especially if adaptation occurs

(Gooch and Schopf, 1972; Nevo, 1978; Eanes, 1987; Fevolden and Garner, 1987; Johannesson and Tatarenkov, 1997; Laudien *et al*., 2003; Pigliucci *et al*., 2006).

Although the taxonomic status (i.e. phylogeny) of the two southern African *Afrolittorina* spp. is known (see Reid, 1989; 2002; Williams *et al.*, 2003; Reid and Williams, 2004), the phylogenetic (evolutionary) relationships and diversity between and within these species have not been investigated. Thus, there is no regional study that has looked at the evolutionary relationships and diversity of the *Afrolittorina* spp., and other littorinids found along the southern African coastline, including South Africa. The only local study was by Grant and Lang (1991) who found low genetic (allozyme) variation in *A. knysnaensis* on a geographical scale. Therefore, the present study is the first regional study to investigate the phylogenetic relationships and diversity of *A. africana* and *A. knysnaensis* from South Africa.

This study uses the mitochondrial cytochrome oxidase subunit I (COI) and nuclear ribosomal 28S rRNA markers to clarify the evolutionary relationships and genetic diversity between and within *Afrolittorina* spp. The phylogeny built on the basis of the DNA sequence data will provide information on whether each species is a distinct genetic entity as suggested by Williams *et al*. (2003). The data gathered here will also help to explain the phylogeographic patterns of distribution and diversity within and among members of *A. africana* and *A. knysnaensis* along the South Africa coastline. It is assumed that the phylogeography of *Afrolittorina* spp. will be similar to that of invertebrates with planktonic larvae. Furthermore, the data also tested if there was a genetic basis for the range of colour morphs found within the distributional ranges of these two species.

2.2. Materials and methods

2.2.1. Study species

Two closely related *Afrolittorina* spp., namely: *A. knysnaensis* (Philippi, 1847) and *A. africana* (Philippi, 1847) were used. See Chapter 1 for information on species distribution ranges and patterns of vertical zonation as well as microhabitat use and aestivation behaviour

2.2.2. Specimen collection and identification

Specimens belonging to *A. africana* and *A. knysnaensis* as well as morphological intermediates (in colour pattern; see above) were collected from 46 localities across species distribution ranges along the southern Africa coasts (see Table 2.1 and Fig. 2.1) from Ponta do Ouro (26°50'S; 32°53'E) in Moçambique in the east coast to Port Nolloth (29°15'S; 16°52'E), on the South African west coast.

During collection, specimens were sorted into species („white" for *A. africana* and „brown" to „black" for *A. knysnaensis*) or intermediates (all other varieties of colours) based on the colour of the shell. Specimens were collected in 70% or absolute (100%) ethanol as the fixative, except for samples which were collected and brought to the laboratory in the aestivation state and later kept in the fixative. Shells of individual snails were gently cracked-open to ensure rapid penetration of the fixative into the tissues. The fixative in all containers was changed after 10-14 days of collection or earlier if necessary, and for long term storage samples were stored in absolute ethanol.

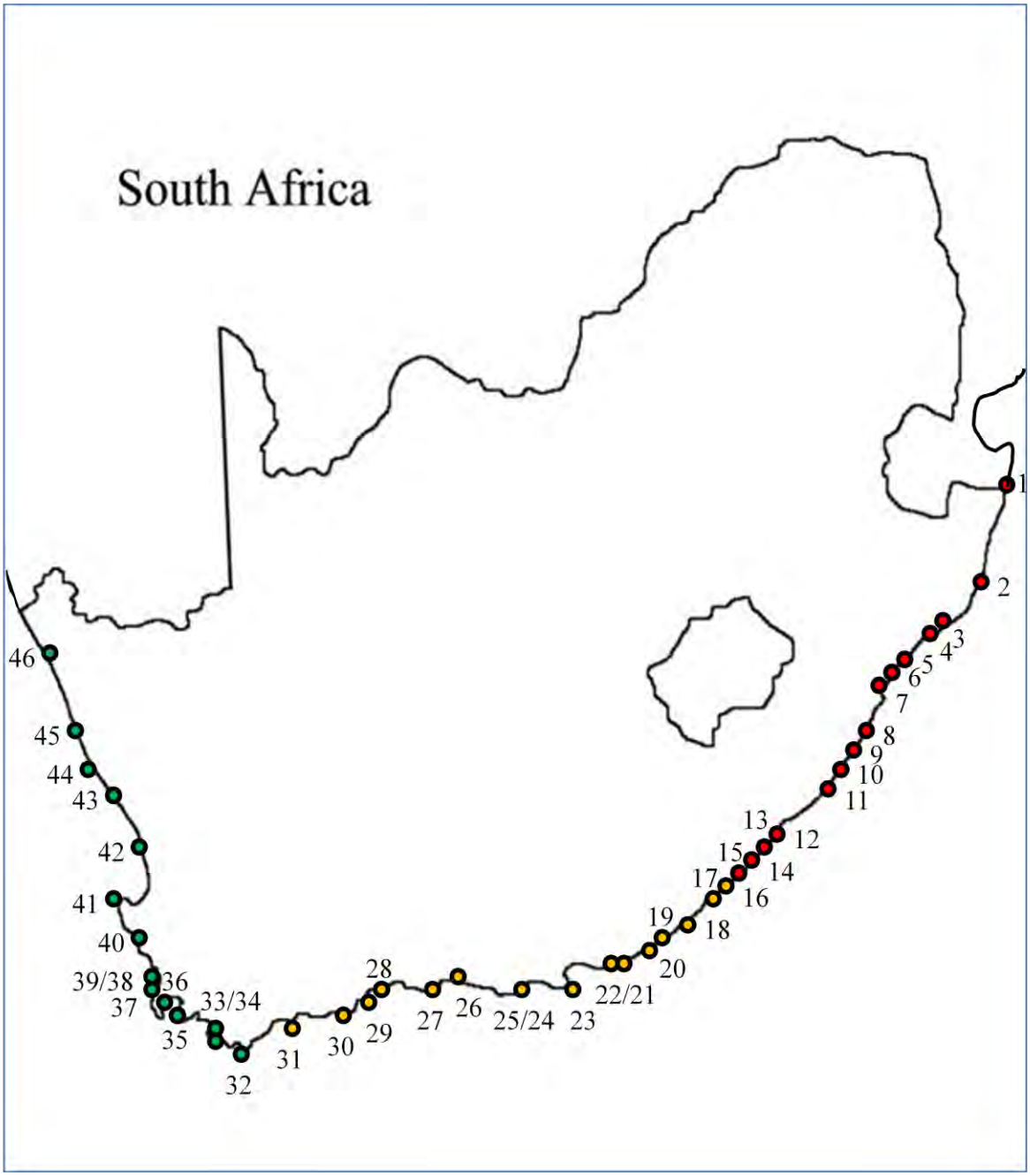

Figure 2.1. Map showing sampling sites (see Table 2.1 for the list of sites) of *Afrolittorina* species along the South African coastline. Different colours (red = subtropical, yellow = warm temperate, and green = cool temperate) indicate sampling sites in different biogeographic regions.

Table 2.1. Mitochondrial (mtCOI) and ribosomal (28S rRNA) sequences for *A. africana* and *A. knysnaensis* from three biogeographic regions and sampling sites within the regions. Empty cells are sites were species and/or sequences were not sampled or obtained.

Region	**Site (Abbreviation)**	**Number of samples per marker and species**			
		mtCOI		**28S rRNA**	
		A. africana	***A. knysnaensis***	***A. africana***	***A. knysnaensis***
Subtropical	1. Ponta do Ouro (PDO)	11			
	2. Mission Rocks (MR)	8		6	
	3. Lona Rocks (LR)	10		-	
	4. Zinkwazi (ZK)	10		5	
	5. Sheffield (BS)	20	2	15	
	6. Ballito (BA/BT)	5		4	
	7. Mhlanga (ML)	13		6	
	8. Park Rynie (PR)	4	3	10	
	9. Shelly Beach (SH)	7			
	10. Ramsgate (RG)	6	11	5	6
	11. Port Edward (PE)	15	3	9	2
	12. Port St. Johns (PJ)	5	9	6	6
	13. Hluleka (PE)	3	1	2	1
	14. Dwesa (DS)	11	5	8	

	15. Shixini (SX)	5	2		
Warm temperate	16. Haga-Haga (HH)	11	12	8	12
	17. Gonubei (GO/GU)	5	8	4	2
	18. Hamburg (HU/HM)	6	8	2	
	19. Fish river (FR)	7	4	9	
	20. Port Alfred (PA)	9	10	10	4
	21. Bushmans river (BR/BU)	7	9	3	5
	22. Cannon Rocks (CRB)	5	6	5	4
	23. Cape Recife (CR)	10	14	6	9
	24. St. Francis Bay (FB)	1			
	25. Jeffrey‟s Bay(JB)	3	10	1	3
	26. Tsitsikamma (TT)	-	5		2
	27. Plettenberg Bay (PL)	3	5		4
	28. Sedgefield (SE)	5	4	5	1
	29. Wilderness (WN)	4	11		2
	30. Harold‟s Bay (HB)		4		4
	31. Still Bay (SB)	1	8	6	5
Cool temperate	32. Cape Agulhas (CA)		8	4	
	33. Franskraal (FR)		9		1
	34. Pringles Bay (PB)		5		
	35. Rooi Els (RE)		8		2

	36. Muizenberg (MU/MZ)		3		
	37. Camps Bay (CB)		4		
	38. Bloubergstrand (BL)		3		
	39. Melkbosstrand (ML)		5		5
	40. Yzerfontein (YZ)		3		2
	41. Paternoster (PN)		9		5
	42. Lamberts Bay (LB)		8		5
	43. Strandfonteinpunt (SF)		12		2
	44. Groensreviersmond (GR)		8		6
	45. Hondeklipbaai (HN)		4		
	46. Port Nolloth (POB)		10		5

2.2.3. DNA extraction, amplification and sequencing

The procedure for DNA extraction, amplification (polymerase chain reaction; PCR) and sequencing followed that of Williams *et al*. (2003) with minor modifications (see below).

2.2.3.1. DNA extraction

Total genomic DNA was extracted from the whole specimens using CTAB buffer and chloro-phenol extraction method described by Doyle and Doyle (1987) with minor modifications. In brief, individual snails were taken from the fixative, gently cracked-open to remove whole tissues, and rinsed in double distilled water (ddH_2O) to remove excess ethanol and shells. Tissues were blot-dried on paper towel, placed in Eppendorf tubes containing 1mL of CTAB (consisting of 0.1M Tris-HCl pH 8.0, 1.4M NaCl, 0.002M EDTA, 2% CTAB, and 1% PVP) extraction buffer pre-heated at 60°C, ground with a plastic pestle, and later incubated overnight at 52°C with 20µl of Proteinase K.

The mixture (extraction buffer and tissues) were further ground using a plastic pestle and mortar, with 1 drop of 0.2% 2-mercaptoethanol added just before use. Thereafter, about 500µl of chloroform: isoamyl alcohol (CIA; 24: 1; v/v) was added, vortexed or shaken vigorously to mix, centrifuged for 1.5 minutes at 14400 g, after which an aliquot of 600µl of the top (aqueous) layer was pipetted into a new, clearly labelled Eppendorf tube. Then, 400µl of ice cold isopropanol was added, the Eppendorf tube was gently rotated to mix, and left to stand in the freezer for about 10 minutes. The contents were centrifuged for 10 minutes at 9200 g, and the supernatant decanted, leaving the pellet of precipitated DNA. The pellets were washed with 750µl of 70% ethanol, air-dried in a fume hood, and finally the pellet (i.e. DNA) was resuspended in 50-300µl ddH_2O. The extracts were screened for the presence of DNA with a GelVue UV Transilluminator GVM20 on 1.5% agarose gel using Ethidium Bromide and SYBR® Green I as an indicator where necessary.

2.2.3.2. DNA amplification (PCR) and purification

Portions of mtDNA (mtCOI) and nuclear (28S rRNA) genes were amplified in 50µl reaction containing 5-10µl of DNA template, 0.1µM of primers (forward and reverse; see Table 2.2), 200µM of deoxyribonucleotide triphosphates (dNTPs), 5µl of 25 mM Magnesium chloride ($MgCl_2$), 5µl of 10x Buffer solution (NH_4), 24-29µl of ddH_2O, and 0.1µl of enzyme (BIOTAQ® or Go AmpliTaq DNA polymerase), with 0.8µg of bovine serum albumin (BSA) added and overlayed with mineral oil where necessary. The PCR amplifications were conducted on a ThermoHybaid Sprint Temperature Cycling System, PC – 960G Gradient Thermo Cycler or AB Applied Biosystems 2720 Thermo Cycler. PCR reactions were performed as follows: an initial denaturation for 3 min at 95 °C, followed by 35 cycles of 45s at 94 °C, 30s at a gene-specific annealing temperature (48-50 °C for mtCOI, and 50-52°C for 28S rRNA), 2-3 minutes at 72 °C, with a final extension of 10 min at 72 °C at the end of PCR run.

The final PCR products were visualised on agarose gel (see above), and successful PCR products were then purified using commercially purchased cleanup kits (Qiagen© QIAquick™ or Promega PCR Preps™) according to the manufactures‟ instructions.

Since the primers for mtCOI and 28S rRNA used by Williams *et al*. (2003) were not successful in amplifying most of the samples, specific internal primers (see Table 2.2) were designed from a few aligned sequences initially obtained (this study and GenBank; Williams *et al*., 2003) using the primer designer software CLM Main Workbench 6.7 (CLC Bio). In addition, the crustacean primer (Decap CO1- R; see Table 2.2) was also useful in amplifying some of the samples (from the northern part of east coasts or subtropical region) which did not amplify with either universal or internal primers. The details of all primers used are presented in Table 2.2.

Table 2.2. Table showing the details of primers [forward (F) and reverse (R)] used to amplify and sequence mtCOI and 28S rRNA gene fragments.

Marker	**Primer**	**Primer Sequence**	**Source/Author**
mtCOI	HCO 2198 (R)	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.*, 1994
	LCO 1490 (F)	GTT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.*, 1994
	Decap CO1- R	AAT TAA AAT RTA WAC TTC TTG	Teske *et al.*, 2006
	Afrolittorina black F	TGG AAC CTT ATA TAT TTT ATT CGG	This study
	Afrolittorina white F	TGG AAC TTT ATA TAT TTT ATT TGG	This study
28S rRNA	LSU5F	TAG GTC GAC CCG CTG AAY TTA AGC A	Littlewood *et al.*, 2000
	LSU1600 R	AGC GCC ATC CAT TTT CAG G	Williams *et al.*, 2003
	Afrolitt internal 1F	AAC AGT TGA ACC CGC C	This study
	Afrolitt internal 1R	GCC TCT ATT CAT TCG CTT TAC C	This study

2.2.3.3. Sequencing

The clean, concentrated products were sequenced in 20µl reaction in either the forward or the reverse direction using 0.5µl of the same primers as used for PCR (see Table 2.2), 2µl of sequence mix, 4µl of 5x sequencing (Applied Biosystems Big Dye Terminator ver.3.1) buffer, 2-5µl of DNA and 12.5-8.5µl of ddH_2O. The sequencing reactions were conducted on a ThermoHybaid Sprint Temperature Cycling System. Sequencing reaction was performed as follows: 5 min at 96 °C, followed by 25 cycles for 15s at 96 °C, 10s at 45°C, and 4 min at 60 °C. The resulting sequence reaction products were precipitated with 50µl of 100% ethanol plus 2µl of 3M Sodium acetate acid (CH_3COONa) and 2µl of 125mM EDTA, cleaned with 150µl of 70% ethanol, and later sequenced in both forward and reverse direction.

Electropherograms (i.e. trace files) were obtained using automated DNA Sequencer (ABI Prism 310; Applied Biosystematics) at Rhodes University‟s Sequencing Unit. See Appendix 2.1 for few representative sequences. Complete and all data sets can be provided on request.

2.2.4. Sequence editing and alignment

The forward and reverse sequences were assembled, checked and edited using Sequencher™ version 4.1 (Gene Codes Corporation), and aligned using McClade version 4.06 (Maddison and Maddison, 2000), with reference to GenBank sequences (Williams *et al*., 2003). *Afrolittorina praetermissa* and *A. acutispira*, which belong to the same genus as the study species were used as outgroups, and their sequences (mtCOI and nuclear 28S rRNA) were extracted from the GenBank database (Williams *et al*., 2003).

The alignments were edited and formatted into different files for different phylogenetic analyses using DAMBE version 5.3.8 (Xia and Xie, 2001).

2.2.5. Phylogenetic and phylogeographic analyses

Data or nucleotide sequences were then used to construct phylogenetic trees using Maximum-Likelihood (ML) and Maximum-Parsimony (MP) analyses. At this time, only unique sequences were used for phylogenetic analyses. Data sets were analysed independently as well as in combination in the case of samples with sequences from both markers. For the combined data set, ML analysis was conducted in a partitioned fashion, with parameters for each gene (mtCOI and 28S rRNA) optimized independently.

Maximum parsimony (MP) analyses were conducted using PAUP* version 4.0b10 (Swofford, 2002). The characters were equally-weighted. The Heuristic search and tree bisection reconnection (TBR) plus branch-swapping option were selected as the criteria to reconstruct phylogenetic tree. The node support was assessed using bootstrap values which were determined using non-parametric bootstrapping with 1000 pseudo-replicates (Felsenstein, 1981). The Maximum likelihood (ML) phylogenetic reconstruction was conducted using RAxML HPC version 7.2.6 (Stamatakis, 2006). Gaps were treated as missing data and uncertainties as polymorphic characters. The program utilises the GTR + I + G substitution model and 1000 bootstrap replicates were run to generate likelihood support values for the branch nodes. The ML trees were all rooted using *Afrolittorina acutispira* which is the member of the same genus as South African species (Williams *et al*., 2003).

2.2.6. Genetic diversity analysis

Genetic heterogeneity was calculated using the software package DnaSP version 5.00.07 (Librado and Rozas, 2009). A partial fragment of the mtDNA COI was used to compare the levels of genetic heterogeneity within *Afrolittorina* spp., and for each species, the input data set comprised 1-25 samples from different populations (see Table 2.1). The levels of genetic heterogeneity were estimated for the following standard molecular indices (Nei, 1987): number of samples (*n*), number of haplotypes (*k*), average nucleotide difference (*II*), polymorphic sites (*S*), haplotype diversity (*Hd*) and nucleotide diversity (π). Neutrality tests were calculated using Fu and Li‟s D (Fu and Li, 1993) statistics.

2.3. Results

2.3.1. Sequence characteristics

The details of both the mtCOI and the 28S rRNA data sets used to reconstruct phylogenetic trees are presented in the table below (Table 2.3).

Table 2.3. Tables showing the details of mtCOI and 28S rRNA data sets used to reconstruct phylogenetic trees.

Data set	**Ingroup taxa**	**Final alignment length in base pairs (bp)**	**Number of variable characters**
mtCOI	65	615	56
28S rRNA	62	745	6

2.3.1. Phylogenetic and phylogeography of Afrolittorina species

There were differences in the phylogenetic trees recovered by the two markers; the mtCOI data set produced clearer, well-resolved (shown by strong nodal support) trees than 28S rRNA data set (see Fig. 2.2-2.5). However, this was not significant since 28S rRNA data set had only 6 variable characters compared to mtCOI which had 56 variable characters (see Table 2.3).

The ML and MP trees reconstructed using the mtCOI data set recovered two major clades which conformed to *A. africana* and *A. knysnaensis* (see Fig. 2.2 and 2.4), both with a strong node support of 100%. On the other hand, the ML and MP phylogenetic reconstruction of

28S rRNA data set revealed different phylogenetic tress (see Fig. 2.3 and 2.5). Except for a few samples, the MP tree recovered two major clades (with 90 and 96% node support each) that conformed to *A. africana* and *A. knysnaensis* (see Fig. 2.3), while the ML tree did not produce such clear clades as shown by weak node support (see Fig. 2.5).

Apart from two *A. africana* outliers that clustered within the *A. knysnaensis* clade (see Fig. 2.6), the ML tree reconstructed using the combined data sets also recovered two major clades which conformed to *A. africana* and *A. knysnaensis* (see Fig. 2.6), both with strong support of 100%.

2.3.3. Genetic diversity of Afrolittorina species

The results of the genetic diversity indices and neutrality test are presented in the Table 2.4. There was a difference in the genetic diversity indices between the two species, with *A. africana* showing significantly higher values than *A. knysnaensis* (see Table 2.4). In addition, both species showed low haplotype and nucleotide diversity (see Table 2.4).

For *A. africana*, 221 individuals from 29 sites revealed a total 31 haplotypes. The dominant haplotype (H1 with 154 individuals) was found at almost all sites, followed by H10 with 14 individuals, H9 with 7 individuals sampled at several sites, site-restricted H24 and H1 with 3 individuals, H5, H11 and H17 two of which are site-restricted, and the remaining site-restricted 23 haplotypes had one individual each.

Likewise for *A. knysnaensis*, 254 individuals from 37 sites revealed a total 22 haplotypes. The dominant haplotype (H1 with 228 individuals) occurred in almost all sites, followed by H15 with 3 individuals, H3 (site-restricted), H5 and H9 which each had 2 individuals, and the remaining site-restricted 17 had one individual each. The Fu and Li‟s neutrality test revealed unexpected significant variation ($p < 0.002$) amongst haplotypes of both species (see Table 2.4).

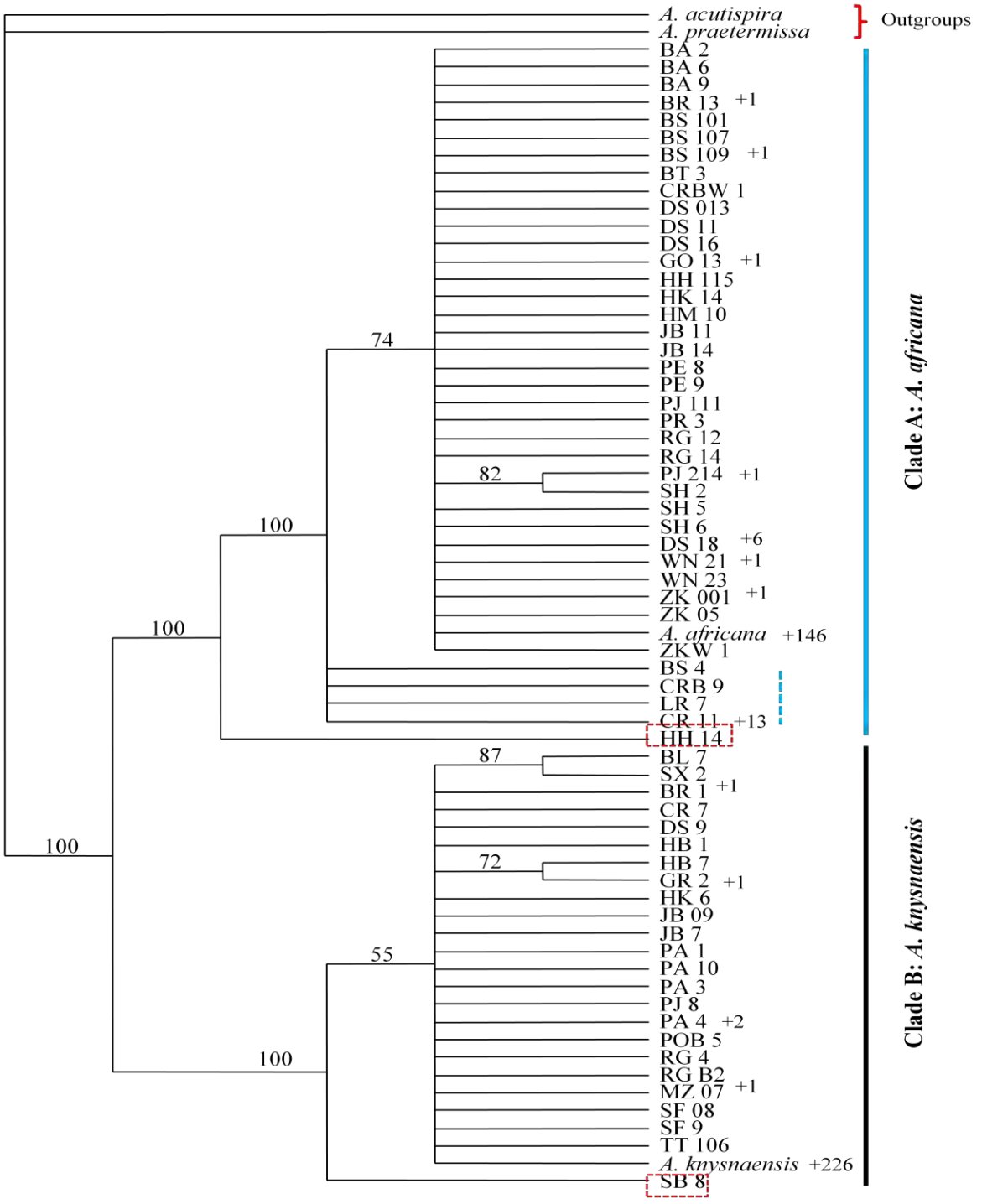

Figure 2.2. Maximum parsimony (MP) tree based on 615 base pairs of 39 and 24 unique [see Appendix 2.2 for list of samples with plus (+) sign] mtCOI sequences including reference sequences of *A. africana* and *A. knysnaensis* plus outgroups, *A. praetermissa* and *A. acutispira*. Solid lines indicate grouping according to species (blue for *A. africana* and black for *A. knysnaensis*); dotted lines and squares indicate outliers. The values at the branch nodes indicate the maximum parsimony support base on 1000 replicates.

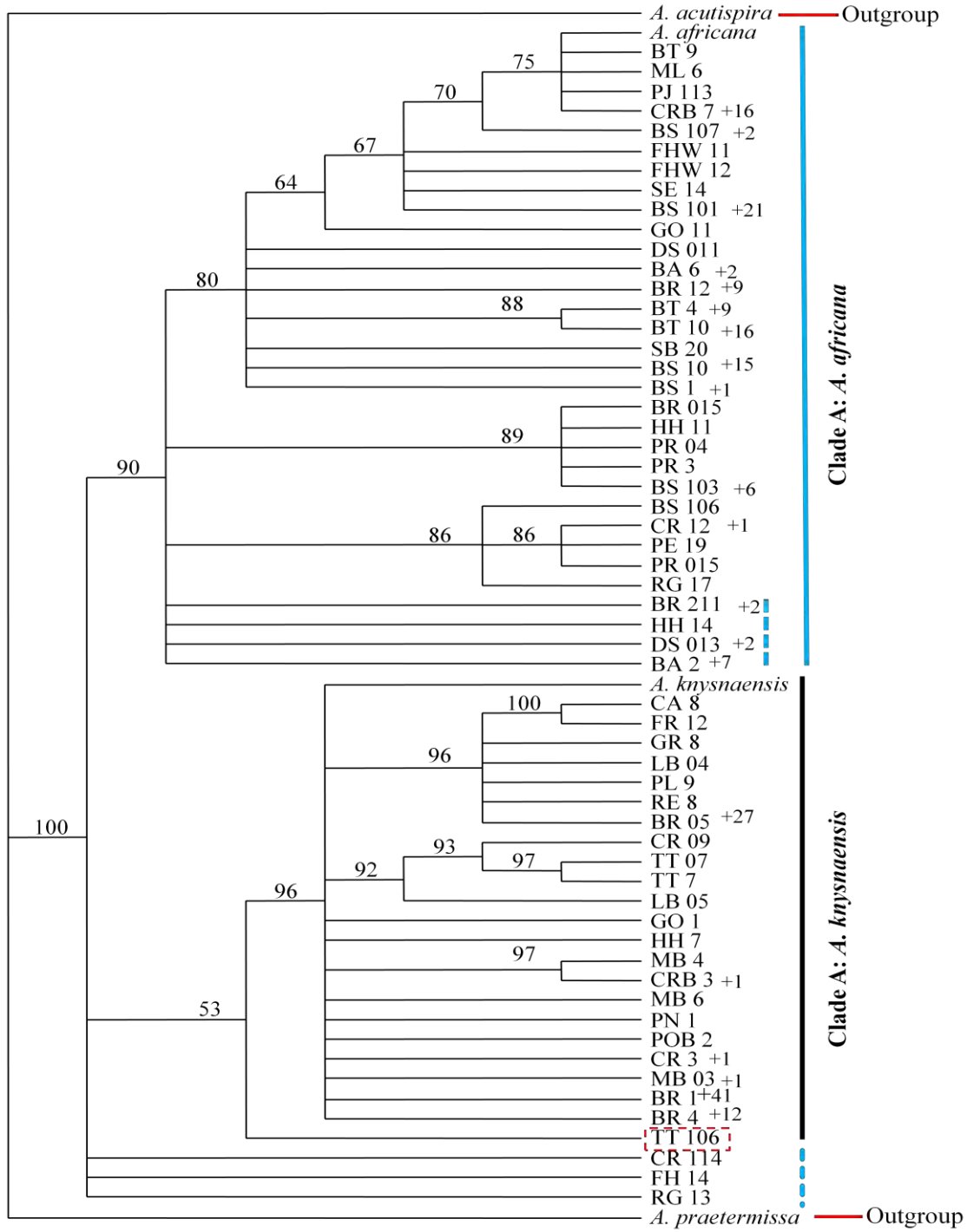

Figure 2.3. Maximum parsimony (MP) tree based on 745 base pairs of 34 and 24 unique [see Appendix 2.2 for list of samples with plus (+) sign] 28S rRNA sequences including reference sequences of *A. africana* and *A. knysnaensis* plus outgroups, *A. praetermissa* and *A. acutispira*. Solid lines indicate grouping according to species (blue for *A. africana* and black for *A. knysnaensis*); dotted lines and squares indicate outliers. The values at the branch nodes indicate the parsimony likelihood support base on 1000 replicates.

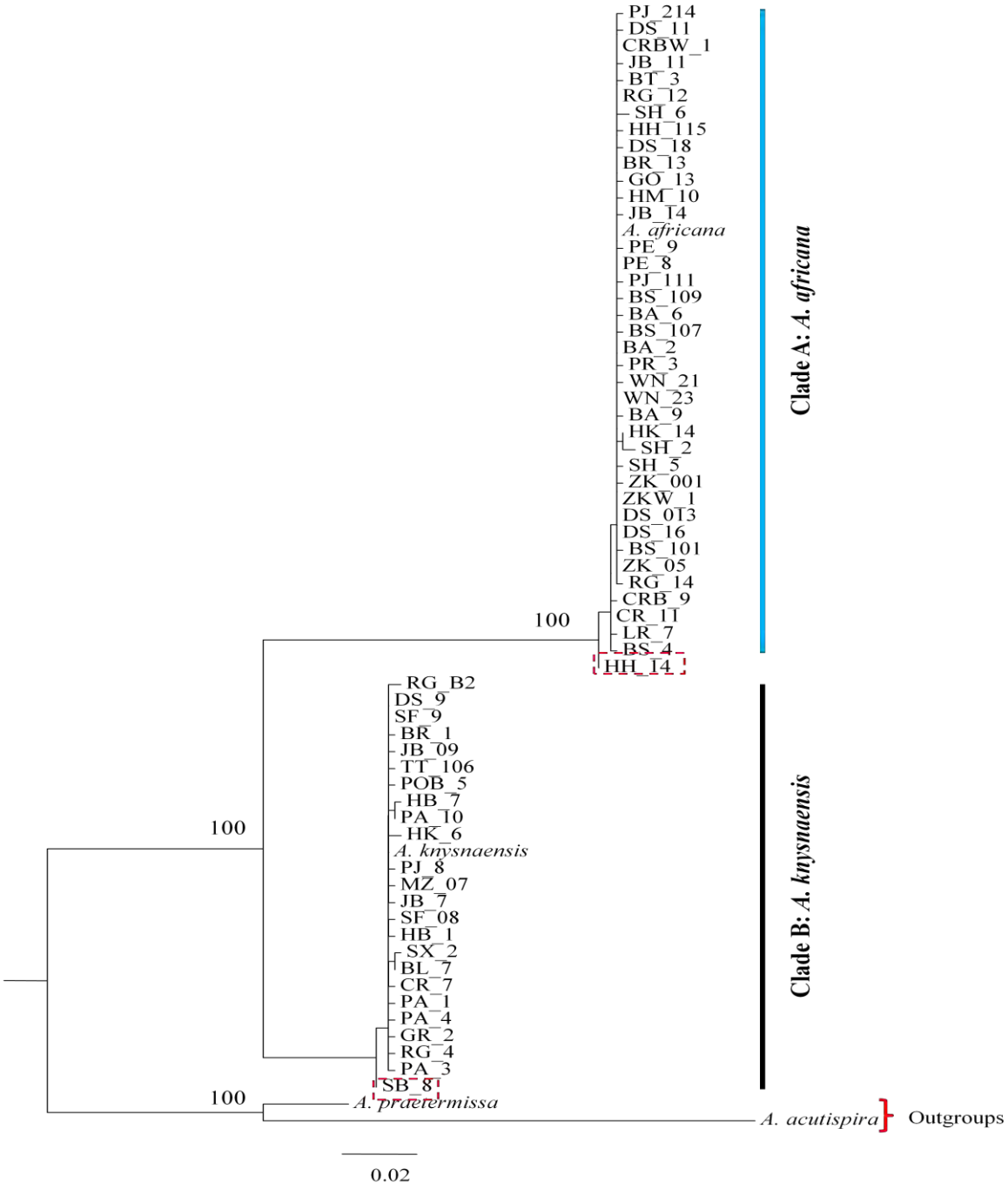

Figure 2.4. Maximum likelihood (ML) tree based on 615 base pairs of 39 and 24 unique (see Fig. 2.2) mtCOI sequences including reference sequences of *A. africana* and *A. knysnaensis* as well as *A. praetermissa* plus *A. acutispira* as an outgroup. Solid lines indicate grouping according to species (blue for *A. africana* and black for *A. knysnaensis*); dotted squares indicate outliers. The values at the branch nodes indicate the maximum likelihood support base on 1000 replicates.

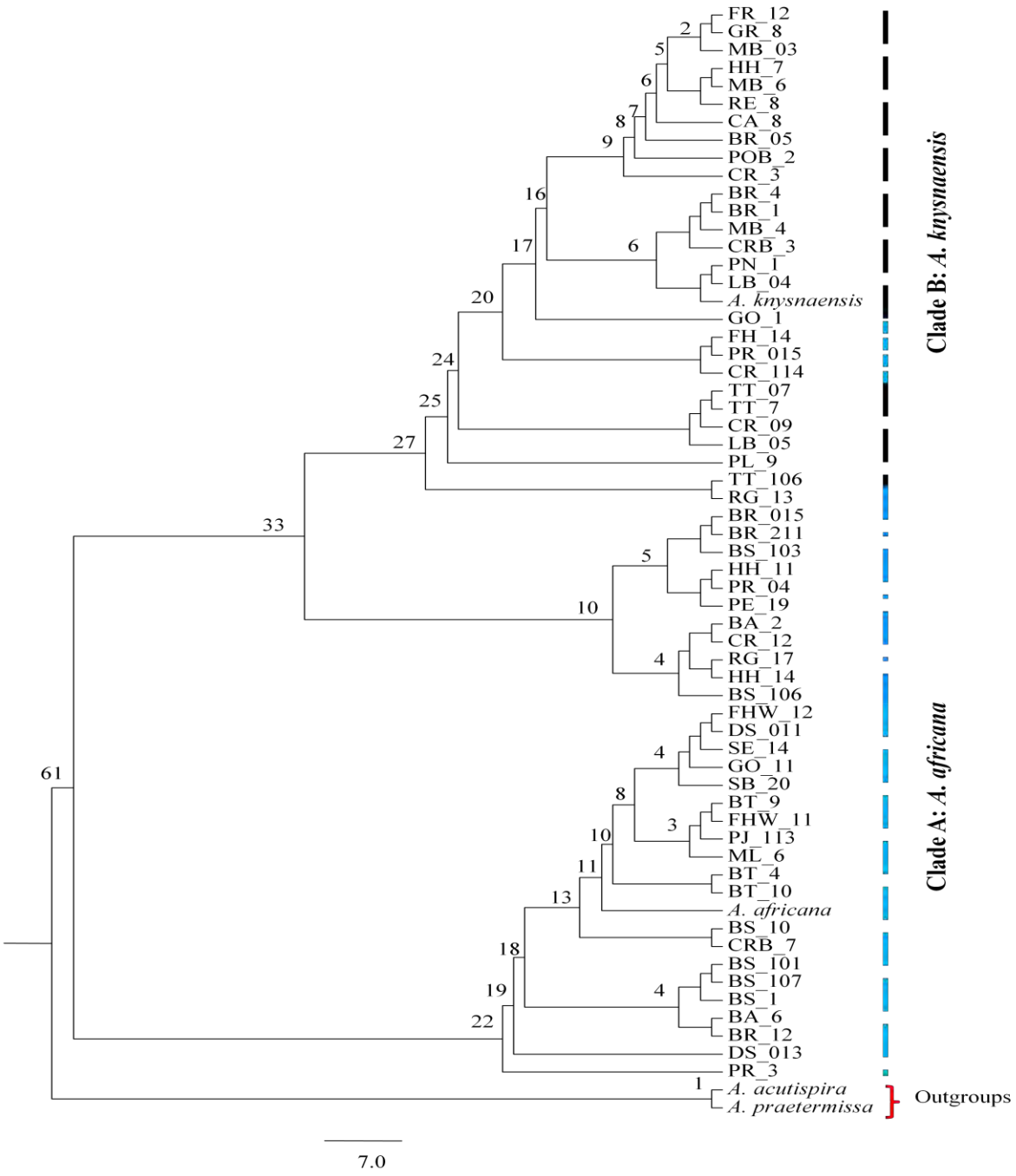

Figure 2.5. Maximum likelihood (ML) tree based on 745 base pairs of 34 and 24 unique (see Fig. 2.3) 28S rRNA sequences including reference sequences of *A. africana* and *A. knysnaensis* as well as *A. praetermissa* plus *A. acutispira* as an outgroup. Dotted lines indicate grouping according to species (blue for *A. africana* and black for *A. knysnaensis*). The values at the branch nodes indicate the maximum likelihood support base on 1000 replicates.

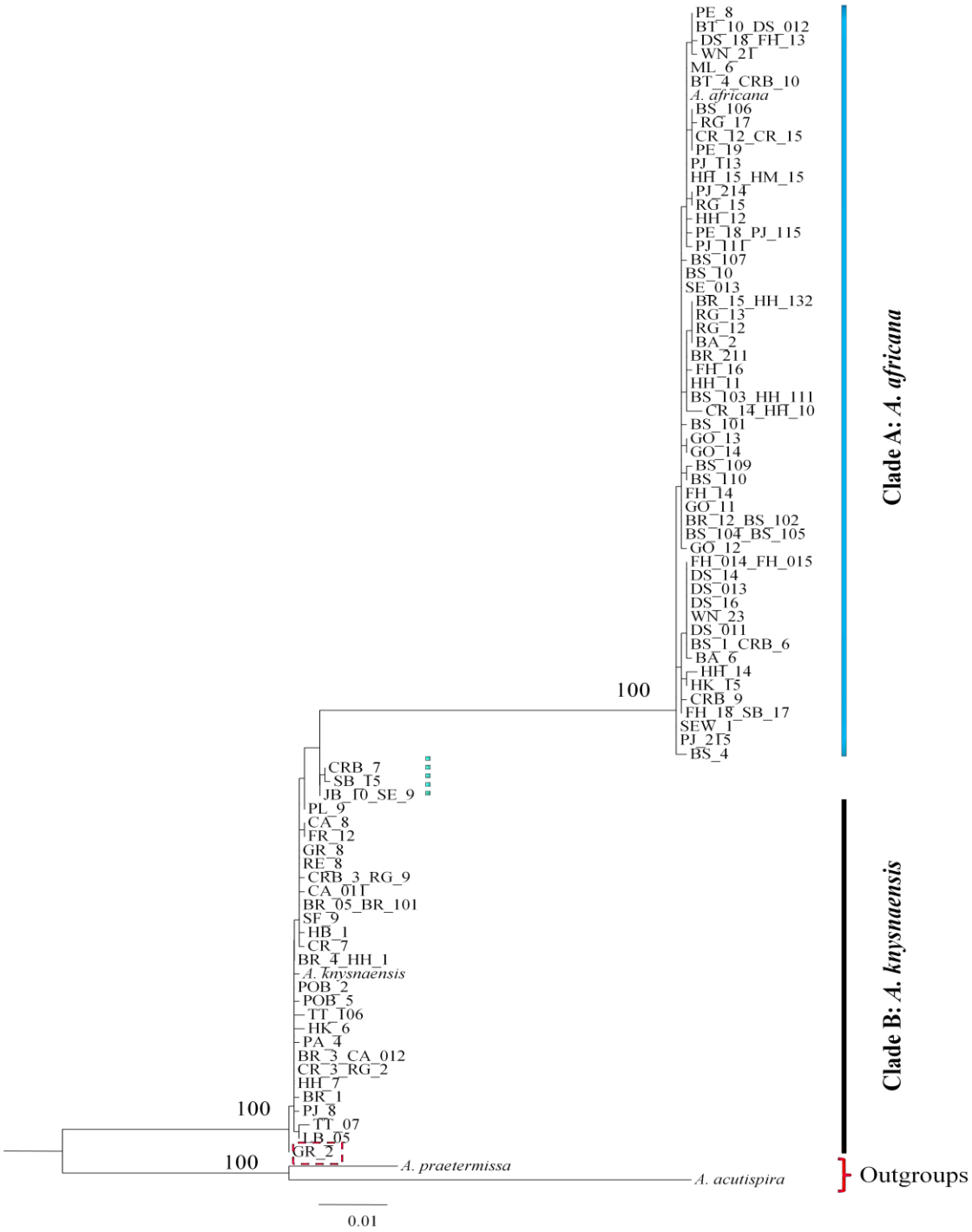

Figure 2.6. Maximum likelihood (ML) tree based on 1360 base pairs of 366 combined mtCOI and 2S rRNA sequences including reference sequences of *A. africana* and *A. knysnaensis* as well as *A. praetermissa* plus *A. acutispira* as an outgroup. Solid lines indicate grouping according to species (blue for *A. africana* and black for *A. knysnaensis*); dotted line and square indicate outliers. The values at the branch nodes indicate the maximum likelihood support base on 1000 replicates.

Table 2.4. Table showing the results of the genetic diversity indices and neutrality tests for both species. Number of samples (*n*), number of haplotypes (*k*), average differences (*II*), Polymorphic sites (*S*), haplotypes diversity (*Hd*), nucleotide diversity (π) Fu and Li‟s D statistics. * and ** indicate significant ($p < 0.002$ and 0.05) differences.

Species	**Region**	***n***	***k***	***II***	***S***	***Hd***	***π***	**Fu and Li's D**
A. africana	**All**	221	31	0.589	29	0.463	0.00096	-6.28583**
	Subtropical	133	22	0.572	21	0.443	0.00094	-5.30171**
	Warm	77	12	0.651	12	0.502	0.00106	-2.69725*
A. knysnaensis	**All**	254	22	0.236	21	0.194	0.00039	-6.18791**
	Warm	159	18	0.289	19	0.225	0.00048	-6.58457**
	Cool	89	7	0.157	6	0.152	0.00026	-3.57932**

2.4. Discussion and conclusions

Even though the phylogeny of the two southern African *Afrolittorina* spp. is known (Hughes, 1979; Reid, 1989; 2002; Williams *et al*., 2003; Reid and Williams, 2004; Reid *et al*., 2012), it is not clear if the two species are distinct species or subspecies. Thus, there is controversy on the classification system of the family Littorinidae, and the two *Afrolittorina* spp. are among those affected. Previous classifications have grouped them within the genera *Littorina* (see Hughes, 1979 and references herein) and *Nodilittorina* (Reid, 1989; 2002), respectively. But the most recent classification has described them under the new genus *Afrolittorina* (Williams *et al.*, 2003; Reid *et al*., 2012).

Both morphological characteristics and molecular techniques have been used to shed light on their classification, but this has resulted in further confusion. Hughes (1979) suggested that the two *Afrolittorina* spp. are a single species which differs in its morphological appearance, and represents a cline which shows a gradual change in colour pattern along its distribution range. This was based on the lack of differences in morphology (shell shape and size) and habitat (occurs in the eulittoral zones and fringe), range of colour morphs and latitudinal distributions (overlaps in the warm temperate region and southern part of subtropical region). In contrast, Rosewater (1970), Reid (1989; 2002), Williams *et al.* (2003) and Reid *et al*. (2010) suggested that the same two species are distinct species based on morphological and DNA sequence data, respectively.

The results of this study confirmed that these are two distinct species, with the brown to black *A. knysnaensis* predominant in the cool-temperate region and the pale blue-grey *A. africana* in the subtropical region. This is largely consistent with the current taxonomy (Williams *et al*., 2003; Reid and Williams, 2004; Reid *et al*., 2012). Thus, both data sets support previous suggestions that the two southern African *Afrolittorina* spp. are genetically distinct species, eliminating the subspecies dilemma and the possibility of a single species cline as suggested by Hughes (1979). Although the mitochondrial (mtCOI) sequence data showed strong support (complemented by the combined sequence data) for the distinction of the two species,

the nuclear (28S rRNA) data showed weak support which might be the result of difficulties of working with this marker. Nevertheless, the results support a strong argument for a close phylogenetic relationship between these two distinct *Afrolittorina* species.

The results also show no evidence of a genetic basis for the range of colour morphs found within the two species distribution ranges, suggesting no past or ongoing hybridization between natural populations of *Afrolittorina* spp. Instead, colour morphs from the east and southeast coasts were *A. africana* while those from the west and southwest coasts were *A. knysnaensis*. Thus, specimens with unusual or intermediate colouration were not hybrids, but rather phenotypic variants of either species. This suggests that either environmental conditions (e.g. temperature gradients or substratum colour) might be responsible for the range of colour morphs. In fact, in the lab colour morphs from the east coast changed to „white" (i.e. *A. africana*) after long storage with the fixative. Future studies should focus on the cause of the variation of colours in *Afrolittorina* spp., which could represent either genetic polymorphism or phenotypic plasticity. Transformation experiments of marked juveniles could verify the hypothesis of environmental conditions as a cause of phenotypic plasticity.

It is known that phenotypes of gastropods, including littorinids, can become genetically adapted or plastically changed in response to environmental factors (see Fevolden and Garner, 1987; Johannesson *et al*., 1993; De Wolf *et al*., 1997; Trussell and Etter, 2001; Johannesson, 2003; Rolán-Alvarez, 2007; Teske *et al*., 2007c, d; Azuma *et al*., 2011; etc). This means that phenotypes can be determined solely by the genotype of a species or by the interaction between genes and the environments (see Pigliucci, 1996; Soares *et al*., 1998; 1999 and references herein). Pigliucci *et al*. (2006) suggested that individual genotypes can produce different phenotypes (i.e. morphs) each fitted for different environmental condition when exposed to different environmental conditions. This allows species or populations to live in different habitats (e.g. biogeographic regions) (see Laudien *et al*., 2003). Several field and laboratory studies on littorinids reported morphological (e.g. shell colour, size and shape) clines in animals of the same species from different habitats, thus environmental gradients (see Rolán-Alvarez *et al*., 1997; Johnson and Black, 1999; Sokolova and Berger, 2000; Wilding *et al*., 2001; Kurihara *et al*., 2006; Quesada *et al*., 2007; Cuña *et al*., 2011; Silva *et al*., 2013). Phifer-Rixey *et al*. (2008) documented replicated clines in shell colour morph

frequencies in the flat periwinkle *Littorina saxatilis* over thermal gradients at two spatial scales, which had effects on shell temperature.

Although this study shows no evidence of hybridization between the two species, hybridization hypotheses might be plausible based on existing knowledge. *A. africana* and *A. knysnaensis* co-exist along the warm temperate region where they even occupy the same microhabitats without any physical or ecological barriers separating the two species (pers. obs.; McQuaid and Scherman, 1988; McQuaid, 1992; d'Errico *et al*., 2008). Studies on littorinids and other marine animals provide evidence of interspecific hybridization and/or introgression, especially when closely related (sister) species or ecotypes co-exist or live in sympatry (see below; Brown, 1995; Rawson *et al*., 2003; Nydam and Harrison, 2011; Zardi *et al*., 2011). Both field and laboratory studies on littorinid snails, especially on species or ecotypes of the genus *Littorina* have shown hybridization and/or introgression (De Wolf *et al*., 1998; Erlandsson *et al*., 1999; Wilding *et al*., 2001; Rolán-Alvarez, 2007). Mikhailova *et al*. (2009) found evidence of possible hybridization between natural populations of the sibling species *Littorina saxatilis* and *L. arcana* living in sympatry. This is supported by the laboratory results of Warwick *et al*. (1990) where females of *L. arcana* hybridized (even though the frequency of viable offspring was lower than either of the parental crosses) with males of *L. saxatilis*.

Sharing of mtDNA COI haplotypes between co-exiting species has also been suggested to be a consequence of either persistent hybridization or episodes of hybridization, or incomplete lineage sorting of ancestral polymorphs (Small and Gosling, 2000; Wilding *et al*., 2000a; 2001; Azuma *et al*., 2011; Díaz- Ferguson *et al*., 2012). For example, the lack of mitochondrial divergence between *Littorina fabalis* and *L. obtusata* led Kemppainen *et al*. (2009) to suggest that there might be some degree of incomplete lineage sorting or introgressive hybridization between these species.

Other studies have shown no evidence of hybridization and/or introgression, and explain this as the results of prezygotic (e.g. mate choice and gamete incompatibility) or postzygotic (e.g. unfit hybrids) reproductive barriers to gene flow (Saur, 1990; Johannesson *et al*., 1995; Erlandsson *et al*., 1999; Johannesson, 2003; Rawson *et al*., 2003; Rolán-Alvarez, 2007;

Slaughter *et al*., 2008; Addison and Pogson, 2009). For example, Johnson (1999) suggested that size assortative mating observed between individuals of *Littorina neglecta* and its congener *L. saxatilis* may have acted as a prezygotic barrier to reproduction between these species. Field and laboratory studies on ecotypes (H and M) of *Littorina saxatilis* have shown that morphs from different shores and microhabitats mate assortatively, but produce unfit hybrids (Hull *et al*., 1996; Erlandsson and Rolán-Alvarez, 1998; Hull, 1998; Pickles and Grahame, 1999; Rolán-Alvarez *et al*., 1999; Quesada *et al*., 2007). In fact, where hybridization occurs and hybrids are produced, they occur in low frequencies leading to the suggestion that there is no hybridization.

If true, the explanation for the lack of hybridization (i.e. hybrids) in *Afrolittorina* spp. could be due to differences in reproductive systems (e.g. penis structure; Reid, 1989; Reid and Williams, 2004), behavioural barriers (e.g. mate choice; not yet investigated) or that they can hybridize but produce unfit offspring. In addition, the divergence time (about 10-47 Ma; see Williams *et al*., 2003) as suggested (see Kemppainen *et al*., 2009) might also explain the lack of hybridization or introgression between these two species; but see Nydam and Harrison (2011). Further investigations using different markers (e.g. microsatellites) as well as field and laboratory studies could help to clarify if there is interspecific hybridization or speciation in *Afrolittorina* spp. Microsatellites have been instrumental in identifying hybrids or the possibility of interspecific hybridization in littorinids and other marine animals (Wilding *et al*., 2002; Panova *et al*., 2006; Kemppainen *et al*., 2009), and can be useful in this regard.

Although most studies on the phylogeography of South African marine invertebrates and fishes have shown that phylogeographic breaks within and among taxa often coincide with known biogeographic boundaries, there are some deviations (Teske *et al*., 2006; 2007a, b, c; 2008; 2009; 2011a; von der Heyden, 2007; 2008; 2009; 2011; 2013; Mmonwa, 2009; 2013 unpub. data; etc). This shows that the prevailing biogeographic boundaries do not affect the phylogeography of different marine animals in the same fashion. In addition, given the species requirements for hard substrate (i.e. rocky shores), long stretches of sandy beaches and sand dunes (which can create unsuitable habitats for study species) found along their distribution ranges might promote population structure as in other animals (Teske *et al*. 2006; 2008; Zardi *et al*., 2007; Mmonwa, 2009 unpub. data).

The results of this study show that there are no phylogeographic breaks or genetic structuring in the study species in contrast to other invertebrates which show phylogeographic patterns and/or breaks that coincide with recognised biogeographic boundaries or limits (see above). The data revealed complete genetic homogeneity across the species distribution ranges, suggesting high levels of gene flow as a result of the effect of prevailing currents on larval dispersal. Thus, the accepted southern African biogeographic boundaries seem to have no impact on the phylogeography of the two littorinid snails examined; in contrast to what has been found in other coastal invertebrates. In fact, biogeographic boundaries may have strong or weak effects on the phylogeographic patterns of different species, and this can also depend on the species‟ mode of dispersal (see Teske *et al*. 2006; 2011a). *Afrolittorina spp*. are believed to have planktonic larval stages (see below), and as such are expected to show no phylogeographic patterns as seen in this study.

To my knowledge, this is one of few findings (Grant *et al*., 1992; Soares *et al*., 1999; Tolley *et al*., 2005; Gopal *et al*., 2006; Neethling *et al*., 2008; Bester-van der Merwe *et al*., 2011) where little or a complete lack of structure or break has been shown. Oosthuizen *et al*. (2004) found a single haplotype in the octopus, *Octopus vulgaris* populations from the east and west coast of South Africa. Ridgway *et al*. (1999) found genetic homogeneity in the bearded limpet *Patella barbara* along the west and east coasts of South Africa. Grant and da Silva-Tatley (1997) found a remarkable genetic similarity in populations of the sandy beach whelk *Bullia digitalis* over 2400 km of the coastline. Generally, these studies show that planktonic eggs and larvae are important in maintaining such genetic homogeneity. Similar results, thus lack of structure and/or breaks have been reported in other littorinids (see Reid *et al*., 2006). Kim *et al*. (2003) found lack of genetic structure among populations of the widely distributed littorinid, *Littorina brevicula* around Korean waters. Silva *et al*. (2013) found low genetic differentiation in two littorinids, the rocky shore *Littoraria glabrata* and the mangrove *L. scabra*, along the East African coast.

In addition, these species showed low genetic variation within their distribution ranges as shown by the occurrence of the dominate haplotype and less private haplotypes, suggesting high larval gene flow in these species. Thus, the current study found low levels of haplotype and nucleotide diversity in both species, even though the neutrality test revealed significant

difference between haplotypes found within species distribution ranges. The subtropical/temperate *A. africana* was characterised by higher level of haplotype and nucleotide diversity compared to its temperate congener *A. knysnaensis*, and this might reflect differences in their ecology rather than their life history since they are predicted to have similar larval development (see below). Thus, the explanation for higher genetic diversity in *A. africana* as compared to *A. knysnaensis* may indicate that genetic heterogeneity as an adaptive strategy to heat stress as suggested in other studies (Noy *et al*., 1987; Ward, 1990; Hawkins, 1995; Schmidt *et al*., 2007). This means that the higher heat tolerance (see Chapter 3-5) in *A. africana* has elicited higher genetic diversity to increase its fitness in heterogeneous conditions in the subtropics.

The study species are believed to be pelagic spawners with planktotrophic larvae which can disperse long distances with the help of currents, inhibiting genetic structuring. Although the duration of the planktonic larvae stage of these species is not known, it is believed that they have long phases of planktonic development which result in high gene flow, thus less genetic variation across their distribution range. In addition, both species are thought to breed throughout the year, and this can help to prevent the effect of seasonal variation in currents on larval dispersal. A previous study by Grant and Lang (1991) also found low genetic (allozyme) variation on a geographical scale in the population genetics of *A. knysnaensis*. However, there are no other studies which have investigated the phylogeographic patterns of littorinids from South Africa.

The low genetic variation found in this study supports the idea that the mode of development in the study species is planktonic. Grant and Lang (1991) suggested that the mode of development in *A. knysnaensis* is planktonic after finding low allozyme variation on a geographical scale. The findings of McQuaid (1981) on the eggs (about 87 μm) size of *A. knysnaensis* further supported the idea of planktonic development in this species. The dimensions of the protoconch and the large capsule gland of the two species (see Reid, 1989; Reid and Williams, 2004) again suggest that they have planktonic development.

There is still a need to investigate the phylogeography and population genetics of *Afrolittorina* spp., and other littorinids found along the South African coastline. Both nuclear

and mitochondrial „neutral" or unlinked markers such as microsatellites, introns, internal transcribed spacers, etc. can be useful in this regard. Thus, to have an accurate picture of phylogeography and population structure within a group, both nuclear and mitochondrial „neutral" markers are needed. These markers, especially microsatellites, have been widely used in phylogeographic and population genetic studies of invertebrates, including littorinids (Wilding *et al*., 2000b; Sokolova *et al*., 2001; 2003; 2004; Simpson *et al*., 2005; Teske and Beheregaray, 2009; Zhan *et al*., 2009; Zulliger *et al*., 2009; etc). However, microsatellites have limitations such as the time and effort required for the isolation and characterization, the failure of cross-species amplification, which depends on the phylogenetic distance between source and target species, and the fact that as yet, they are scarce and incomplete, and for many taxa totally absent (see Winnepenninckx and Backeljau, 1998; Sokolova *et al*., 2001; 2004; Panova *et al*., 2008). Therefore, most researchers working at phylogeographic and/or population genetic levels have exclusively used and/or are still using mitochondrial DNA sequences (see Wilding *et al*., 2000b; Teske and Beheregaray, 2009; Panova *et al*., 2011; Teske *et al*., 2011a, b, c) as in the case in this study.

In summary, the results show that there are two distinct species, with a brown to black *A. knysnaensis* predominant in the cool-temperate region and a pale blue-grey *A. africana* in the subtropical region. These results support the previous morphological and recent molecular distinction of the two species (Reid, 1989, 2002; Williams *et al*., 2003; Reid and Williams, 2004; Reid *et al*., 2012). The data also show no evidence of a genetic basis behind the colour morphs as the intermediate morphs clearly grouped as one species or the other. This suggests the absence of either past or ongoing interspecific gene flow (hybridization) and/or speciation in these species. Thus, it is probable that there are reproductive barriers preventing the gene pools of the two species amalgamating or mixing. Therefore, it is possible that the cause of different colour morphs within each species is the result of the conditions (i.e. phenotypic plasticity) in their microhabitats. Furthermore, there was low genetic variation within each species, suggesting that there is high gene flow among populations; supporting the suggestion of planktonic development in these species. This is in agreement with previous phylogeographic studies of South African marine taxa, where species with planktonic larva showed low genetic variation compared to those with non-planktonic larva. Thus, high dispersal of planktonic larvae coupled with the effects of dispersal by currents results in low genetic diversity as there is high gene flow among populations.

CHAPTER 3. Thermal tolerance of littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from temperate and subtropical regions of South Africa

3.1. Introduction

The effects of temperature and desiccation on the behaviour, survival and physiological performance of animals, and the subsequent influence of physiological tolerances or adaptations to temperature and desiccation on the distribution and abundance of animals have been extensively studied (see Evans, 1948; Vernberg, 1959; McMahon, 1990; Bertness *et al.*, 1999; Menge *et al*., 2007; Sokolova *et al*., 2012). This is because temperature and/or desiccation are among the most important environmental factors that affect the distribution and abundance of marine, estuarine and open sea animals, particularly ectotherms (see Huey and Stevenson, 1979; McQuaid and Branch, 1984; Segnini de Bravo *et al.*, 1998; Muñoz *et al.*, 2005; Chan *et al*., 2006; Iacarella and Helmuth, 2012).

Environmental temperature affects an animal‟s performance (including activity) and fitness (including survival) through its effect on body temperature (see Cornelius, 1972; Huey and Kingsolver, 1989, 1993; Angilletta Jr. *et al.*, 2002; Martin and Huey, 2008; Pincebourde *et al*., 2008). This is because physiological performance increases with temperature until declining above optimum or near lethal temperatures (see Clarke, 1993a; Huey and Berrigan, 2001; Peck *et al*., 2007; Ivanina *et al*., 2009; Tattersall *et al*., 2012). On the other hand, desiccation too affects the behaviour (i.e. activity) and physiology of animals (see Stillman and Somero, 1996; Lang *et al*., 1998; Bates and Hicks, 2005) through its effects on body water and gaseous exchange (see Sandison, 1967; Shick *et al*., 1988; McMahon, 1990; Stenseng *et al*., 2005; Gardeström *et al*., 2007). Thus, temperature and desiccation are critical structuring forces for animal populations, especially intertidal ones.

Various field and laboratory studies show that the distribution and abundance (vertically or horizontally) of intertidal animals are influenced by a wide variety of physical (abiotic) and biological (biotic) factors. These include abiotic factors such as temperature, salinity and wave action, which can be modified by complex topography (see Connell, 1961; Wallace, 1972a; Wolcott, 1973; McMahon and Britton, 1991; Chapman and Underwood, 1994; 1996; Kaehler and Williams, 1996; 1997; Boulding and Harper, 1998; etc) and biotic factors such as competition, predation and food availability (see Connell, 1961; 1972; Menge, 1976; Underwood and McFadyen, 1983; Little and Williams, 1989; Jones *et al*., 1994; Duncan and Szelistowski, 1998; Chapman, 2000; Rochette and Dill, 2000; Burnaford, 2004; etc). These abiotic and biotic factors, either alone or in synergy, determine the distribution and abundance of animals in the intertidal (see Etter, 1988; Bustamante and Branch, 1996; Bustamante *et al*., 1997; Soto and Bozinovic, 1998; Dahlhoff *et al*., 2002).

However, abiotic factors have a greater effect on the distribution of high shore organisms than biotic interactions. Therefore, it is generally accepted that abiotic factors set species‟ upper limits while biotic factors set the lower limits (see Wethey, 1984; Britton, 1992; Yamada and Boulding, 1996; Harley and Helmuth, 2003; Miller *et al*., 2009; Perez *et al*., 2009; Lima *et al*., 2011). Because of the profound effects of environmental conditions, physiological adaptations (e.g. thermal tolerance, metabolic adjustments, heat shock protein production, etc) are critical in setting the distribution patterns and limits of intertidal organisms (see Stillman and Somero, 1996; 2000; Tomanek and Somero, 2000; Somero, 2002; 2005; 2010; Tomanek, 2002; 2008; 2010; Madeira *et al*., 2012b, c; etc).

Temperature and desiccation are the two main factors that affect distribution and abundance of littorinids and other intertidal gastropods (see below). This is because many littorinids live highest on the shore where they experience long period of exposure, and thus temperature and desiccation stress during low tides. In fact, some are supralittoral and can be exposed to aerial conditions for weeks or months (see Jones and Boulding, 1999; Backeljau *et al*., 2001; Muñoz *et al.*, 2008; Lee and Boulding, 2010; Marshall and McQuaid, 2010; Judge *et al*., 2011). In addition, the degree and duration of environmental stresses increase from low to high shore levels (see Suryanarayanan and Nair, 1979; McMahon and Wilson, 1981; McMahon, 1990; Menge *et al*., 2007; Muñoz *et al.*, 2008). At high shore levels, aerial

conditions may not only become more extreme but are also characterized by high temporal instability.

As a result of their regular exposure to temperature and desiccation stress, littorinids and other intertidal animals have developed various survival strategies. When heat and desiccation stress increase, mobile animals actively choose suitable microhabitats and escape from unfavourable conditions (see McMahon, 1990; Britton and Morton, 2003; Harley *et al*., 2009; Chapperon and Seuront, 2011a, b; Judge *et al*., 2009; 2011). Shelled gastropods can withdraw into the shell and isolate themselves inside by tight closure of the shell aperture with their operculum (see Reese, 1969; Garrity, 1984; McMahon, 1990; Jones and Boulding, 1999; Iacarella and Helmuth, 2011). These behavioural responses are also evident in other intertidal molluscs and crustaceans (see Barnes *et al*., 1963; Garrity, 1984; McMahon, 1990; Williams and Morritt, 1995) and limit exposure of the animal to adverse environmental conditions. In the case of snails, withdrawal into the shell can be used to minimise physical contact with the substratum, thus reducing heat uptake.

Other behavioural thermoregulation mechanisms include evaporative and convective cooling (see Lewis, 1963; Britton and Morton, 2003; Marshall and Chua, 2012), formation of aggregations (see Reese, 1969; Feare, 1971; Soto and Bozinovic, 1998; Stafford *et al*., 2007; 2008; but see Stafford and Davies, 2004; Chapperon and Seuront, 2012), use of sheltered microhabitats such as crevices, cracks and biogenics (see Atkinson and Newbury, 1984; Britton, 1995; Stafford and Davies, 2004; Judge *et al*., 2009; Cartwright and Williams, 2012), orientation of the shell to avoid direct exposure to sunlight (see McMahon, 1990; 2001b; Marshall and Chua, 2012; Marshall and Ng, 2013), removing the foot from the substratum and attachment of the shell by a mucus thread to reduce contact with the substratum (see McMahon, 1990; Emson *et al.*, 2002), and restricting activity to periods of reduced stress (Lang *et al*., 1998; Emson *et al.*, 2002; Bates and Hicks, 2005; Cartwright and Williams, 2012), amongst others.

When conditions become too harsh, some animals, including some littorinids, can enter a dormant state (i.e. aestivation) (see McMahon, 1990; Emson *et al.*, 2002; Judge *et al*., 2011) during which they depress their metabolic rate and enter a new hypometabolic state, which

extends the time an animal can survive on stored energy supplies (see Sokolova and Pörtner, 2001b; Pörtner, 2002b; Storey, 2002; Anestis *et al*., 2007; Sokolova *et al*., 2012). Other animals, such as bivalves, switch to anaerobic metabolism, which although highly inefficient and costly, allows the animal to close off (e.g. through valve closure) from the external environment to avoid deleterious conditions (Widdows *et al*., 1979; Storey and Storey, 1990; Anestis *et al*., 2007; Nicastro *et al*., 2010). Because heat stress is often linked to desiccation (see Ottaway, 1973; McMahon, 1990; Bustamante *et al*., 1997), metabolic adjustments can include water conservation abilities and desiccation tolerance (see McMahon, 1988a; Britton, 1992; 1993; Sokolova and Pörtner, 2001b; Ji *et al*., 2008). Other physiological adaptations include thermal regulation, tolerance and acclimation (see McMahon, 1990; Clarke, 1993a; Horowitz, 2001; 2002; Camacho *et al.*, 2006), metabolic and heart rate adjustments (see McMahon, 1990; Horowitz, 2001; 2002; Sokolova and Pörtner, 2001b; Nguyen *et al*., 2011).

Some species induce a so-called heat shock response, which gives rise to a strong and transient induction of genes responsible for the production of heat shock proteins and increases thermal tolerance (Feder and Hofmann, 1999; Pörtner, 2002b; Tomanek, 2002; Tomanek and Sanford, 2003; Finke *et al.*, 2009). Other cellular level responses are increased heat stability of key metabolic enzymes (Hull *et al.*, 1999; Stillman and Somero, 2001; Somero, 2004), modification of enzymes (Somero, 1978; 1995; 2004; Schmidt *et al*., 2007; Dong and Somero, 2009) as well as enzyme-substrate and enzyme-modulator interactions (Somero and Hochachka, 1968; Somero, 1969; Newell *et al.*, 1980), higher mitochondrial density and capacity (see Sommer *et al.*, 1997; Guderley and St-Pierre, 2002; Pörtner, 2002b; Fangue *et al.*, 2009), and structural or membrane stability (see Somero, 2004; Rais *et al.*, 2010), a balanced suppression of energy demand and supply pathways (see Storey, 2002; Sokolova *et al*., 2012) and a decline in protein synthesis (Guppy and Withers, 1999; Somero, 2002; Sokolova *et al*., 2012). There are also morphological adaptations such as shell size, shape, colour, presence of opercula and sculpturing and/or ornamentation (see Britton, 1995; Lang *et al.*, 1998; Sokolova and Berger, 2000; Bates and Hicks, 2005; Harley *et al*., 2009).

Thus, classically, two main strategies are used to survive heat and desiccation stress: behavioural and physiological, and during rapid increases in heat stress, animals may use behavioural, physiological or both types of adaptation. The importance of a behavioural

adaptation to thermal stress is that it can allow regulation of body temperatures as animals approach their physiological limits (see Huey and Kingsolver, 1989; Eshky and Ba-Akdhah, 1992; Soto and Bozinovic, 1998; Angilletta Jr. *et al.*, 2002; Miller and Denny, 2011; Cartwright and Williams, 2012). In fact, thermoregulatory behaviour is a homeostatic mechanism which tends to maintain internal temperatures favourable for physiological processes (see Díaz *et al*., 2002). Above the thermal limits for physiological associated locomotory behaviour and feeding, animals rely on physiological adaptations to tolerate or resist heat stress (see McMahon, 1990; Sokolova and Pörtner, 2001b; Horowitz, 2001; 2002; Miller and Denny, 2011). Physiological mitigation of heat stress is critical for most intertidal ectotherms since they are sessile or have low mobility (see McMahon, 1988a, b; 1990; Halpin *et al*. 2004; Zardi *et al*., 2011; Marshall and Chua, 2012).

Thermal tolerance is among the most critical of physiological mechanisms and many studies have examined temperature tolerances or the effect of temperature on lethal limits of intertidal organisms, including littorinids as a way of understanding how animals tolerate their environment (see below). This is especially important given the current scenarios of future climate change (see Pörtner, 2002a, b; Helmuth *et al*., 2010; Somero, 2010; Madeira *et al*., 2012a, b). Thermal tolerance of intertidal organisms is commonly assessed from two attributes (traits); 1) heat coma temperature (HCT), at which neuromuscular coordination is lost but animals recover when temperature is lowered, and 2) median lethal temperature (LT_{50}) following exposure to temperatures from which animals cannot recover (see below; McMahon, 1990; Clarke *et al.*, 2002a, b, c; Díaz *et al*., 2002). Studies on thermal tolerance have established that it can differ for animals from different regions or latitudes, taxa, shore levels, habitats, etc. In general, tropical species show higher tolerances than their counterparts from subtropical and temperate regions, respectively. For example, within the genus *Echinolittorina*, thermal tolerances are high for tropical species followed by subtropical and temperate species or conspecifics (see Table 3.1). This is true for other snails/gastropods (see Table 3.1; Ansell and McLachlan, 1980; Backeljau *et al*., 2001; Sorte and Hofmann, 2005; Kuo and Sanford, 2009), bivalves (Compton *et al*., 2007; Morley *et al*. 2009; Zardi *et al*., 2011), echinoderms (Byrne *et al*., 2010), tunicates/bryozoans (Sorte *et al*., 2011), and crustaceans (Vernberg, 1959; Stillman and Somero, 1999; Stillman and Tagmount, 2009; Kelley *et al*., 2011) as well as intertidal fish (Fangue *et al*., 2006; Madeira *et al*., 2012a).

Table 3.1. Heat coma (HCT) and lethal (LT_{50}) temperatures of some littorinid snails of the family Littorinidae from tropical, subtropical and temperate regions.

Taxon		Distribution		Tolerance temperatures (°C)		Reference
Genus	**Species**	**Bioregion**	**Vertical**	**HCT**	**LT_{50}**	
Echinolittorina	*E. malaccana*	Tropical	Eulittoral fringe	46.8	59.0	Cleland and McMahon, 1986
				-	50.04	Lee and Lim, 2009
				-	56.5	Marshall *et al*., 2011
	E. vidua	Tropical	Eulittoral to lower eulittoral fringe	44.5	56.5	Cleland and McMahon, 1986
				-	48.1	Lee and Lim, 2009
				-	54.7	Marshall *et al*., 2011
	E. peruviana	Tropical/Temperate	Eulittoral zones and fringe	37	-	Muñoz *et al*. 2005
	E. natalensis	Subtropical	Eulittoral fringe	37.2	56.4	This study
Nodilittorina	*N. leucosticta*	Tropical	Eulittoral fringe	-	60.0	Suryanarayanan and Nair, 1979
	N. pyramidalis	Tropical	Littoral fringe	48.5	56.5	Stirling, 1982
			Littoral fringe	46.5	-	Cleland and McMahon, 1986
			Eulittoral fringe	46.3	-	McMahon, 2001b
	N. millegrana	Tropical	Littoral fringe	46.0	56.5	Stirling, 1982
	N. exigua	Tropical	Littoral fringe	44.8	-	Cleland and McMahon, 1986
			Eulittoral fringe	44.8	-	McMahon, 2001b
	N. natalensis	Tropical	Littoral Fringe	46.0	53.5	Stirling, 1982
	N. unifasciata	Tropical	Eulittoral zones	41.3	-	McMahon, 1990
			Eulittoral fringe	41.1	-	McMahon, 2001b
Littorina	*L. undulata*	Tropical	Eulittoral zone	-	55.0	Suryanarayanan and Nair, 1979
	L. krausii	Tropical	Littoral fringe	45.0	53.0	Stirling, 1982
	L. saxatilis	Tropical/Temperate	Littoral fringe	37	45	Evans, 1948
				32	40	Sandison, 1967
			Eulittoral fringe	38.2	-	McMahon, 2001b
				35.0		Davenport and Davenport, 2005
	L. saxatilis "H"	Tropical/Temperate	Eulittoral fringe	31.52	41.3	Clarke *et al*., 2000b
				31.63/31.96	-	Backeljau *et al*., 2001
	L. saxatilis "M"	Tropical/Temperate	Eulittoral fringe	31.72	43.8	Clarke *et al*., 2000b
				33.00/31.97	-	Backeljau *et al*., 2001
	L. saxatilis "B"	Tropical/Temperate	Eulittoral zone	30.76	-	Clarke *et al*., 2000b

	L. arcana	Tropical/Temperate	Eulittoral fringe	33.13/32.70	-	Backeljau *et al*., 2001
				31.71	41.5	Clarke *et al*., 2000b
	L. compressa	Tropical/Temperate	Eulittoral fringe	34.16	-	Backeljau *et al*., 2001
	L. littorea	Tropical/Temperate	Eulittoral	39	46	Evans, 1948
			Eulittoral zone	31	40	Sandison, 1967
			Eulittoral zone	-	40-41	Fraenkel, 1960
			Eulittoral zone	30.16	43.8	Clarke *et al*., 2000b
				33.43/31.48	-	Backeljau *et al*., 2001
			Eulittoral	32.0	-	McMahon, 2001b
			Eulittoral zone	35.3	-	Davenport and Davenport, 2005
	L. fabalis	Tropical/Temperate	Eulittoral	30.60	-	Clarke *et al*., 2000b
			Eulittoral zone	29.9	-	Davenport and Davenport, 2005
	L. obtusata	Tropical/Temperate	Eulittoral zone	30.66	40.2	Clarke *et al*., 2000b
				35.93/32.06	-	Backeljau *et al*., 2001
			Mid Eulittoral zone	29.6	-	Davenport and Davenport, 2005
			Lower eulittoral	28.3	-	McMahon, 2001b
	L. brevicula	Tropical	Eulittoral zone	39.9	-	Cleland and McMahon, 1986
			Eulittoral fringe	40.1	-	McMahon, 2001b
	L. neglecta	Tropical/Temperate	Eulittoral zone	30.63	-	Clarke *et al*., 2000b
	L. littoralis	Tropical/Temperate	Eulittoral zone	36	44.3	Evans, 1948
				30	40	Sandison, 1967
	L. neritoides	Tropical/Temperate	Eulittoral zone	38	46.3	Evans, 1948
				35	42	Sandison, 1967
Littoraria	*L. glabrata*	Tropical/Subtropical	Eulittoral fringe	37.4	53.8	This study
	Littoraria spp.	Tropical/Subtropical	Eulittoral fringe	-	47.5	Lee and Lim, 2009
				43.9	-	McMahon, 2001b
Afrolittorina	*A. africana*	Subtropical	Eulittoral zone	35.3	51.4	This study
		Warm-temperate	Eulittoral fringe	34.6	51.1	This study
	A. knysnaensis	Warm-temperate	Eulittoral fringe	32.8	50.1	This study
		Cool-temperate	Eulittoral fringe	33.1	50.0	This study
		Cool-temperate	Eulittoral fringe	-	48.6	See Evans, 1948

"H", "M" and "B" represent high-shore, mid-shore and barnacle-dwelling ecotypes of *L. saxatilis*. HCT = heat coma temperature; LT_{50} = lethal temperature.

Likewise, eulittoral fringe species show higher tolerances than eulittoral zone and subtidal species, respectively (see Table 3.1; Cuculescu *et al*., 1998; Stillman and Somero, 1999; 2000; Backeljau *et al*., 2001; Sorte and Hofmann, 2005; Miller *et al.*, 2009; Nguyen *et al*., 2011; Madeira *et al*., 2012a). In addition, the differences in thermal tolerance between low and high intertidal species are greatest for temperate species (Stillman and Somero, 1996; 1999; Stillman, 2002; Compton *et al*., 2007). Members of the family Littorinidae show particularly high tolerances (Table 3.1; McMahon, 1990; 2001a; Nguyen *et al*., 2011). Thus, there are species-specific ranges of tolerance (Díaz *et al*., 2002), with tropical and high shore species consistently demonstrating higher tolerances than temperate and low shore species (McMahon, 1990; Britton, 1992; Stillman and Somero, 1996; 1999; Davenport and Davenport, 2005; Nguyen *et al*., 2011). But the specific temperatures that are tolerated differ depending upon whether a species is studied in summer or winter, or acclimated in the laboratory. Differences in thermal tolerances can also be explained by differences in the conditions animals experience in their microhabitats (see Stillman and Somero, 1996; Nakano and Iwama, 2002; Stillman, 2002; Morley *et al*., 2009; Sorte *et al*., 2011; Madeira *et al*., 2012b; Vinagre *et al*., 2012). For example, Sanders *et al*. (1991) found that the limpet *Collisella scabra* which inhabits the exposed high intertidal zone had a greater tolerance to acute heat shock than *C. pelta* which lives in the more protected upper midtidal region.

Differences in thermal tolerances can relate not only to extrinsic and intrinsic factors, but also to combinations of, or interactions between these factors. This is because multiple factors, rather than single factors (e.g. temperature) are encountered in the natural environment (see Backeljau *et al*., 2001; Roelofs *et al*., 2008; Nicastro *et al*., 2010; Zippay and Helmuth, 2012). For example, salinity, oxygen, carbon dioxide (CO_2) and chemicals as well as activity, size, sex, nutrition and health can significantly influence an animal‟s response to temperature. Salinity can have different effects on thermal tolerance of animals when in combination with temperature (see Hicks, 1973; McMahon and Russell-Hunter, 1981; Li and Brawley, 2004; Re *et al*., 2005; 2006). For example, Todd and Dehnel (1960) found that salinity had a marked effect on the temperature tolerance of two grapsid crabs, *Hemograpsus nudus* and *H. oregonensis*. Dehnel (1960) found that animals of the above species when acclimated at a combination of high temperature and salinity showed higher tolerance than those acclimated to both low temperature and salinity. Sherman and Eichrodt (1982) found that a combination of low salinity and temperature was most stressful and resulted in higher mortality than other

combinations. Thus, tolerance of high temperatures was best under high temperature and high salinity (see Nagabhushanam and Sarojini, 1969).

As for salinity, oxygen levels affect thermal tolerance. In the whelk *Nucella lapillus*, thermal tolerance increased under hyperoxic conditions and decreased under hypoxic conditions; while in *Littorina littorea* oxygen levels did not affect thermal tolerance (Davenport and Davenport, 2007). Several studies on invertebrates and fishes have shown that oxygen concentration influences temperature tolerance via oxygen limitation (Frederich and Pörtner, 2000; Mark *et al*., 2002; Peck, 2002; Pörtner *et al*., 2004a; Lannig *et al*., 2004; Jansen *et al*., 2009; etc). This is also true for high temperature-induced systemic hypoxia (Pörtner *et al*. 2000; Pörtner, 2001; 2002a, b; 2012; Peck *et al*., 2002; Anestis *et al*., 2008; Kassahn *et al*., 2009). Carbon dioxide levels as well as ocean acidification also influence temperature tolerance via oxygen limitation (Pörtner, 2008; 2012; Walther *et al*., 2009; Lannig *et al*., 2008; 2010; Christensen *et al*., 2011). For example, Metzger *et al*. (2007) found a 5°C decrease in the upper thermal limits of aerobic scope of the edible crab *Cancer pagurus* exposed to elevated CO_2.

The effects of size are contradictory, with some studies suggesting that an animal‟s size affects thermal tolerance, while others did not (see Todd and Dehnel, 1960; Jensen and Armstrong, 1991; Backeljau *et al*., 2001; Ospína and Mora, 2004; Peck *et al*., 2009b; Nguyen *et al*., 2011; Madeira *et al*., 2012b). Smaller animals seem more tolerant in some species, while adults are more tolerant in others. Clarke *et al*. (2000a, b) found that larger individuals of *Littorina littorea* showed heat coma at significantly lower temperatures than juveniles, while Hicks and McMahon (2002b) found that smaller individuals of the invasive mussel *Perna perna* were less temperature tolerant than larger individuals. Lee and Boulding (2010) found that body size did not significantly affect thermal tolerance in the intertidal snail, *Littorina keenae*.

High aerobic scope (i.e. activity) results in a greater physiological capacity to cope with elevated temperature, leading to higher tolerance limits (see Pörtner *et al*., 2000; Pörtner, 2001; 2002a, b; 2010; Peck *et al*., 2009b; Storch *et al*., 2009; Nguyen *et al*., 2011).

Nutritional status (e.g. starvation) can also have effects on an animals‟ ability to cope with heat stress (see Dahlhoff, 2004; McCue, 2010; Terblanche *et al*., 2011; Zardi *et al*., 2011; Fitzgerald-Dehoog *et al*., 2012) and thus tolerance limits. Infection (which also depend on the type and intensity) by macroparasites such as trematodes is known to lead to reduced resistance to extreme (high and low) temperatures (see Berger and Kharazova, 1997; Curtis, 2002; Granovitch *et al.*, 2000; Meißner and Schaarschmidt, 2000; Bates *et al*., 2011). For example, infected individuals of the snail *Biomphalaria glabrata* had lower thermal tolerance than uninfected individuals under the same temperature treatments (Lee and Cheng, 1971). Chemicals or pollutants combined with high temperature result in a reduction of thermal tolerance (see Lannig *et al*., 2006; Sokolova and Lannig, 2008).

The effect of acclimation or previous thermal history (see Nagabhushanam and Sarojini, 1969; Vernberg, 1969; Cuculescu *et al*., 1998; Zakhartsev *et al*., 2003; Dong *et al*., 2008a; Middlebrook *et al*., 2008; Sunday *et al*., 2012) and season (see Todd and Dehnel, 1960; Newell *et al*., 1971; Backeljau *et al*., 2001; Hopkin *et al*., 2006; Stillman and Tagmount, 2009; Sunday *et al*., 2012) as well as parental thermal history (see Stillman and Somero, 2000; Li and Brawley, 2004; Byrne *et al*., 2010; Zerebecki and Sorte, 2011) can also contribute to differences in thermal tolerances. Clarke *et al*. (2000a, b) found both acclimation (at elevated temperatures) and previous thermal history influenced heat coma temperatures in *Littorina littorea*. A brief or prior heat shock (sudden exposure) to moderate temperature can induce increased thermal tolerance also called „induced tolerance‟ during subsequent thermal stress (see Parsell *et al*., 1993; Stillman and Somero, 1999; Hopkin *et al*., 2006; Li *et al*., 2007; Dong and Dong, 2008), and this is linked to the up-regulation of heat shock proteins and/or stability of proteins or enzymes (see Feder and Hofmann, 1999; Pörtner and Knust, 2007; Ulrich and Marsh, 2009; etc). However, sudden exposure to elevated temperatures can also cause mortality.

Differences in experimental methods (design and protocols) can also explain some of the differences observed. For example, Clarke *et al.* (2000b) and Lee and Lim (2009) determined the lethal thermal limits of *Littorina* spp., *Littoraria* spp. and *Echinolittorina* spp. in water, while most studies were done on dry (i.e. aestivating) animals. Thus, as a result of differences in thermal conductivities between media (see Madeira *et al*., 2012a), body temperature can

respond much quicker to temperature changes in water than in air leading to differences in tolerances. However, Sandison (1967) found that the heat coma and lethal temperatures of intertidal gastropod snails were higher in air than water. Jones *et al*. (2009) found a difference of 0.7°C in June and 4.8°C in November in the thermal limits of *Mytilus edulis* in water and air, respectively.

Most studies have tested the effects of prolonged exposure to temperature lasting for hours or days (see Evans, 1948; Newell *et al*., 1971; Pörtner and Helmuth, 2007; Miller *et al.*, 2009; Dilly *et al*., 2012), without taking into account the possible effects of short-term exposure to sudden heat stress, which can be particularly frequent in the intertidal environment. The latter is probably more useful when making comparisons with field conditions (see Joyner-Matos *et al*., 2009; Terblanche *et al*., 2011). For example, *Echinolittorina malaccana* and *E. vidua* had lethal temperatures of 50.04 and 48.10°C respectively when exposed for 1 hour at particular temperatures (Lee and Lim, 2009). In contrast, Marshall and McQuaid (2010) found lethal temperatures of 59.0 and 56.5°C for the same species when exposed for 5 minutes at particular temperatures.

Most studies have determined temperature tolerance limits using static (constant) methods, while others have used dynamic (ramping) methods, resulting in different results (see below). In addition, the rates at which temperature is increased (i.e. rate of heating) have different effects on thermal tolerance of animals from different environments (see Evans, 1948; Ospina and Mora, 2004; Mora and Maya, 2006; Angilletta Jr., 2009; Nguyen *et al*., 2011; Richard *et al*., 2012). Although some results are contradictory, slow rates generally result in lower tolerance limits than fast rates (Reese, 1969; Segnini De Bravo *et al*., 1998; Chown *et al*., 2009; Peck *et al*., 2009b; Nguyen *et al*., 2011). However, slower rates can also provide sufficient time for hardening, a form of phenotypic plasticity that protects cells from subsequent exposure (see references in Terblanche *et al*., 2007) resulting in higher tolerance limits. In addition, stresses due to starvation and/or desiccation can arise during ramping (see Terblanche *et al*., 2007; 2011), and this can affect animals‟ ability to handle heat stress.

Differences in metrics (e.g. sublethal versus lethal) used to measure tolerances can also result in different results (see McMahon, 1990; Clarke *et al*., 2000a, b, c; Terblanche *et al*., 2007; Sunday *et al*., 2012). Repeated or multiple exposure to heat stress or events is known to result in a decrease in thermal tolerance in other studies (see Jones *et al*., 2009, Clarke *et al*., 2000a), though other studies show the opposite (see Buckley *et al*., 2001; Middlebrook *et al*., 2008). In a study on heat coma in *Littorina littorea*, Clarke *et al*. (2000a) found that the temperature when heat coma sets in decreased significantly with repeated daily but not weekly exposure. In addition, the effect of start temperature which can differ by investigators when species or populations from different thermal environments are examined is poorly understood (see Terblanche *et al*., 2007). Thus, the wide variety of methods and protocols used might have contributed to the differences reported in various studies.

Overall, differences in the tolerances of littorinid snails and other intertidal ectotherms seem to relate to differences in their biogeography, ecology, and phylogeny. McMahon (1990) suggests that tropical littorinids fill a completely new niche in the eulittoral fringe and that this requires a completely different physiology to other littorinids. He further suggests that eulittoral fringe rocky shore species have different adaptive physiological attributes that allow them to cope with temperature stress. For example, most exhibit foot withdrawal to prevent heat conduction from the substratum, aestivation in air, high thermal tolerances and can use mucus to cement a small area of the lip of the shell to the substratum to minimize heat uptake and increase their capacity for heat dissipation. All of these attributes distinguish them from eulittoral species which might benefit from behavioural mechanisms such as evaporative cooling and formation of aggregations to regulate body temperature. Thus, eulittoral fringe species are better able to regulate heat uptake and cope with heat and desiccation stress than their eulittoral and low shore counterparts.

Littorinids are characteristic of high shore levels worldwide (see Reid, 1989; 1996a, b; 2002; Chapman and Underwood, 1994; McQuaid, 1996a, b; Lee and Boulding, 2010; etc) and are extremely tolerant of temperature and desiccation (see McMahon, 1990; 2001b; Backeljau *et al*., 2001; Emson *et al*., 2002; Marshall and McQuaid, 2010; etc). Tolerance of marine intertidal animals to temperature and desiccation correlates to their position in the intertidal

zone and their geographical distribution. Thus, the ability of littorinids to cope with and survive high temperatures and desiccation is related to their distribution patterns.

Although much is known about thermal and desiccation tolerance of littorinids, very little is known about the thermal and desiccation tolerance of South African littorinids from temperate and subtropical regions. A previous study by McQuaid and Scherman (1988) found a 1°C difference in lethal thermal limits (LT_{50}) between the two *Afrolittorina* spp. collected in the warm temperate bioregion, with higher tolerance in *A. africana* than *A. knysnaensis*. To my knowledge, no studies have looked at the temperature (i.e. heat) and desiccation tolerance of the subtropical *E. natalensis* and *L. glabrata*.

Therefore, the study aims to compare thermal tolerance of *Afrolittorina* spp. particularly where their distribution overlaps in the warm temperate region of South Africa and they can co-exist on the same shores. In addition, the tolerance of these *Afrolittorina* spp. was compared to that of the subtropical *E. natalensis* and *L. glabrata*. Heat coma and lethal temperatures were assessed using approaches followed by McMahon (1990) and Clarke *et al.* (2002a, b, c) with minor modifications, and it was hypothesised that the tolerance of *Afrolittorina* spp. from warm temperate regions will be similar, but different from that of *E. natalensis* and *L. glabrata* from the subtropical region. Furthermore, *A. africana* can be found in the subtropical region of the country and it was hypothesised that the tolerance of individuals from the subtropical region would differ from that of individuals from the warm temperate region; likewise for *A. knysnaensis* from the warm and cool temperate regions.

3.2. Materials and methods

3.2.1. Study species

Four littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* were used, namely: *A. knysnaensis*, *A. africana*, *E. natalensis* and *L. glabrata*. See Chapter 1 for species distribution ranges and patterns of vertical zonation as well as microhabitat use and aestivation behaviour.

3.2.2. Collection and transportation

Specimens of *A. africana*, *A. knysnaensis*, *E. natalensis* and *L. glabrata* were collected from natural rocks at different sites (see Fig. 3.1; Table 3.2) along the South Africa‟ coast between late 2009 and early 2011 during winter and summer months in order to investigate the effect of season on thermal tolerance. The selected sampling sites ranged from Ballito in the subtropical region to Strandfonteinpunt in the cool temperate region of South Africa (see Fig. 3.1). Individuals of each species were collected from the upper levels occupied (i.e. eulittoral fringe and eulittoral zone) depending on the site and level/s occupied by each species. Large and small individuals of each species that were feeding or had fed within 12 hours (assumed to have fed since they were collected while wet immediately after or during high tides) were returned to the laboratory in plastic bags placed inside an insulated cool box.

3.2.3. Handling and treatment conditions

On arrival at the laboratory, specimens were washed in seawater, allowed to emerge from their shells and to reattach to 2 L lidded plastic containers in seawater before being exposed to air at room temperature (18-22ºC), when they exhibited behavioural emergence. Thus, specimens were allowed to rehydrate for at least 3 hours for same day use or overnight if

used later, after which active individuals were selected for experimental treatments. Selected animals were blotted dry with paper towel and dried using a fan at room temperature. Specimens were kept on dry paper towel at room temperature (18-22ºC) immediately before use.

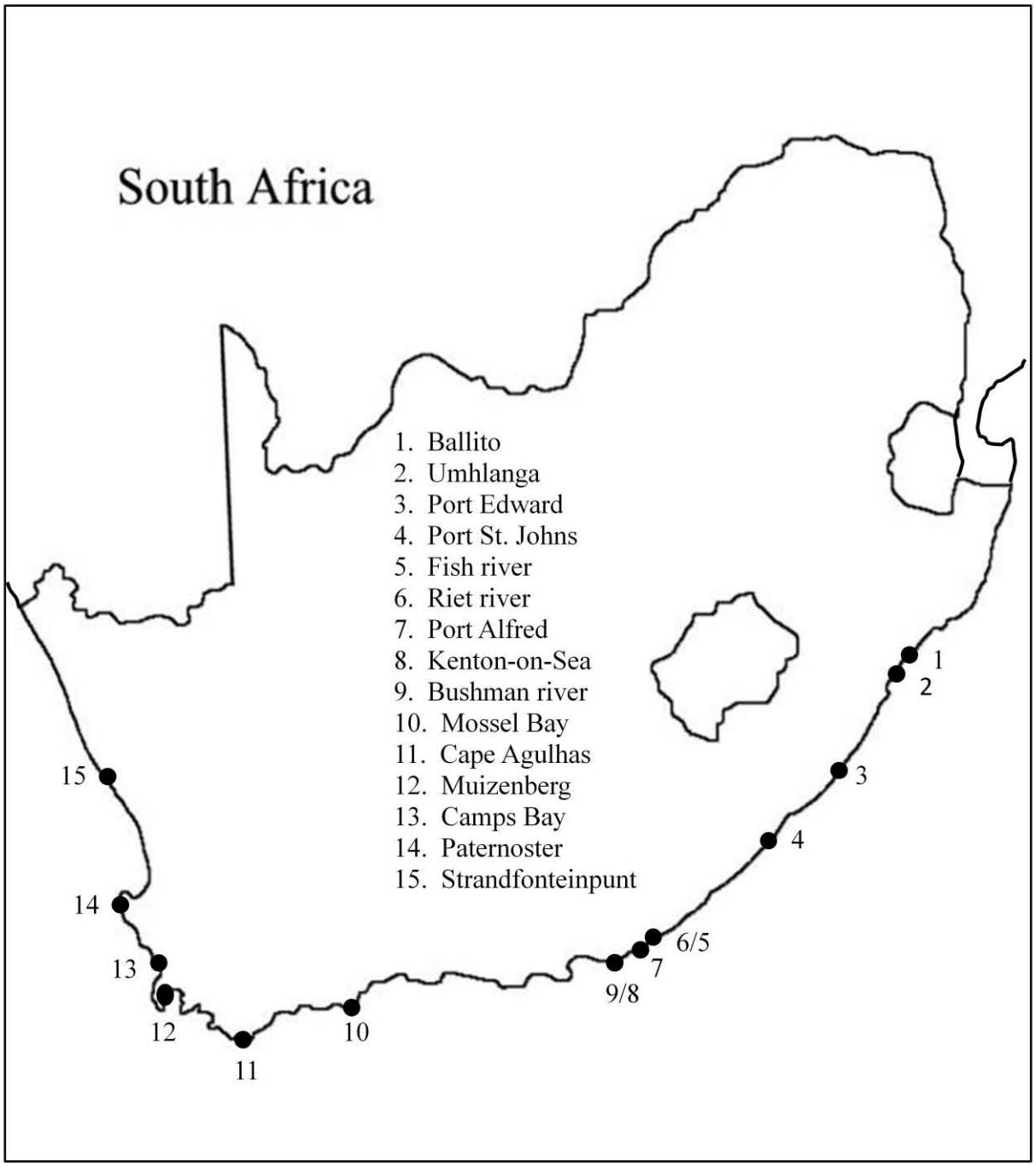

Figure 3.1. Map of South Africa showing sampling sites (see Table 3.2) for littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* used for thermal tolerance experiments.

Table 3.2. Sampling sites for littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from South Africa used for thermal tolerance experiments.

Bioregion	**Site (abbreviation)**	**Species sampled**	**Experiment**	
			Heat coma	**Lethal limits**
Subtropical	1. Ballito (BA)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*	Yes	Yes
	2. Umhlanga (ML)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*	Yes	Yes
	3. Port Edward (PE)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*	Yes	Yes
	4. Port St. Johns (PJ)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*	Yes	Yes
Warm temperate	5. Fish river (FR)	*A. knysnaensis*; *A. africana*	Yes	Yes
	6. Riet river (RR)	*A. knysnaensis*; *A. africana*	Yes	Yes
	7. Port Alfred (PA)	*A. knysnaensis*; *A. africana*	Yes	No
	8. Kenton-on-Sea (KOS)	*A. knysnaensis*; *A. africana*	Yes	No
	9. Bushman river (BU)	*A. knysnaensis*; *A. africana*	Yes	Yes
	10. Mossel Bay (MBB)	*A. knysnaensis*; *A. africana*	Yes	Yes
Cool temperate	11. Cape Agulhas (CA)	*A. knysnaensis*	Yes	Yes
	12. Muizenberg (MU)	*A. knysnaensis*	Yes	No
	13. Camps Bay (CB)	*A. knysnaensis*	Yes	Yes
	14. Paternoster (PN)	*A. knysnaensis*	Yes	Yes
	15. Strandfonteinpunt (SF)	*A. knysnaensis*	No	Yes

To investigate the effect of acclimation on heat coma temperatures, specimens were acclimated at each of four different temperatures (20, 25, 30 and 35°C) for 14 days without feeding. In brief, 10 small and 10 large aestivating snails were placed in 20 ml dry plastic containers, and the containers holding the snails were then floated in a temperature-controlled digital waterbath (Labcon, SA) set at the appropriate acclimation temperature using Polystyrene holders. To maintain a uniform distribution of heat, a separate heating head (mgw Lauda, Germany) fitted with a stirrer was used for circulation of water in the waterbath. Water temperature and air temperature inside the containers were monitored using a Fluke 54II Thermometer (Fluke Corporation, USA) fitted with a T-type thermocouple (Fluke Corporation and Cromega) and/or a thermometer.

3.2.4. Determination of heat coma temperatures

Mean heat coma temperatures (HCT) were determined using a slightly modified version of the protocol used by McMahon (1990) and Clarke *et al.* (2002a, b, c) amongst others. The heat coma temperature in littorinid snails and other gastropods is defined as the temperature at which normal nervous function is lost, and is manifested by a cessation of activity such as locomotion, a ventral medial curling of the lateral edges of the foot and an inability to remain attached to the substratum (see Sandison, 1967; McMahon, 1990; Clarke *et al.*, 2002a, b, c; Lee and Boulding, 2010; etc). Thus, heat coma is a „non-lethal" condition characterised by loss of nervous integration.

In this study, I measured heat coma temperatures by recording the temperature at which snails (1) were no longer able to locomote and tentacle movement ceased, and (2) showed ventral curling of the foot, being unable to remain attached to the sides of the test tube or cotton plugs. This combination of criteria was used because individual snails displayed different behavioural responses during heat exposure. For example, some individuals remained motionless after attaching to the test tubes. This could occur even before they were transferred to the waterbath as well as during or after equilibration. In addition, some individuals never fell off as they were securely attached by a transparent mucus thread or film after withdrawing the foot into their shells. This is the same behavioural mechanism

used to escape unfavourable and stressful environmental conditions or to minimize contact with the substratum in the field (see Reese, 1969; McMahon, 1990; Emson *et al.*, 2002; Miller, 2008).

For each trial, 10 large and 10 small individuals of each species were placed in 50 ml test tubes containing 45 ml of seawater and allowed to attach to the test tube walls and actively locomote or crawl. For *Afrolittorina* spp. and *E. natalensis*, large was >9mm, for *L. glabrata* they were >15mm; while for all species small was < 5mm. For adults of *L. glabrata* which were larger, 10 individuals were placed in 80 ml test tubes filled with 75 ml seawater and allowed to attach. Individuals that did not attach during this time were discarded. Porous cotton plugs where pushed down the openings of the test tube to block the water surface. This kept the snails immersed and prevented them from escaping during heating.

At the start of an experiment, test tubes containing snails were placed on a wooden test-tube rack which was then placed in a temperature-controlled digital waterbath set to 20ºC, and animals were allowed to equilibrate for 20-30 minutes. Four to eight test tubes were placed into the experimental waterbath so that all tested snails could be simultaneously watched during the course of the experiment. A separate heating head fitted with a stirrer was used to maintain uniform distribution of heat through regular circulation of water. Temperature in the water bath and inside a test vial were monitored using a Fluke 54II Thermometer fitted with a T-type thermocouple and/or a thermometer inserted into a separate empty test tube.

Waterbaths were adjusted manually to raise the water temperature by 1°C every 5 minutes, and test tube temperatures were monitored as described. This rate of increase in temperature has been found to make the lag between test tube water and snail tissue temperatures negligible (see Broekhuysen, 1940 in Lee and Boulding, 2010). The number of individuals entering heat coma was recorded after every 1°C increase in temperature. For each heat coma determination, observations started at 20°C, the equilibration temperature, and continued until every individual snail showed one of the criteria used for the diagnosis of heat coma.

Once all animals had entered heat coma or temperature had reached 45°C, test tubes were taken out of the waterbath and allowed to cool for at least five minutes at room temperature (19-22°C). The snails were gently placed into labelled Petri dishes with about 10 ml of seawater to allow recovery at ambient temperatures. This allowed determination of whether the snails recovered from heat coma and became active again. After 15 or 30 minutes, snails were inspected for survival and those with their foot extended and attached to the substratum, or those which responded to poking with blunt forceps were scored as alive. As expected, no snails had died from heat stress.

3.2.5. Lethal thermal limit (LT_{50}) determination

Acute upper lethal thermal limits were determined as lethal temperatures (LT_{50}; temperature at which 50% mortality of population occurs) using slightly modified protocols from McMahon (1990) and Clarke *et al.* (2002b). In this study, I measured lethal temperatures by recording the temperature at which snails were unable to (1) to attach to the Petri dishes and (2) respond to poking with blunt forceps after 12-24 h recovery following high temperature exposure.

Subsamples of 10 aestivating snails of each species (large or small as defined above) were placed in 20 ml plastic vials on a polystyrene holder. At the start of an experiment, the polystyrene holder, together with lidded vials containing snails, was placed in a digital waterbath set to 20ºC to equilibrate for 20-30 minutes. Circulation of water and monitoring of temperature were done as described above.

The waterbath was switched manually to raise the water temperature, initially at 5°C increments over 10 minute intervals to reach 40 or 45°C, after which temperature was increased at the rate of 1°C every 10 minutes. At 1°C intervals, starting at 45 or 50°C, three randomly selected vials were removed from the waterbath. The vials were allowed to cool for 5 minutes to ambient laboratory temperature (18-22°C), and then the snails were gently

placed into 8 cm lidded Petri dishes with about 10 ml of seawater to allow recovery. This allowed determination of whether the snails recovered from heat stress and became active again or were dead. After 12 or 24 hours, animals were inspected and those with their foot extended and attached to the substratum, or those which responded to poking with a blunt forceps were scored as alive, the remainder were assumed to be dead.

3.2.6. Data and statistical analysis

The data are presented as means ± SD, and figures were drawn using Excel. Statistical analyses were performed using Statistica 10 (Statsoft). General Linear Models Factorial ANOVA (Statistica 10, Statsoft) was used to determine differences using heat coma temperature and lethal temperature as dependent variables and species, size, season and treatment as fixed independent variables. Significance differences between and within a species, size, season or treatment were determined using different ANOVAs (two or three way-ANOVA), and significant results were explored using Tukey tests.

3.3. Results

When comparing the two metrics used to determine thermal tolerances, it was found that heat coma temperatures were always about 15-20°C lower than lethal temperatures (see Table 3.3). This was expected as heat coma temperatures represent temperatures that induce changes in the snails‟ behavioural responses (i.e. withdrawal into the shell and attachment of the shell to the substratum with mucus films), whereas lethal temperatures represent temperatures at which death sets in. Both methods showed similar trends with higher tolerances in species collected from the subtropics to lower tolerances in those from temperate shores (see Fig. 3.2 and 3.3).

3.3.1. Heat coma (HCT) temperatures and lethal thermal limits (LT_{50})

There was a difference in the two criteria (1) cessation of activity and (2) ventral curling of foot, hanging or falling used to score heat coma temperatures. Animals stopped activity (crawling and moving tentacles) about 3-5°C before ventral curling of the foot, hanging or falling. However, both criteria showed similar trends (see Fig. 3.2A) and thus complement each other. The first criterion was difficult to apply when dealing with animals that tended to aggregate and/or remain inactive after attaching to the test tube walls. This was observed when adults of *Afrolittorina* spp., and sometimes those of *E. natalensis* formed aggregations and did not locomote until ventral curling of the foot manifested. Since all the criteria yielded similar trends, interpretation of the results is presented as mean heat coma temperatures based on the second set of criteria, ventral curling of the foot, hanging or falling.

Likewise, when comparing the two methods (attachment and poking) used to score lethal thermal limits, it was clear that the attachment method always showed lower values than the poking method, indicating that the ability to remain attached was lost before the ability to respond to tactile stimulation. Despite a few exceptions, the two methods yielded similar trends (see Fig. 3.3A) and thus complemented each other. In addition, most studies have used the poking method, and to allow comparison of my data with those from other studies, the

results are presented as mean lethal temperatures based on the poking method. However, the poking method will be unreliable when interpreting animal responses to heat stress in nature. For example, most animals in this study showed weak responses (very slow retraction of the foot or operculum) after poking, and in most cases if not all, the same animals did not recover (e.g. attach and crawl) after hours or even days at room temperature. Thus, if the weak response is manifested in nature, animals will stand a very high chance of being swept away by waves during high tides and be effectively ecologically dead if not physiologically dead.

3.3.1.1. Are there phylogenetic differences in HCT and LT_{50} of the studied species?

Two-way ANOVA showed clear differences in the heat coma and lethal temperatures of the four species investigated (see Fig. 3.2B and 3.3B). In addition, there was no significant interaction between the two factors (i.e. species and size) for either heat coma or lethal temperatures. For heat coma, the two exclusively subtropical *Echinolittorina* and *Littoraria* species did not differ significantly, but had significantly ($F_{3.55} = 50.24$; $p < 0.001$) higher heat coma temperatures than the subtropical/temperate *Afrolittorina* spp. (see Fig. 3.2B). *L. glabrata* showed the highest heat coma temperatures followed by *E. natalensis* > *A. africana* > *A. knysnaensis* (Tukey test, see Table 3.3). Unexpectedly, juveniles (small) of all species showed significantly ($F_{1.55} = 51.41$; $p < 0.001$) higher heat coma temperatures than adults (large) (see Fig. 3.2B and Table 3.3).

As for heat coma, the two exclusively subtropical *Echinolittorina* and *Littoraria* species showed significantly ($F_{3.64} = 104.56$; $p < 0.001$) higher lethal limits than the subtropical/temperate *Afrolittorina* spp. (see Fig. 3.3B). *E. natalensis* showed the highest lethal temperatures followed by *L. glabrata* > *A. africana* > *A. knysnaensis*, (Tukey test, see Table 3.3). Except for *L. glabrata* where adults and juveniles showed similar lethal temperatures, adults of other species showed significantly ($F_{1.64} = 4.15$; $p < 0.001$) higher lethal temperatures than conspecific juveniles (see Fig. 3.3B and Table 3.3).

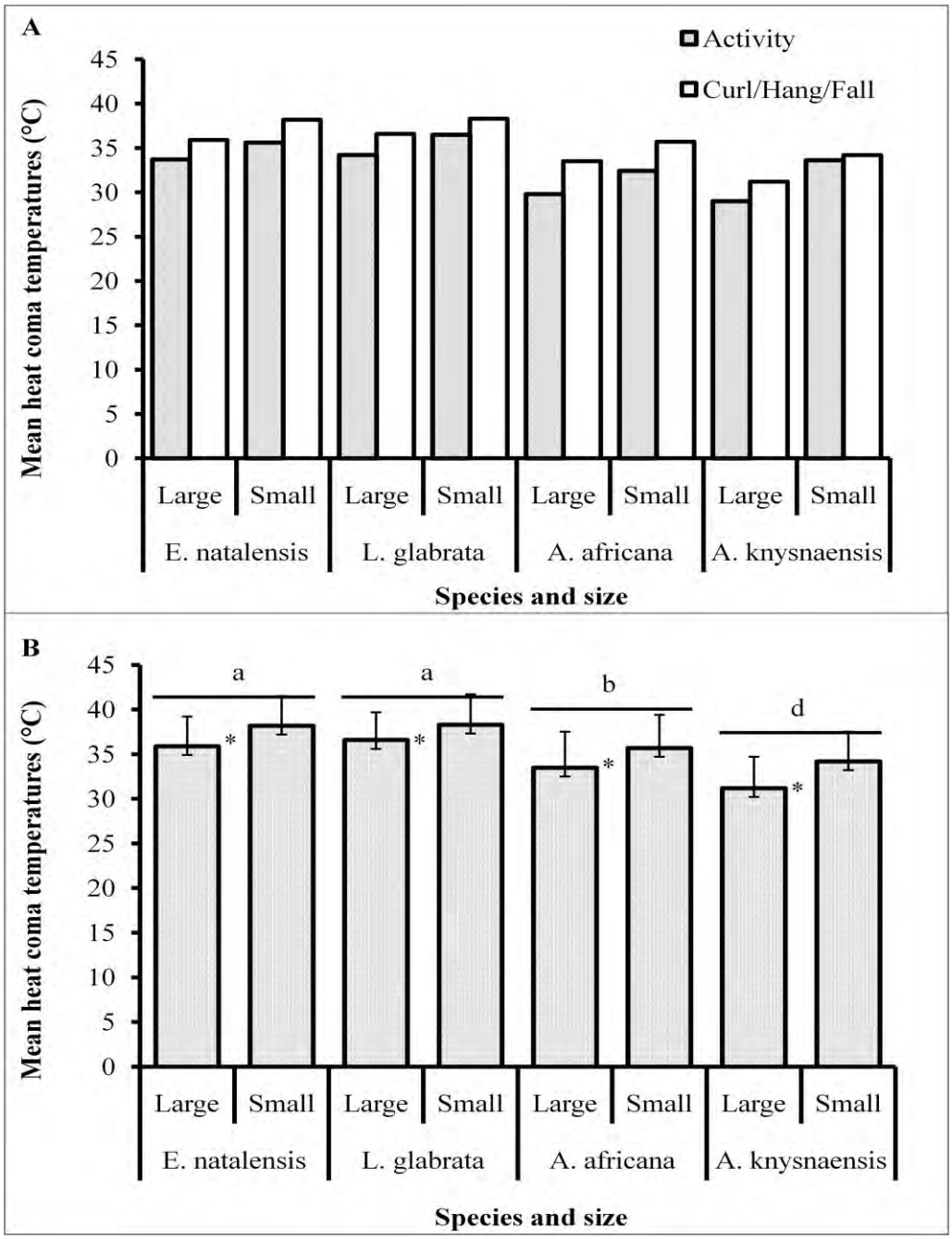

Figure 3.2. Mean heat coma temperatures of *E. natalensis*, *L. glabrata*, *A. africana* and *A. knysnaensis* from South Africa. (A) results using two different criteria, (1) cessation of activity and (2) ventral curling of foot, hanging or falling used to score heat coma temperatures; (B) enlarged data from criterion (2). Histograms are means + SD of different measurements. Different letters and asterisks (*) indicate significant differences between and within species respectively as determined using two-way ANOVA ($p < 0.05$).

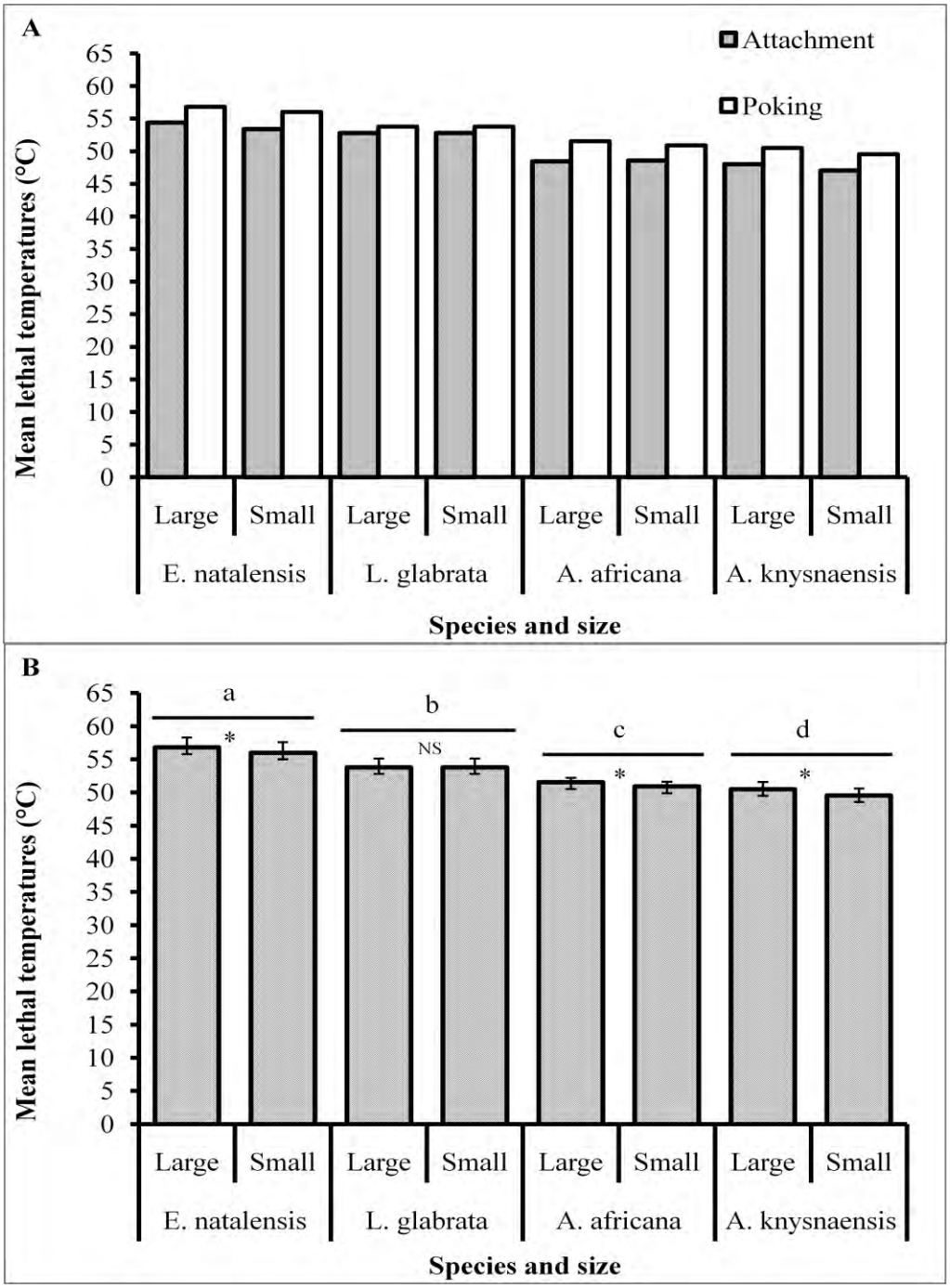

Figure 3.3. Mean lethal temperatures of *E. natalensis*, *L. glabrata*, *A. africana* and *A. knysnaensis* from South Africa. (A) results using two different methods, (1) attachment and (2) poking used to score lethal thermal limits; (B) enlarged data from method (2). Histograms are mean + SD of different measurements. Different letters and asterisks (*) indicate significant differences between and within species respectively as determined using two-way ANOVA ($p < 0.05$); NS = non-significant.

Table 3.3. Mean heat coma (HCT) and lethal (LT_{50}) temperatures of littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from South Africa. Values are means + SD; Large and Small are defined in main text.

Taxa	Temperatures (°C)			
	Heat coma (HCT)		Lethal (LT_{50})	
	Large	Small	Large	Small
E. natalensis	35.9±3.3	38.2±3.3	56.8±1.5	56.0±1.6
L. glabrata	36.6±3.1	38.3±3.4	53.8±1.3	53.8±1.3
A. africana	33.5±4.0	35.7±3.7	51.5±0.7	50.9±0.7
A. knysnaensis	31.2±3.5	34.2±3.3	50.5±1.1	49.6±1.0

3.3.1.2. Do species from the same region show the same HCT and LT_{50}?

Two-way ANOVA showed differences in heat coma and lethal temperatures among species within the subtropics and the warm temperate region (see Fig. 3.4 and 3.5). In the subtropics, the eulittoral fringe to eulittoral zone *E. natalensis* and *L. glabrata* showed significantly ($F_{2.22} = 9.90$; $p < 0.001$) higher heat coma temperatures than the eulittoral to low shore *A. africana* (Tukey test, see Fig 3.4A). Unexpectedly, *L. glabrata* adults showed non-significantly higher heat coma temperatures than adult *E. natalensis* (Tukey test, see Fig. 3.4A and Table 3.4).

In addition, there was a significant ($F_{1.22} = 19.18$; $p < 0.001$) difference between adults and juveniles of all species (see Fig. 3.4A). In the warm temperate region, there was a significant interaction ($F_{1.25} = 6.40$; $p < 0.05$) between species and size on heat coma temperatures of *Afrolittorina* spp. Juveniles showed significantly ($F_{1.25} = 34.11$; $p < 0.001$) higher tolerances than adults for both species (see Fig. 3.4B); but the effect was stronger for *A. knysnaensis*. For both size classes, *A. africana* showed significantly ($F_{1.25} = 52.10$; $p < 0.001$) higher heat coma temperatures than *A. knysnaensis* (see Fig. 3.4; Table 3.4).

Table 3.4. Mean heat coma temperatures (± SD) of large and small littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from different regions of South Africa. Large and Small are defined in main text.

Taxa	**Heat coma temperatures (°C)**					
	Subtropical		**Warm temperate**		**Cool temperate**	
	Large	**Small**	**Large**	**Small**	**Large**	**Small**
E. natalensis	35.9±3.3	38.2±3.3				
L. glabrata	36.6±3.1	38.3±3.4				
A. africana	32.8±3.4	35.8±2.8	33.9±2.9	35.5±1.9		
A. knysnaensis			31.2±2.9	34.3±2.4	31.1±3.4	34.9±2.5

As for heat coma, in the subtropics, the eulittoral fringe to upper eulittoral *E. natalensis* showed significantly ($F_{2, 24} = 41.23$; $p < 0.001$) higher lethal temperatures than the eulittoral fringe *L. glabrata* and the eulittoral *A. africana* (Tukey test, see Fig 3.5A; Table 3.5). With the exception of *L. glabrata*, for which adults and juveniles showed very similar lethal temperatures, adults showed higher lethal temperatures than juveniles (Tukey test, see Table 3.6), though the effect was not significant (see Fig. 3.5A).

On the other hand, in the warm temperate region for both sizes, *A. africana* showed significantly ($F_{1.20} = 9.10$; $p < 0.01$) higher lethal thermal limits than *A. knysnaensis* (Tukey test, see Fig. 3.5B). In addition, adults of both species showed significantly ($F_{1.20} = 9.10$; $p < 0.01$) higher lethal temperatures than juveniles (Tukey test, see Fig. 3.5).

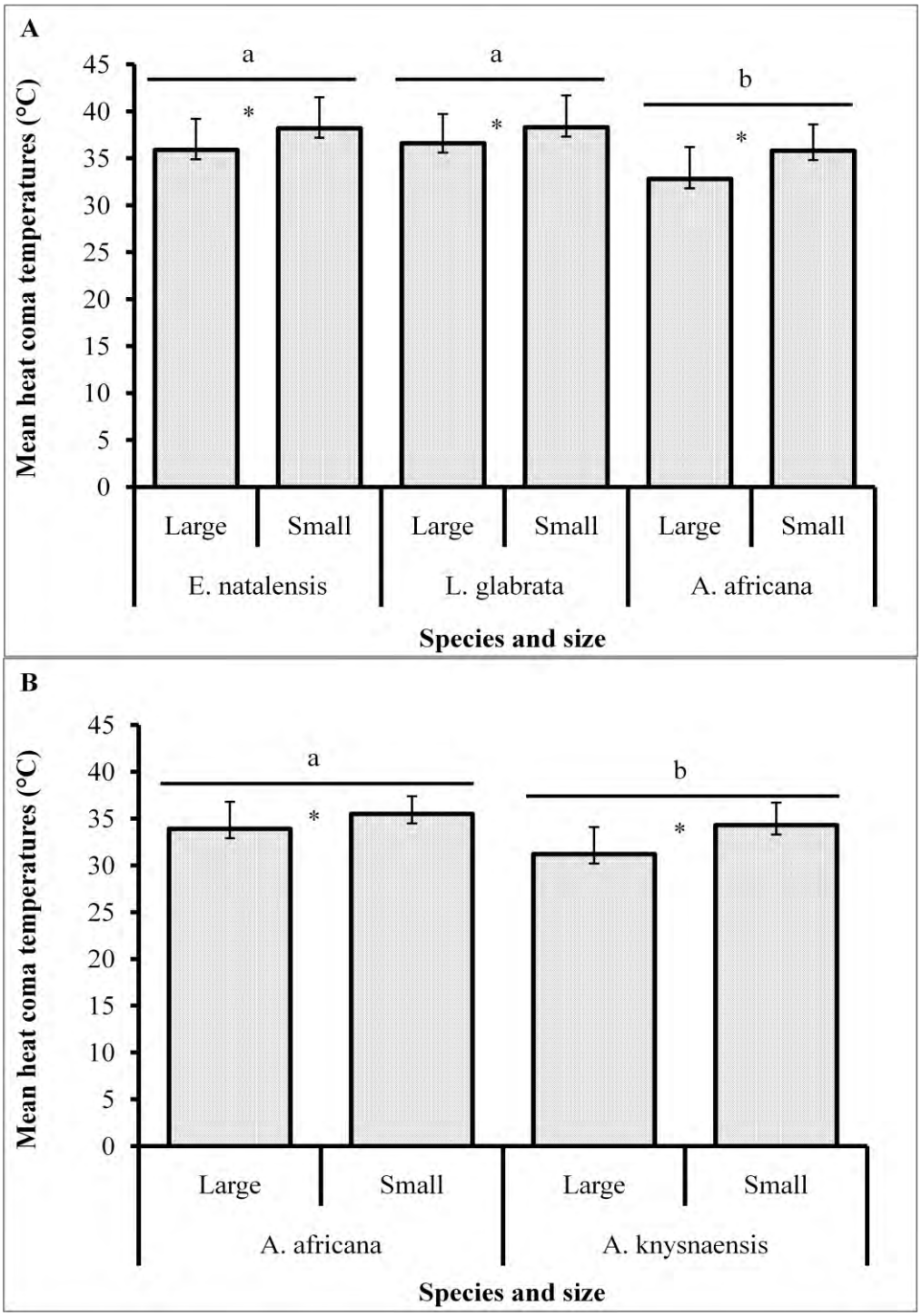

Figure 3.4. Mean heat coma temperatures of (A) *E. natalensis*, *L. glabrata* and *A. africana* from subtropical and (B) *A. africana* and *A. knysnaensis* from warm temperate regions. Histograms are mean + SD of different measurements. Different letters and asterisks (*) represent significant differences between and within species respectively as determined using two-way ANOVA ($p < 0.05$).

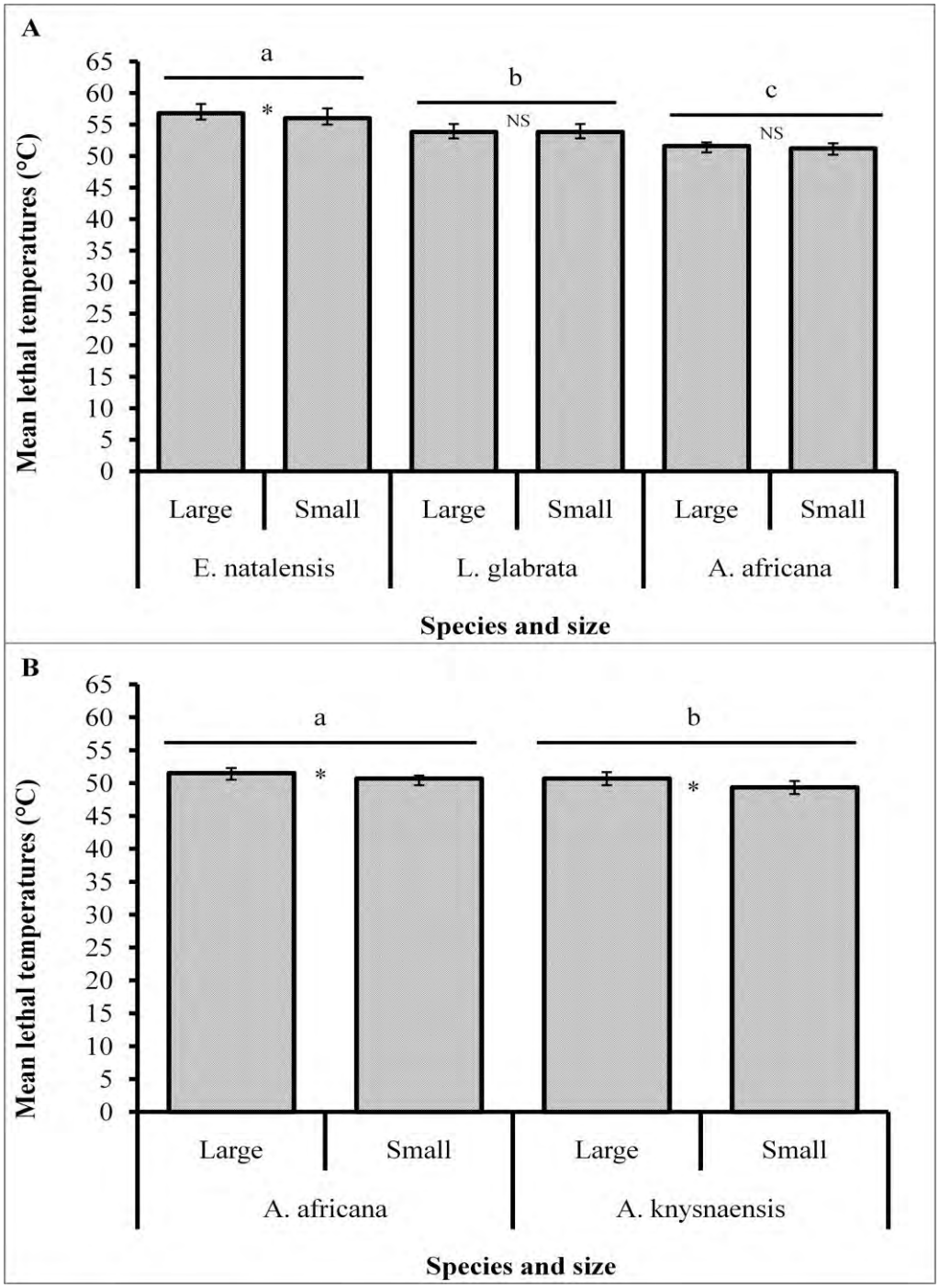

Figure 3.5. Mean (+SD) lethal temperatures (LT_{50}) of (A) *E. natalensis*, *L. glabrata* and *A. africana* from subtropical and (B) *A. africana* and *A. knysnaensis* from warm temperate regions. Histograms are mean + SD of different measurements. Different letters and asterisks (*) represent significance differences between and within species respectively as determined using two-way ANOVA ($p < 0.05$); NS = non-significant.

Table 3.5. Mean lethal temperatures (± SD) of large and small littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from different regions of South Africa. Large and Small are defined in main text.

Taxa	**Lethal temperatures (°C)**					
	Subtropical		**Warm temperate**		**Cool temperate**	
	Large	**Small**	**Large**	**Small**	**Large**	**Small**
E. natalensis	56.8±1.5	56.0±1.6				
L. glabrata	53.8±1.3	53.8±1.3				
A. africana	51.6±0.6	51.2±0.8	51.5±0.8	50.7±0.5		
A. knysnaensis			50.7±1.0	49.3±1.0	50.2±1.1	49.7±1.1

3.3.1.3. Is HCT and LT_{50} affected by region?

Comparing the heat coma and lethal temperatures of *A. africana* from subtropical and warm temperate regions and of *A. knysnaensis* from cool and warm temperate regions revealed that there were differences in thermal tolerances of conspecifics from different regions, but these differences were not significant (see Fig. 3.6 and 3.7; Table 3.4 and 3.5). In *A. africana*, adults from the warm temperate region showed unexpectedly, but not significantly ($F_{1.19}$ = 3.53; $p > 0.05$), higher heat coma temperatures than those from the subtropics and *vice versa* for juveniles (see Fig. 3.6A and Table 3.4).

On the other hand, juveniles of *A. knysnaensis* from the cool temperate region showed unexpectedly, but not significantly ($F_{1.16}$ = 0.37; $p > 0.05$), higher heat coma temperatures than their warm temperate counterparts, while there was very little difference in heat coma temperatures for adults from the two regions (see Fig. 3.6B and Table 3.4). Within regions, however, heat coma temperatures were significantly ($F_{1.19\ \&\ 1.64}$ = 18.56 and 40.66 for *A. africana* and *A. knysnaensis*, respectively; $p < 0.001$ in both cases) lower for adults than for conspecific juveniles (see Fig. 3.6).

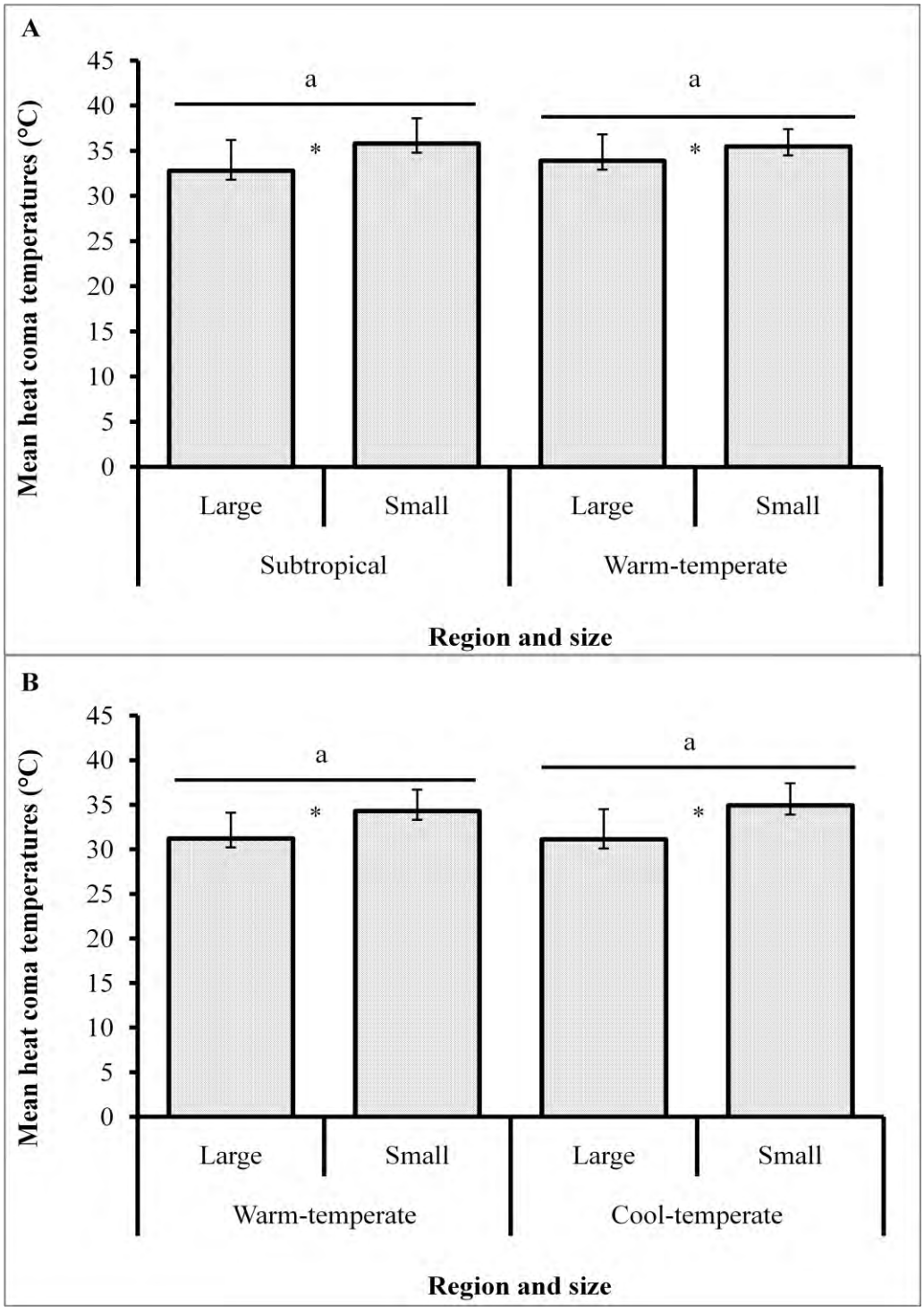

Figure 3.6. Mean heat coma temperatures of (A) *A. africana* from subtropical and warm temperate and (B) *A. knysnaensis* from warm and cool temperate regions. Histograms are mean ± SD of different measurements. Asterisks (*) indicate significance differences between sizes within regions as determined using two-way ANOVA ($p < 0.05$).

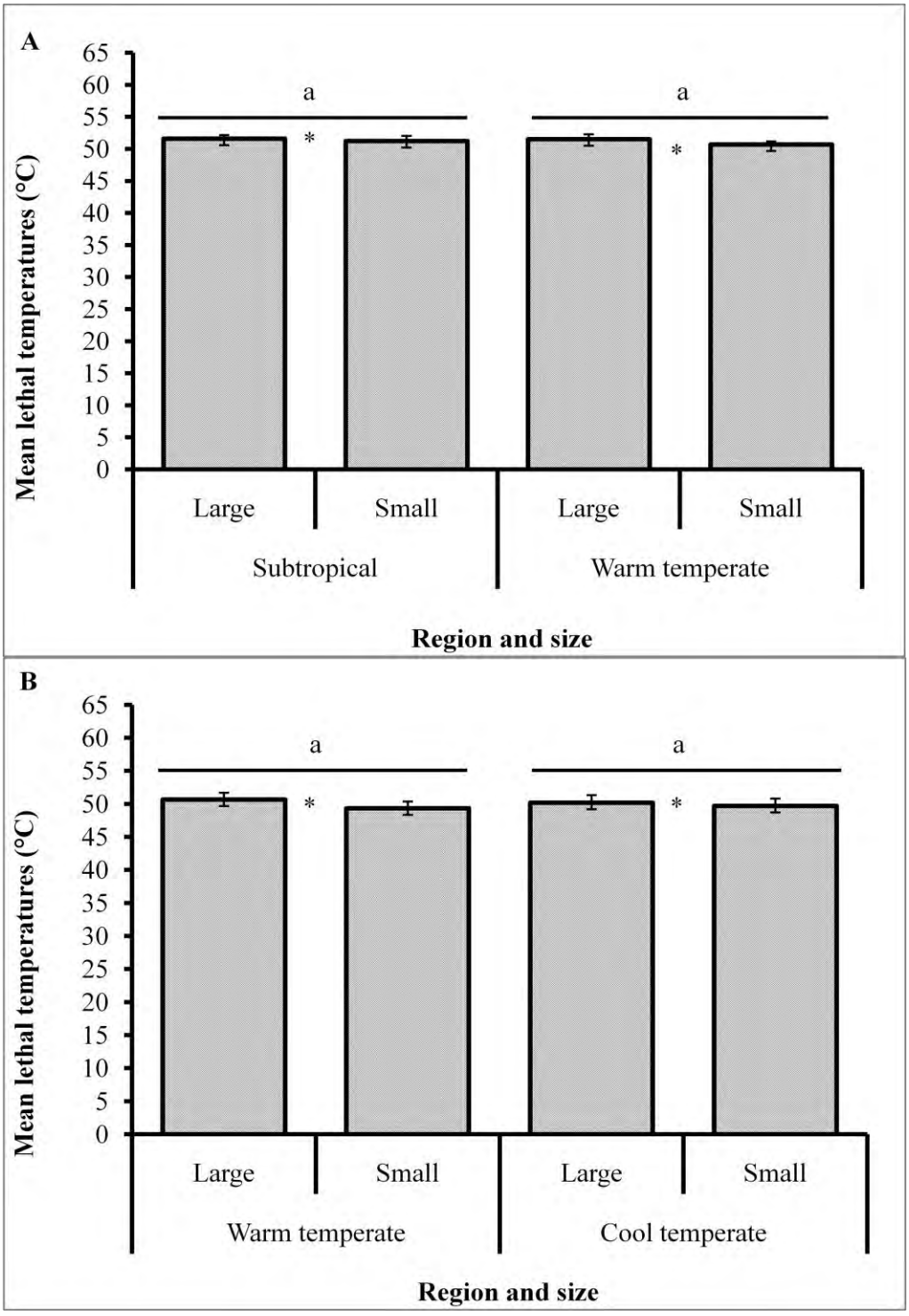

Figure 3.7. Mean lethal temperatures of (A) *A. africana* from subtropical and warm temperate and (B) *A. knysnaensis* from warm and cool temperate regions. Histograms are mean ± SD of different measurements. Asterisks (*) indicate significance differences between sizes within regions as determined using one-way ANOVA ($p < 0.05$).

For lethal temperatures, *A. africana* from the subtropics showed higher lethal temperatures than those from the warm temperate region as expected; though the difference was marginally non-significant ($F_{1.18} = 1.11$; $p = 0.05$; see Fig. 3.7A and Table 3.5). On the other hand, juveniles of *A. knysnaensis* from the cool temperate region showed unexpectedly higher lethal temperatures than their warm temperate counterparts, while adults showed the expected reverse pattern (see Table 3.5). Nevertheless, these differences were not significant ($F_{1.26} = 0.02$; $p > 0.05$; see Fig. 3.7B and Table 3.5). Within regions, adults of all species showed significantly ($F_{1.18\ \&\ 1.26} = 4.23$ and 5.53 for *A. africana* and *A. knysnaensis*, respectively; $p < 0.05$ in both cases) higher lethal temperatures than juveniles (see Fig. 3.7).

3.3.1.4. Does acclimation (laboratory) and acclimatization (season) affect HCT and LT_{50}?

For all species, laboratory acclimation at different temperatures for 14 days had little effect on heat coma temperatures (see Fig 3.8.1-2; Table 3.6). In addition, there was no trend in heat coma temperatures for animals acclimated at different temperatures (see Fig 3.8.1-2). Although not of direct relevance to this question, statistical analyses showed significant difference between species, sizes and treatments (see Table 3.8) with freshly collected animals showing significantly (mostly lower) different heat coma temperatures than laboratory acclimated animals (see Fig 3.8.1-2).

Although lethal temperatures were higher for summer compared to winter (see Fig. 3.9 and Table 3.7), the effect of seasonal acclimatization was not significant ($F_{1.56} = 3.15$; $p > 0.05$; see Fig. 3.9). As expected, there was a significant ($F_{3.56} = 94.24$; $p < 0.001$) difference between species with higher tolerances in *E. natalensis* followed by *L. glabrata* > *A. africana* > *A. knysnaensis*, regardless of season (see Fig. 3.9). In addition, adults of all species showed non-significantly ($F_{1.56} = 3.37$; $p > 0.05$) higher lethal temperatures than juveniles, regardless of season (see Fig. 3.9; Table 3.8).

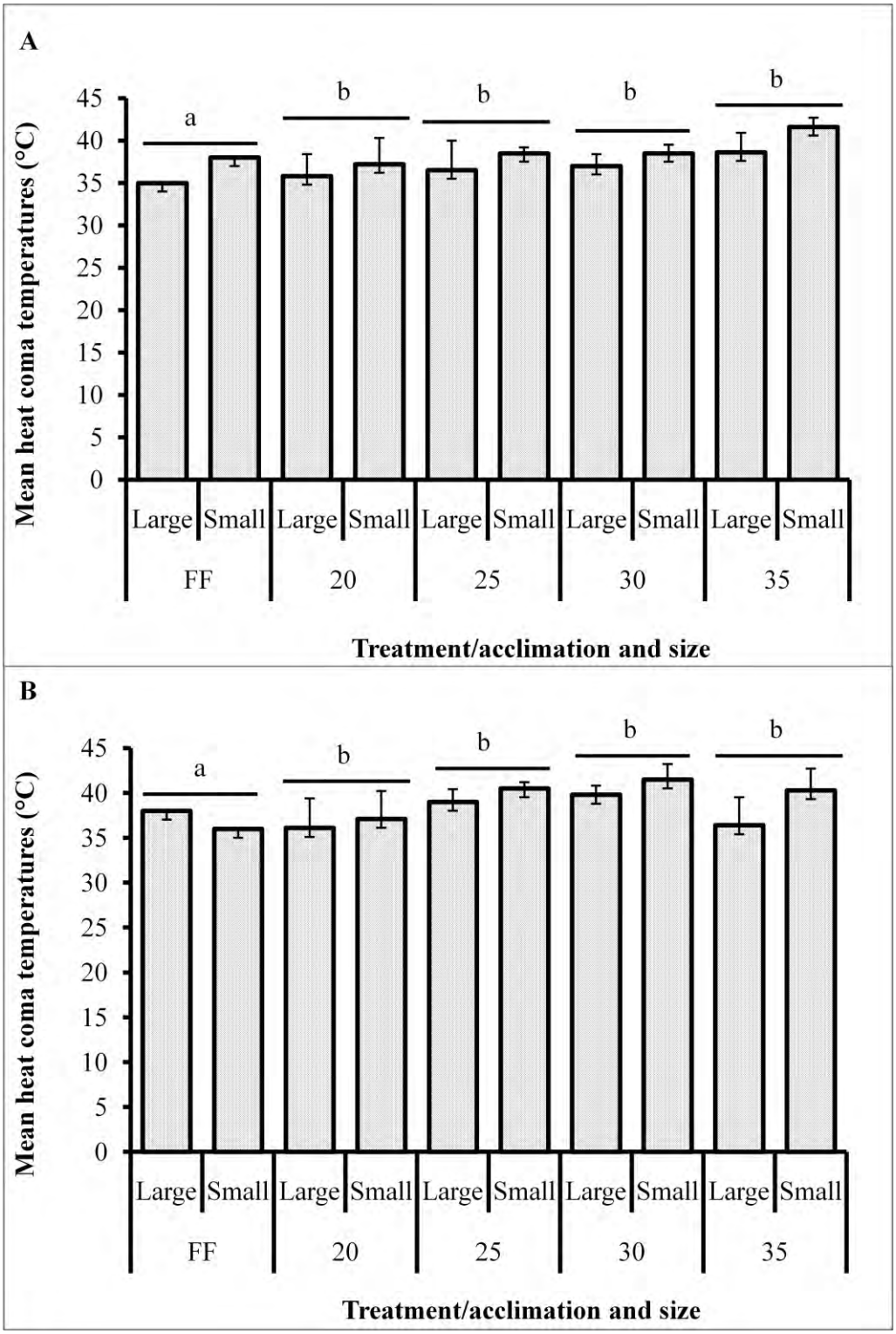

Figure 3.8.1. Mean heat coma temperatures of field fresh and laboratory acclimated (A) *E. natalensis* and (B) *L. glabrata*. Histograms are mean + SD of different measurements. Different letters represent significance differences between treatments as determined using one-way ANOVA ($p < 0.05$).

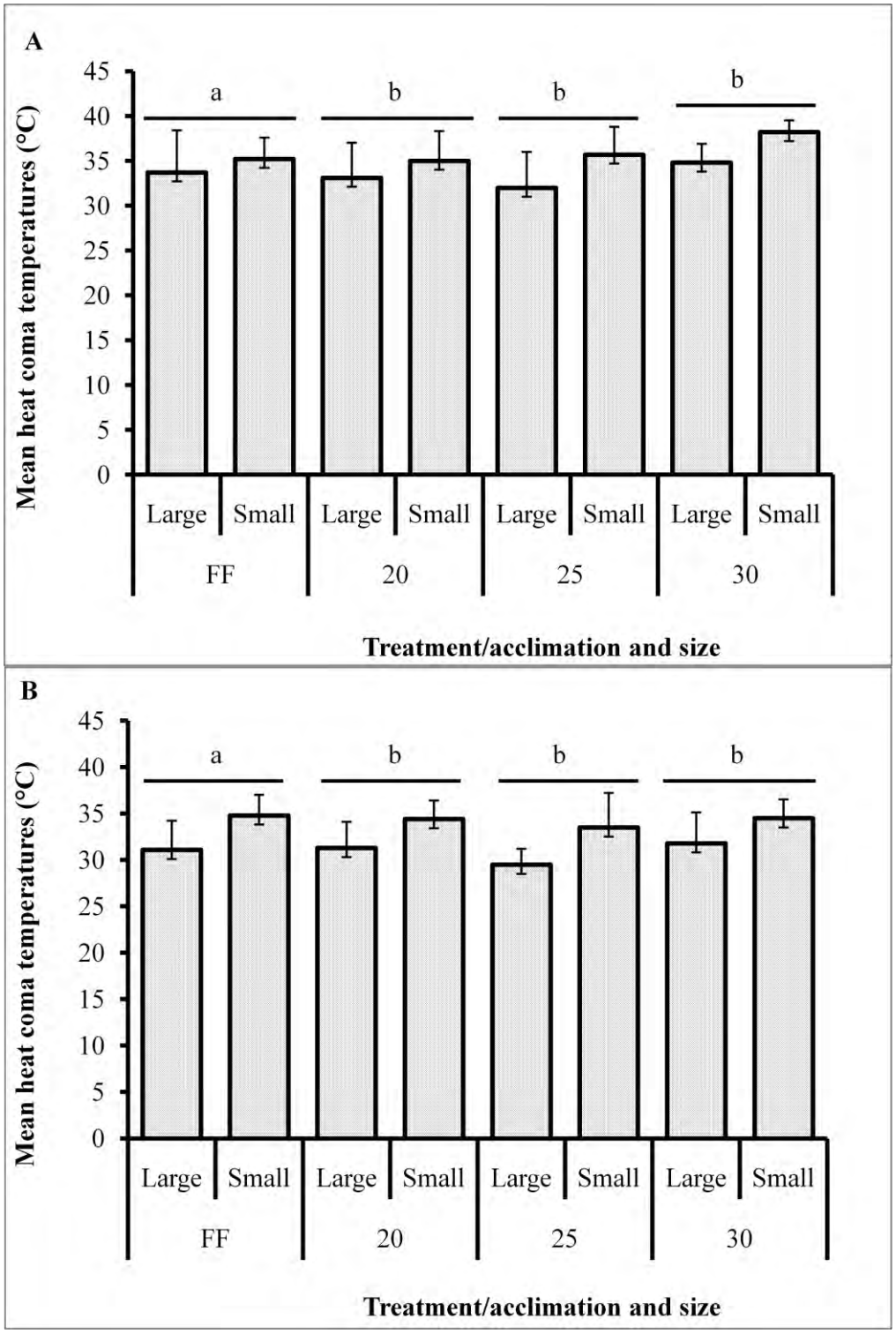

Figure 3.8.2. Mean heat coma temperatures of field fresh and laboratory acclimated (A) *A. africana* and (B) *A. knysnaensis*. Histograms are mean + SD of different measurements. Different letters represent significance differences between treatments as determined using one-way ANOVA ($p < 0.05$).

Table 3.6. Mean heat coma temperatures (+ SD) of field fresh and laboratory acclimated large and small littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from South Africa. Large and Small sizes are defined in the text.

Taxa	**Acclimation temperatures (°C)**									
	FF		**20**		**25**		**30**		**35**	
	Large	**Small**	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**
E. natalensis	35.0±00	38.0±0.0	35.8±2.6	37.2±3.1	36.5±3.5	38.3±0.7	37.0±1.4	38.5±1.0	38.6±2.3	41.6±1.1
L. glabrata	38.0±0.0	36.0±0.0	36.1±3.3	37.1±3.1	39.0±1.4	40.5±0.7	39.8±1.0	41.5±1.7	36.4±3.1	40.3±2.4
A. africana	33.7±4.7	35.2±2.4	33.1±3.9	35.0±3.3	32.0±4.0	35.7±3.1	34.8±2.1	38.2±1.3		
A. knysnaensis	31.1±3.1	34.8±2.2	31.3±2.8	34.4±2.0	29.5±1.7	33.5±3.7	31.8±3.3	34.5±2.0		

Table 3.7. Mean (± SD) lethal temperatures of seasonally acclimatized large and small littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from South Africa.

Taxa	Season			
	Summer		Winter	
	Large	Small	Large	Small
E. natalensis	57.0±2.0	56.0±2.0	56.5±0.5	56.0±1.0
L. glabrata	54.3±1.5	54.0±1.7	53.0±0.0	53.5±0.5
A. africana	51.8±0.4	51.2±0.4	51.2±0.8	50.6±0.7
A. knysnaensis	50.6±1.0	49.7±1.1	50.3±1.0	49.4±1.1

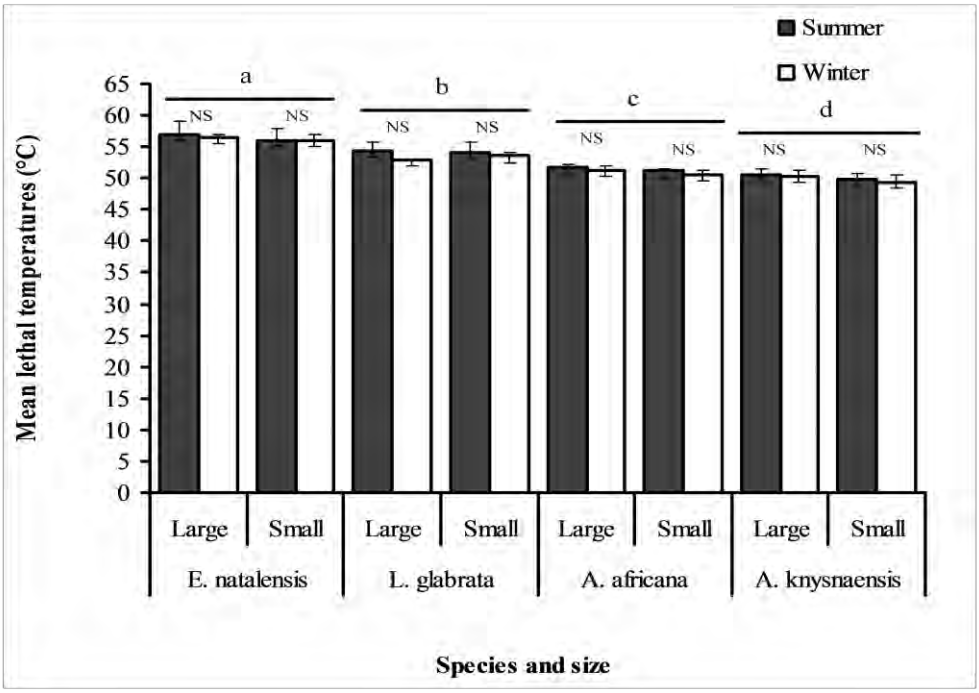

Figure 3.9. Mean lethal temperatures of summer and winter field acclimatized *E. natalensis*, *L. glabrata*, *A. africana* and *A. knysnaensis*. Histograms are mean ± SD of different measurements. Different letters represent significance differences between species as determined using three-way ANOVA ($p < 0.05$); NS = non-significant.

Table 3.8. Three way-ANOVA results on the effect of laboratory acclimation (HCT) and seasonal acclimatization (LT_{50}) of *Afrolittorina* spp. *Echinolittorina natalensis* and *Littoraria glabrata* from SA.

Variables	**HCT**				**LT_{50}**			
	Degree of freedom	**Mean Square**	**F- ratios**	**P values**	**Degree of freedom**	**Mean Square**	**F- ratios**	**P values**
Species	3	237.6	28.75	***0.000000***	3	114.3	94.2	***0.000000***
Size	1	135.4	16.39	***0.000065***	1	4.1	3.4	0.071888
Treatment	3	53.3	6.45	***0.000297***	1	3.8	3.2	0.081156
Interactions:								
Species*Size	3	8.2	0.99	0.398857	3	0.6	0.5	0.712718
Species*Treatment or Season	9	14.2	1.71	0.084691	3	0.3	0.2	0.876228
Size* Treatment or Season	3	3.4	0.14	0.0937961	1	0.4	0.3	0.560699
Species*Size*Treatment or Season	9	16.3	0.22	0.0991754	3	0.1	0.1	0.952703

Bold letters and Italics indicates significant ($p < 0.05$) effects; * represent where interaction was done.

3.4. Discussion and conclusions

Temperature (habitat or environmental) is one of the most important environmental factors that affect the distribution and abundance of animals, particularly ectotherms (see Dahlhoff and Somero, 1993a; Tomanek and Helmuth, 2002; O'Connor *et al*., 2007; Helmuth *et al*., 2010; Hofmann and Todgham, 2010; etc). This is because ectotherm body temperatures and performance are strongly under the influence of environmental temperature (see Sagarin *et al.*, 1999; Angilletta Jr. *et al*., 2002; Helmuth *et al.*, 2005; 2006a, b; Pörtner and Knust, 2007; Pincebourde *et al*., 2008). The influence of temperature change on an animal‟s performance is of particular relevance to intertidal animals, especially those from the temperate regions where there are strong seasonal variations in temperature (see Stillman, 2003; Lesser and Kruse, 2004; Bijlsma and Loeschcke, 2005; Jones *et al*., 2009; Peck *et al*., 2009a, b; Lannig *et al*., 2010). In addition, the magnitude of global warming is predicted to be much greater in temperate regions and higher latitudes (see Oviatt, 2004; Jentsch *et al*., 2007; Helmuth *et al*., 2010; Caddy-Retalic *et al.*, 2011; Wernberg *et al*., 2011). This may be especially problematic for intertidal organisms such as littorinids as they live in environments that are already harsh and fluctuating.

As a result, there are calls to understand and predict how species will respond to climate change (Pörtner *et al*., 2004b; Helmuth *et al.*, 2002; 2006b; 2010; Fitzhenry *et al*., 2004; Harley *et al*., 2005; Parmesan, 2007; Lannig *et al*., 2010). Climate change related heat events are expected to pose additional thermal problems particularly for organisms in ecosystems (e.g. intertidal) already subjected to local warming (see Cuculescu *et al*., 1998; Tebaldi *et al*., 2006; Mislan *et al*., 2009; Stillman and Tagmount, 2009; Lagos *et al*., 2011; Madeira *et al*., 2012b, c). Thus, with the anticipated effects of climate change where the mean air and sea surface temperatures as well as solar radiation have risen and are predicted to rise in the coming years, there is concern over how animals, especially intertidal ectotherms, will respond to or tolerate extreme and fluctuating environmental temperature stress.

Many littorinids live at the highest levels (i.e. fringes) of the intertidal zone and have to cope with periodic events of extreme heat and cold as well as desiccation during low tides. Littorinid snails have certain abilities that allow them to survive harsh conditions in the littoral zones (see McMahon, 1990; Marshall and Chua, 2012). These include adaptation mechanisms such as high thermal tolerance (i.e. resistance adaptation; see Vernberg, 1969), metabolic adjustments, and enhanced production of heat shock proteins (see Suryanarayanan and Nair, 1979; Sokolova and Pörtner, 2001b; Emson *et al.*, 2002; Marshall *et al*., 2011). As for other ectotherms (see Somero, 2002; 2010), studies on thermal tolerances of littorinids show that tolerances differ for animals from different regions, shore levels, microhabitats, and species. Although there is no study which has compared intra- and interspecific tolerances of tropical, subtropical and temperate littorinids species, Table 3.1 shows tolerances decrease from tropical to subtropical and temperate regions. In addition, most of these studies have shown geographical or population differences in tolerances.

For example, Lee and Boulding (2010) found evidence of latitudinal difference in heat coma temperatures of intertidal snails, *Littorina keenae* from different regions; though the difference was weak. Clarke *et al.* (2000c) found that populations of *L. obtusata* from South Wales showed higher heat coma temperatures than those from south-west Ireland and the east coast of Scotland. Sokolova *et al*. (2000c) found a higher tolerance for White Sea populations of *L. saxatilis* than those from the North Sea, and this is not surprising as the White Sea is much colder than the North Sea. Sandison (1967) found the heat coma and lethal temperatures to be higher for populations of gastropods including littorinids of the genus *Littorina* from Cardigan Bay than those from Port Seton; however the results for the Cardigan Bay populations come from a different study.

Likewise, eulittoral fringe species show higher tolerances than eulittoral zone and low shore species. In a study of *Littorina* species from different regions, Clarke *et al*. (2000a, b, c) found that eulittoral fringe species show higher tolerances than eulittoral species. This is true for species of the genera *Echinolittorina*, *Nodilittorina* and *Littoraria* (see Table 3.1). These differences can be explained as adaptations to the different microhabitat conditions and acclimation to different thermal regimes. In addition, the variability in tolerance values reported for each species in the literature appears to be due to the effect of season,

acclimation and/or thermal history as well as the methods followed in different studies (see below).

The four species of littorinid snails studied here have distinct geographical and vertical distribution patterns that are hypothesized to reflect differences in their tolerances to temperature, assuming that temperature is the main factor in determining their distribution patterns. Their thermal tolerance as estimated by heat coma and lethal temperatures was as expected, with significantly higher tolerances in the two exclusively subtropical species than subtropical/temperate species. These differences can be explained as adaptations to the different conditions the animals experience in their habitats, though it is only possible to separate this from the effects of species identity by comparing conspecifics from different regions (see below).

In the subtropics, *E. natalensis* and *L. glabrata* occupy the eulittoral fringes where they are subjected to higher levels of heat stress than *Afrolittorina* spp. which are dominant in eulittoral zones of subtropical/temperate regions. Thus, the differences in geographic and vertical distribution and the conditions experienced can explain the differences in thermal tolerances found in these species. Although *Afrolittorina* spp. also occur in the eulittoral fringes, the fact that they are of temperate origins (cool environments) (see Hartnoll, 1976; Reid, 1989; 1996; Williams *et al*., 2003; Reid and Williams 2004) suggests a phylogenetic influence resulting in them being less tolerant to heat stress than the subtropical species, which are of tropical origins (see Hartnoll, 1976; Reid, 1989; 1996; 2007; Inness-Campbell *et al*., 2003; Torres *et al*., 2008; Williams and Reid, 2004; Reid *et al*., 2010). This is also supported by the fact that *A. africana* is restricted (presumably by heat stress) to lower levels on the shore in the subtropics where it also adopts different habitat use, preferring shallow pools and their edges.

It is well known that whole organism thermal tolerance limits closely reflect differences in habitat temperature that result from different latitudinal and vertical distribution patterns (see Stillman and Somero, 1996; Cuculescu *et al*., 1998; Clarke *et al*., 2000c; Tomanek and Somero, 2000; Backeljau *et al*., 2001; Tomanek and Helmuth, 2002; Tepler *et al*., 2011). On

comparing thermal tolerances of bivalves from different regions, Compton *et al*. (2007) found that species from tropical Roebuck Bay, Australia had higher lethal temperatures than those from the temperate Wadden Sea, Netherlands. Stillman and Somero (1999, 2000) found the upper thermal tolerances of porcelain crabs of the genus *Petrolisthes* correlate with species‟ maximum habitat temperatures. This was also true for dogwhelks of the genus *Nucella* (Sorte and Hofmann, 2005). In the tropics, Stirling, (1982) found a difference in the upper tolerance of prosobranchs gastropods from Hong Kong (22°N) and Tanzania (7°N), that was explained by greater seasonal fluctuations and/or differences in geomorphology and microclimate in Hong Kong. In tropical intertidal zones, animals are subjected to periods of emersion of several hours in which intense solar radiation may raise surface temperatures as high as 45 to 50°C (Garrity, 1984; Williams and Morritt, 1995; Marshall *et al.*, 2010; Cartwright and Williams, 2012). Although the subtropics might not experience conditions as extreme as in the tropics, one could expect the subtropical species to show higher tolerances than temperate species, as was seen in this study.

Differences between species were also seen within regions. In the subtropics, the eulittoral fringe to upper eulittoral *E. natalensis* and *L. glabrata* showed higher thermal tolerance than the eulittoral *A. africana*. It is well known that gastropods including littorinid snails that occupy different positions on the shore are subjected to varying degrees of thermal stress brought upon by contrasting effects of solar radiation and tidal inundation (see Sandison, 1967; Suryanarayanan and Nair, 1979; McMahon, 1990; Gracey *et al*., 2008; Mislan *et al*., 2009). The species inhabiting the eulittoral fringes can be exposed to dry air and intense solar radiation for periods of hours to days or even months (see McMahon, 1990; Emson *et al*., 2002; Marshall *et al*., 2010; 2011; Marshall and Chua, 2012). *E. natalensis* (second highest) and *L. glabrata* (highest) live higher on the shore and experience greater heat stress during low tides than *A. africana* which not only occurs lower on the shore, but exploits more benign habitats than in temperate regions. Although in the subtropical region *L. glabrata* lives higher on the shore than the other two species, like *A. africana*, it relies on benign habitats. This contrasts with *E. natalensis* which also lives on the high shore, but lives in the open on unshaded dry rocks, so that habitat use by these two eulittoral species is different.

Within the warm temperate region, the two *Afrolittorina* spp. showed different thermal tolerances, with *A. africana* showing higher tolerances than *A. knysnaensis*. This again reflects their geographical distributions; *A. africana* extends just into the subtropical parts of the coast, while *A. knysnaensis* is found in the cool and warm temperate regions (see McQuaid and Scherman, 1988; McQuaid, 1992; Sinclair *et al*., 2004; d'Errico *et al*., 2008). The two overlap extensively in the warm temperate region where they even co-exist and use the same microhabitats (see McQuaid, 1992; d'Errico *et al*., 2008). Thus, the slight (1-2°C) difference in tolerance was expected. Previous studies by McQuaid and Scherman (1988) also found a small (1°C) difference between these two species.

Situations where species overlap in distribution and show different tolerances can be explained by different microhabitat use (see Vernberg and Vernberg, 1970; Stirling, 1982; Garrity, 1984; Stillman and Somero, 1996), but in this case shell colour may also be important (see below; Phifer-Rixey *et al*., 2008; Miller and Denny, 2011). The brown-black shell of *A. knysnaensis* is expected to absorb more radiation and heat up to a greater degree than the light-coloured *A. africana* (see McQuaid and Scherman, 1988; McQuaid, 1992; 1996a), resulting in the former experiencing higher temperatures in the field. Markel (1971) found the dark-coloured *Littorina aspera* absorbed more solar radiation and had a higher lethal temperature than the light-coloured *L. modesta*. On studying the effect of carapace colour on heat tolerance in the fiddler crab *Uca pugilator*, Wilkens and Fingerman (1965) found that dark individuals had higher heat tolerances than pale ones. Even though black or dark bodies are known to absorb a larger fraction of solar radiation, the heat gained remains near the surfaces and is easily removed by either re-radiation, convection or air cooling (see Lewis, 1963; Helmuth, 2002; Britton and Morton, 2003; Phifer-Rixey *et al*., 2008; Marshall and Chua, 2012). This might have been the case in *A. knysnaensis* since the body temperature of both species did not differ despite their colour differences (unpub. data).

There was also a difference between the two eulittoral fringe species, with higher limits for *E. natalensis* than *L. glabrata*. This was expected since *L. glabrata* prefers shaded and more humid microhabitats such as crevices and pits which offer protection from direct sunlight. Microhabitats such as pits and crevices can decrease heat stress levels and reduce rates of evaporation (see Garrity, 1984; Britton, 1995; Stafford and Davies, 2004). As for other

littorinids such as *Cenchritis (Tectarius) muricatus* (see Emson *et al.*, 2002), *L. glabrata* is also found on tufts of grass and other vegetation (pers. obs.) which would avoid the high temperatures of rock surfaces. *E. natalensis* however is mostly found on dry rock surfaces where it is subjected not only to intense solar radiation from direct sunlight, but also to heat conducted from the substratum. Thus, differences in microhabitat conditions correlate with the discrepancies in heat tolerance between these eulittoral fringe species.

Apart from microhabitat effects, deviations in tolerances of particular species from the general relationship with distribution on the shore can be related to differences in shell colour and morphology, and reported thermoregulatory capacities (see Stirling, 1982; Marshall and Chua, 2012). For example, *E. natalensis*, like many *Echinolittorina* spp., has a highly sculptured shell which is regarded as an adaptation to reduce radiant heat uptake (see McQuaid, 1992; Marshall and Chua, 2012), whereas *L. glabrata* has a very thin, smooth shell which can expose internal tissues to intense solar radiation. In addition, *E. natalensis* can also benefit from convective cooling as reported in other *Echinolittorina* and littorinid species (see Marshall and Chua, 2012). No previous data on heat coma and lethal temperatures are available for these two species for comparison with my findings.

Since species identity and regions are largely confounded, I could only compare populations of *Afrolittorina* spp., each of which occurs in two regions. In the case of *A. africana*, I predicted that populations from the subtropics would show higher tolerances than warm temperate populations and for *A. knysnaensis* that cool temperate populations would show lower tolerances than warm temperate populations. Thus, since there is latitudinal difference between sampling sites, it is possible that acclimation to temperature as has been reported in other studies (see Sandison, 1967; Stirling, 1982; Lee and Boulding, 2010; Sorte *et al*., 2011; Zardi *et al*., 2011), may account for tolerance differences between regions. In fact, there were no major effects of region on heat tolerances for either species of *Afrolittorina*, and this may reflect the fact that these species are exposed to terrestrial conditions for most of the time, while the regions are identified mainly on the basis of sea surface temperature (SST) (see Maree *et al*., 2000; Harrison, 2002; 2004; Sinclair *et al*., 2004).

Along the southern African coast, day time air temperatures, which frequently exceed 35°C, and substratum temperatures, often in excess of 45°C for fully heated rocks, rise well above SST, which varies between 20 and 27°C on the subtropical east coast, 15 and 22°C in warm temperate south coast, and from 10 to 19°C on the cool temperate west coast (see Darbyshire, 1966; Roy *et al*., 2001; Sinclair *et al*., 2004; Harrison and Whitfield, 2006). This means that animals are exposed to two forms of heating, either directly by solar radiation or indirectly by conductive transfer from the substrata to which they are attached (see Wethey, 2002; Britton and Morton, 2003; Broitman *et al*., 2009; Marshall *et al.*, 2010; Chapperon and Seuront, 2011a, b). Animals‟ body temperatures (up to approximately 43°C) were always higher than the surrounding air but slightly lower than that of the rocks (unpub. data).

The lack of an effect of region on the tolerances of *A. africana* is reflected in its within-shore distribution. This species occurs at the very top of the eulittoral fringe in the warm temperate region, but in the subtropical region it is found only lower on the shore; this may also be true for *A. knysnaensis*. The sampling sites for both species in each region were only a few (2-3) degrees apart, and as such one would not expect differences between populations as seen in this study. An alternative explanation for the lack of regional difference in *Afrolittorina* spp. may be the lack of genetic diversity (variation) among populations (see Chapter 2; Grant and Lang, 1991) as in other studies. After finding weak evidence of latitudinal difference in heat coma temperatures of *L. keenae* snails from different regions, Lee and Boulding (2010) suggested it as a result of high gene flow between populations. Kuo and Sanford (2009) found evidence of the presence of thermally tolerant genotypes in different parts of an intertidal snail‟s range. Future studies are needed to investigate the effect of region on thermal tolerance of the study species, especially those which are found in more than one region and/or populations separated by several (5 and above) degrees of latitude. In addition, there are possibilities that members of the study species might be more stressed at their range edges and/or hotspot areas as in other animals or species (see Sorte and Hofmann, 2004; Osovitz and Hofmann, 2007; Roelofs *et al*., 2008; Barshis *et al*., 2010; Somero; 2010; Wernberg *et al*., 2011), resulting in tolerance differences.

Other studies have found differences in thermal tolerances between populations from different bioregions or populations (see Vernberg and Vernberg, 1970; Stillman and Somero,

1999; Sorte and Hofmann, 2005; Fangue *et al*., 2006; Sunday *et al*., 2012). For example, Clarke *et al.* (2000c) found a difference in heat coma temperatures of *Littorina obtusata* from different bioregions, with populations from South Wales showing higher tolerances than those from south-west Ireland and east coast of Scotland. Similarly, populations of *Littorina* spp. from South Wales had higher heat coma temperatures than those from Northeast England (Backeljau *et al*., 2001). In *L. littorea*, populations from Ireland had significantly higher lethal tolerance than those from Scotland; while for the whelk *Nucella lapillus*, lethal tolerance was higher for Scottish than Irish populations (Davenport and Davenport, 2005). Sokolova *et al*. (2000c) also found a difference in temperature tolerance of the gastropod *L. saxatilis*, with higher tolerances for White Sea than North Sea populations. Sandison (1967) found that populations of gastropods including *Littorina* spp. from Cardigan Bay had higher tolerances than those from Port Seton; however, results were not from same study.

Similar effects have been found for other marine animals such as gastropods molluscs, echinoderms, crustaceans and fishes (see below). For example, Sorte *et al*. (2011) found that populations of the subtidal epibenthic species from the east coast of the United Sates which experience higher habitat temperatures had higher thermal tolerances than those on the west coast which experience lower temperatures. They also showed (after repeated exposure) that thermal tolerance varied between western and eastern Atlantic populations. Zippay and Hofmann (2010) found that veligers of *Nucella ostrina* from northern latitudes in Washington State had lower lethal temperatures than those from central sites in California. Kuo and Sanford (2009) on the other hand found that newly laboratory hatched *N. canaliculata* from central California had lower lethal temperatures than those from Oregon. The authors suspected that the difference was due to differences in period of exposure; some northern sites experience longer exposure to stressful midday low tides than southern sites due to variation among regions in the timing of low tides (see Helmuth *et al*., 2002; 2006a).

Timing of low tide exposure is only one of many environmental factors that contribute to variation in thermal stress among sites and regions. Persistent regional differences in tidal regimes, climate, and other environmental factors (e.g. air temperature, solar radiation, etc) may act as selecting forces that influence the physiology of intertidal species with broad

latitudinal ranges (see Helmuth *et al*., 2006a; Kuo and Sanford, 2009). As the whole coast of South Africa experiences regular semi-diurnal tides, tidal effects will not be relevant here.

Large differences in thermal tolerances have been reported in other littorinids and intertidal gastropods; and in most cases the differences were related to the conditions animals experience in their regions, shore levels and microhabitats. It is well known that warm water species from the tropics have higher tolerances than cool water species from temperate regions (see Table 3.1; Suryanarayanan and Nair, 1979; McMahon, 1990). The thermal tolerances of species in this study are above those reported for *Littorina* spp. from temperate regions (Clarke *et al.*, 2000a, b, c; but see Backeljau *et al*., 2001) and low compared to those of tropical species (Suryanarayanan and Nair, 1979; Lee and Lim, 2009; Marshall and McQuaid, 2010; Marshall *et al.*, 2011). The tropical *E. vidua* and *E. malaccana* had heat coma temperatures of 44.5 and 46.8°C (Cleland and McMahon, 1986), and lethal thermal limits of 56.5 and 59°C respectively (see Marshall and McQuaid, 2010; Marshall *et al.*, 2011). In *Littorina* species from subtropical and temperate regions, heat coma temperatures were around 30-32°C (Clarke *et al.*, 2000a, b, c; Sokolova and Pörtner, 2003) while their lethal thermal limits were 40-43°C (Clarke *et al.*, 2000a, b, c). Muñoz *et al.* (2005) found a heat coma temperature of 37°C in *E. peruviana* in the temperate region which marks its southern distribution limit.

Likewise, species that live highest in the intertidal (i.e. eulittoral fringes) show higher tolerances than their low shore and subtidal counterparts (see below). Although Clarke *et al*. (2000b) found heat coma temperatures for their study *Littorina* species to be similar, lethal limits were lowest for *L. obtusata*, the species that is found lower on the shore. Markel (1971) found that the high shore *L. aspera*, which experiences higher tissue temperatures in the field, had higher lethal limits than sympatric *L. modesta*, found on the lower shore to subtidal. In the tropics, Stirling (1982) found a difference in thermal tolerances between species, with higher tolerance for eulittoral fringe species than eulittoral zone and subtidal species. Also in the tropics, Suryanarayanan and Nair (1979) found a higher tolerance for the high shore *Nodilittorina leucosticta* than the low shore *Littorina undulata*.

Such differences in tolerances between zones have also been found in other animals. Studies on sympatric crabs have found higher tolerances for high shore species than their lower shore and subtidal counterparts (Cuculescu *et al*., 1998; Stillman and Somero, 1999; Stillman, 2002). In Jensen and Armstrong (1991), the lower eulittoral to subtidal *Petrolisthes eriomerus* showed higher sensitivity to thermal stress than the mid to high intertidal *P. cinctipes*. Moreover, there was a size difference with smaller animals showing a greater resistance of emersion at 25°C than larger ones. In Lagos *et al*. (2011), *P. laevigatus* which inhabits the upper intertidal had greater tolerance to high temperatures when exposed to air than the lower intertidal to subtidal *P. violaceus*.

Although there are many papers dealing with temperature tolerances in intertidal animals (see above), differences in experimental methods and protocols as well as criteria for determining limits or lethality make comparisons among studies difficult. For example, some studies investigated thermal tolerance using chronic methods where temperature was raised at a slow rate (i.e. 1°C in hours or a day); while other studies have used acute methods where temperature was raised at a faster rate (i.e. 1°C in 5 minutes) as in the current study. In Fraenkel (1960) for instance, individuals of *Littorina littorea* were exposed for 1 hour at a particular temperature to determine their lethal temperature. On studying the effect of the rate of temperature increase on the heat tolerance of blenny fish *Acantemblemaria hancocki*, Mora and Maya (2006) found that slow rates resulted in higher tolerances than rapid rates. This is because with slow increase in temperature, animals have enough time to acclimate to new temperature and increase their thermal tolerances. However, the opposite effect can also be observed (see Angilletta Jr., 2009; Nguyen *et al*., 2011). Hicks and McMahon (2002b) found that the lethal thermal limits of the brown mussel *Perna perna* were about 30°C when temperature was increased by 1°C in a day and 45°C when temperature was increased by 1°C in a minute.

It may have been more appropriate to measure heat coma temperatures for the studied species in air rather than in water as for lethal temperatures, because these snails are unlikely to be immersed in nature even during high tides with the exception of those found submerged in pools (pers. obs.). However, measurement of heat coma temperatures have been done in water in most studies (see Fraenkel, 1968; McMahon, 1990, 2001b; Clarke *et al.*, 2000a, b).

There is also evidence which suggests that heat coma temperature in water is positively correlated with that in air for littorinid gastropods (see Sandison, 1967). Therefore, I also measured heat coma temperatures in water so that my data could be directly compared to those of previous studies. It would have been good to test tolerances, both heat coma and lethal temperatures, of my species in both media (water and air). In addition, the criteria used to judge heat coma and lethal temperatures can make comparisons of data impossible (see Fraenkel, 1960; Stirling, 1982). For example, as seen here, studies that use lack of activity to judge heat coma will give different results from those using closure of the operculum as a criterion.

Differences between class sizes, with small individuals (expected to be juveniles) showing higher heat coma temperatures but lower lethal temperatures than large individuals (expected to be adults) was expected, and can be explained by their positions on the shore. Juveniles of *Afrolittorina* spp. are found lower on the shore where they are frequently wetted by incoming tides while adults occupy higher levels and are only wetted by waves‟ splashes during high tides (pers. obs.; but see below). Coupled with regular wetting by tides, juveniles can run risks of exposure to high temperature to increase feeding time. The higher heat coma in juveniles in this study might be explained by high activity (i.e. slightly different behaviour to adults) and the benefits of evaporative cooling lower on the shore.

On the other hand, the higher lethal limits of adults were also expected since they are found at the highest levels on the shore where they are exposed to intense solar radiation for longer than juveniles. Although based on individuals from a single site (St. Abss), larger individuals of *Littorina littorea* showed significantly lower heat coma temperatures than juveniles (Clarke *et al*., 2000a, b). Stirling (1982) suggested that low shore species (animals) may have low lethal temperatures relative to heat coma temperatures since they are unlikely to experience extreme temperatures, while for high shore species high lethal temperature will be more important than high heat coma temperatures.

In contrast, Sandison (1967) suggested heat coma to be the most important factor affecting the zonation of littorinids and this appears to be true here, when I compare the tolerance and

distributions of juveniles and adults of *Afrolittorina* spp. However, juveniles are not always found lower on the shore (see Vermeij, 1972; Boulding and Van Alstyne, 1993; Saier, 2000; Emson *et al.*, 2002). For example, juveniles of *A. knysnaensis* have been described as generally occurring higher on the shore than adults (McQuaid, 1981a, b; d'Errico *et al.*, 2008), though this was in the cool temperate region where heat stress may be less critical. See Vermeij (1972) for size gradients in *A. africana* and other littorinids and molluscs. In summary, my results suggest that the basis for resisting heat stress may differ between large and small individuals. Such size-specific differences may account for different distribution patterns on the shore with larger individuals found higher on the shore while smaller ones are restricted to the lower levels. Indeed when we look at my results, larger specimens of all species showed higher lethal temperatures than smaller animals.

The two subtropical species ranked differently for heat coma and lethal temperatures. For both size classes, *L. glabrata* showed higher heat coma temperatures than *E. natalensis*, but the reverse was true for lethal temperatures. Again, this may be linked to their preferences for different microhabitats, *L. glabrata* preferring shaded and humid environments and *E. natalensis* dry rock surfaces. *L. glabrata* might also benefit from its behaviour, observed in both laboratory and field, of crawling and escaping to avoid heat stress. A delay in succumbing to heat coma may allow them to seek protected microhabitats. This means that if heat coma temperature was the main factor controlling vertical zonation, *L. glabrata* would be expected to occupy a higher level than *E. natalensis*; *visa versa* for lethal temperature.

Situations where heat coma temperature is hypothesised to be the main factor controlling vertical zonation have been found in littorinids (see Sandison, 1967), but in this study lethal temperatures seems to be important. In fact the vertical distributions of *L. glabrata* and *E. natalensis* widely overlap in the eulittoral fringes; but the zones of maximum abundance of these species are well separated. *L. glabrata* is more abundant in the uppermost eulittoral fringe and *E. natalensis* is found in abundance in the middle eulittoral fringe (pers. obs.). However, it must be noted that other factors (e.g. predation) may be responsible for *L. glabrata* occurring further up the shore than *E. natalensis*.

Effects of acclimation and acclimatization on thermal tolerances.

The phenomenon of temperature acclimation (physiological adaptation or capacity adaptation; Vernberg, 1969), leading to shifts in tolerance limits is more common in animals that experience fluctuations in conditions such as temperature and humidity (e.g. temperate species) than those that experience relatively constant conditions (e.g. tropical species) (see Segal, 1961; Huey and Bennett, 1990; Somero, 2002; Stillman, 2003; Jones *et al*., 2009; Pörtner, 2010; Sunday *et al*., 2012). The lack of an acclimation response by tropical or polar organisms presumably relates to the absence of any significant seasonal temperature variation in these regions (see Vernberg, 1969; Bijlsma and Loeschcke, 2005; Clarke and Gaston, 2006; Peck *et al*., 2009a, b; Chapperon and Seuront, 2011a, b; Nguyen *et al*., 2011). In addition, tropical and polar species often live close to their upper thermal limits, and as such have narrower thermal windows than temperate species (see Stillman and Somero, 1996; 1999; Compton *et al*., 2007; Chapperon and Seuront, 2011a, b; Christensen *et al*., 2011; Nguyen *et al*., 2011). Because of the high temperatures routinely experienced, tropical species have thermal tolerance limits that are as high as could be reached through acclimation so that no further acclimation is possible.

This is true for intertidal species, especially eulittoral fringe ones, which experience high fluctuations and extremes of temperature than low intertidal species (Cuculescu *et al*., 1998; Stillman and Somero, 2000; Stillman, 2002; 2003; Somero, 2002; 2005; Compton *et al*., 2007; Nguyen *et al*., 2011). These explanations may well apply to some molluscs such as gastropods and bivalves that only experience slight seasonal fluctuations in temperature (see Vernberg and Vernberg, 1969; Tomanek, 2008; Somero, 2010), such as those on the eastern seaboard of South Africa. Sea surface temperatures vary between 20 and 27°C on the subtropical east coast, and from 10 to 19°C on the cool temperate west coast (see above). Air temperatures are diurnally and seasonally variable reaching 30-35°C in summer and falling to 3°C in the subtropical and to 0°C along the west and south coast during winter (see Kruger and Shongwe, 2004; Sinclair *et al*., 2004).

Studies on acclimation show conflicting outcomes, some suggesting that acclimation occurs, while others suggest little or no acclimation in thermal tolerances. Many studies have found that littorinids and other marine invertebrates from different phyla have limited capacity or are unable to acclimate their thermal tolerances (see Hamby, 1975; Huey and Bennett, 1990; Stillman and Somero, 1999; Stillman, 2002; 2003). In the brown mussel *Perna perna*, acclimation was not pronounced, suggesting limited capacity for temperature acclimation (Hicks and McMahon, 2002b). In contrast, other studies have shown that acclimation can lead to higher thermal tolerances, with noticeable shifts in lethal temperature limits (see Segal, 1961; Backeljau *et al*., 2001; Díaz *et al*., 2002; Somero, 2002; Li and Brawley, 2004; Kelley *et al*., 2011). Clarke *et al*. (2000a) found significantly higher heat coma temperatures (overall shifts of 3 and 5.8°C) in individuals of *Littorina littorea* acclimated at higher temperatures (16 and 20°C respectively) than those acclimated at 12°C. Hamby (1975) also found a significant shift in heat coma in individuals of the common Atlantic littorinid, *L. littorea*. In *L. littorea* and *Monodonta lineata*, lethal temperatures were profoundly influenced by thermal acclimation (Newell *et al*., 1971).

Sorte *et al*. (2011) suggested that the four populations of the intertidal *Littorina* spp. can acclimate as shown by the absence of geographical differences in temperature tolerance. Braby and Somero (2006) found an increase in high critical temperatures of mussels, *Mytilus* spp., acclimated at 14 and 21°C, respectively. Cuculescu *et al*. (1998) found significantly higher heat coma temperatures in crabs, *Carcinus maenas* and *Cancer pagurus*, acclimated at 22°C than those acclimated at 8°C, with greater acclimation ability in *C. pagurus* than *Carcinus maenas*. In addition, for both species, winter caught animals showed significantly lower tolerances than summer and autumn caught animals, indicating the influence of season. Except for laboratory acclimation of critical thermal maxima which increased with acclimation temperature, Fangue and Bennett (2003) found that March acclimatized animals had critical maxima of 37.3°C as compared to 41.8°C for July acclimatized animals. In general, thermal tolerances are higher in summer and lower in winter (see Vernberg and Vernberg, 1970)

In this study, animals acclimated at different temperatures (20, 25, 30 and 35°C, respectively) showed no change in heat coma temperatures. This was irrespective of the effect of season on

lethal temperatures with summer acclimatized animals showing higher tolerances than winter acclimatized animals. Although not significant, variation in lethal temperatures (differences of 1-3°C) with respect to season may reflect some level of acclimation. It must be noted that seasonal acclimatization in this study may be due to acclimatization to other factors (e.g. salinity and food availability) in the field that can affect responses to heat stress (see Nagabhushanam and Sarojini, 1969; Fitt *et al*., 2001); but which were not simulated in the laboratory. Although a seasonal effect on heat coma was not investigated in this study, it is possible that more profound seasonal changes can occur in heat coma than in the lethal temperature. In *Littorina littorea*, heat coma was more subject to change by acclimation than lethal temperature as shown by a shift of heat coma temperature by about 8.5°C while lethal temperature shifted by only about 1-2°C (see Stirling, 1982). The effects of acclimation are seen in seasonality of temperature tolerance in *Littorina* spp. as season can have an effect on heat coma temperatures, which can vary seasonally (Clarke *et al*., 2000b; Backeljau *et al*., 2001). Cuculescu *et al*. (1998) found an effect of season on heat coma temperatures of the crabs *Carcinus maenas* and *Cancer pagurus*, with significantly lower tolerances in winter-caught animals than summer- and autumn-caught animals. This was also true for marine crustaceans where heat coma temperatures were higher in summer-acclimatized animals than winter-acclimatized ones (Hopkin *et al*., 2006).

The lack of heat coma acclimation in this study was unexpected and raises questions as to what causes an inability to acclimate. One possibility is that the acclimation period (14 days) used in this study was too short (see Backeljau *et al*., 2001), but in many studies acclimation for as little as 14 days was found to be sufficient to lead to proper acclimation (see Todd and Dehnel, 1960; Clarke *et al*., 2000a). For example, Hamby (1975) found that 14 days was enough to induce acclimation on *Littorina littorea*, resulting in a shift in heat coma temperatures; while further acclimation for 50-54 days had no effect. Alternatively, since the study littorinids are generally found very high on the shores, it might be that these animals were already acclimatized to high temperatures. This is supported by the results of Sorte *et al*. (2011) where populations of *L. saxatilis* did not acclimate after three weeks (21 days).

In addition, high shore intertidal species are assumed to be already living close to their thermal limits, and may have more limited capacities to increase their thermal tolerance limits

than subtidal species (see above). For instance, Stillman and Somero (1999) found that of the three temperate porcelain crabs, *Petrolisthes spp.* studied, the intertidal species was not able to adjust its lethal limits to the same extent as the subtidal species. This can be explained by differences in media due to the position on the intertidal zone. For example, thermal limits of *Mytilus edulis* in air were the same in June and November; but in water there was acclimation to a slightly higher value (29.8°C) in June than November (25.7°C; Jones *et al*., 2009).

The thermal history of an organism is known to influence thermal acclimation (see Cuculescu *et al*., 1998). Clarke *et al*. (2000a, b) found no effect of acclimation period on heat coma of animals acclimated at 12°C, and suggested that the specimens might have already been field acclimatized. However, the authors did not investigate the effect of period at other temperatures (16 and 20°C) which would have implied the importance of acclimation time. Other causes of lack of acclimation in this study could be the cost of acclimation and maintenance of high lethal limits (see Somero, 2002; Clarke, 2003; Stillman, 2002; Sokolova *et al*., 2012). As other investigators (see Hawkins *et al*., 1987; Hofmann and Somero, 1995; Tomanek, 2008; 2010) have emphasized that acclimation comes at a cost because the synthesis of heat shock proteins (which are involved in thermal acclimation and tolerance; Cuculescu *et al*., 1998; Dong *et al*., 2008a; Gracey *et al*., 2008; Sørensen, 2010) requires more energy (see Stillman, 2002; Whiteley and Faulkner, 2005; Sokolova and Lannig, 2008; Tomanek, 2008; 2010; Fitzgerald-Dehoog *et al*. 2012).

The ability to acclimate to temperature change is an important adaptation, because it signifies that some animals can alter their thermal tolerances during a time of rising temperature in the summer months and during global warming (see Newell *et al*., 1971; Kingsolver and Huey, 1998; Tomanek, 2008; Byrne *et al*., 2010; Pörtner, 2002b; 2010). Thus, acclimation is an important criterion in defining an animal‟s ability to survive environmental change, via the buffering of temperature effects. This is because acclimation is a critical short-term response to rapid and severe environmental changes which are expected in the near future (see Sokolova and Pörtner, 2003; Helmuth *et al*., 2005; Brown and Cossins, 2011; Martin *et al*., 2011) since it allows organisms to shift their thermal optimum (see Horowitz, 2001; Kassahn *et al*., 2009; Silvestre *et al*., 2012).

After comparing thermal acclimation capacities of differently distributed species of porcelain crabs of the genus *Petrolisthes*, Stillman and Somero (1999) and Stillman (2002, 2003) concluded that species with poor abilities to acclimate to temperature change (lower latitudes and high tidal zone species) are likely to be the most vulnerable to future warming scenarios (see Somero, 2005; 2010; Tomanek, 2010; Christensen *et al*., 2011). This can be true for my study species since they live high in the intertidal zone. This suggests that the two eulittoral fringe as well as the eulittoral zone species are likely to show changes in distribution pattern (vertical and geographical) with a small increase in temperature in coming years as predicted. This is supported by restriction of *A. africana* (also *A. knysnaensis*) to lower levels on shore in subtropics (pers. obs.).

In summary, the results of this study show that there are differences in thermal tolerance of the studied species and the differences seem to reflect differences in biogeography, ecology and phylogeny. The two subtropical species, which occupy the eulittoral fringe, showed higher tolerances than the two subtropical/temperate species which are found in the eulittoral zones. This agrees with the hypothesis that temperature tolerances in marine animals show a decrease from tropics to polar regions in both eulittoral fringe and lower shore species. It has also been established that there is little or no such difference as we move from 0 to 30 degrees latitude (tropics to subtropics), with the difference being more obvious from 30 to 60 degrees of latitudes (temperate and polar) (see McMahon, 2001b). By demonstrating the existence of fixed physiological differences between species from different geographic regions, this study provides evidence that environmental (temperature) adaptation at the organism level is important for the maintenance of dissimilar biogeographies.

The results also indicate that littorinids can tolerate high temperature stress, and are therefore well suited to life in the intertidal zones where temperature and other stresses are extreme and can change abruptly. Thus, it can be concluded that, in the short term, littorinids are tolerant of the high temperatures than they are likely to experience on the shore, and that they can also survive temporary exposure to supernormal temperatures. An understanding of animals‟ temperature tolerances or thermal limits, and the plasticity or flexibility of those limits enables us to make some inferences about what will happen to their distributions and abundances during climate change. Although my results suggest that littorinids have high

tolerances to temperature, it is clear that these animals are already living close to their thermal limits as shown by their limited capacity to adjust those tolerances, and the fact that distribution within-shores alters with region. As such, these animals may be vulnerable to small change in environmental temperatures. Thus, in the event of global warming, the distribution of littorinids and other intertidal ectotherms may be more affected than those of subtidal ones.

CHAPTER 4: Temperature-heart function relation of aestivating littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from temperate, subtropical and tropical regions

4.1. Introduction

Various laboratory and field studies have examined responses of physiological processes such as heart rate (the focus of this chapter) and oxygen consumption to abiotic factors such as temperature, oxygen, salinity, light; carbon dioxide, pollutants and chemicals as well as biotic factors such as sex, size, weight, food availability, nutritional status and activity (see below; Table 4.1; Brown, 1979; Höjesjö *et al*., 1999; Isla and Perissinotto, 2004; Langenbuch and Pörtner; 2004; Kemp *et al*., 2009; Marsden *et al*., 2011; etc), amongst others. It is clear from these studies that physiological processes are influenced by a wide variety of extrinsic and intrinsic factors (Newell, 1973; Laird and Haefner Jr., 1976; Aagaard, 1996; McMahon, 1999; Crear and Forteath, 2000; Nicholson, 2002). This is true for heart rate and oxygen consumption which are linked to abiotic factors including temperature, oxygen levels, salinity, chemicals, and many more (see Table 4.1; below). For marine species particularly intertidal ectotherms, temperature, oxygen levels and salinity are the three main factors that affect metabolic rates (see Table 4.1; above; Newell, 1973; DeFur and Mangum, 1976).

Although the effects of changes in the above three variables on the metabolic rates of marine animals have received much attention and are well documented (see Table 4.1), no general consensus on response has emerged as marine animals show diverse metabolic responses(see below; Table 4.1). Some studies suggest regulation of metabolic rate in response to changes in these factors, while others indicate partial independence and still others direct dependence (see below). This shows that animals‟ metabolic rates are complex and vary throughout the biosphere as a result of the diversity of physiology and energy demands of animals as well as geometric and environmental constraints or resource limitations (Newell, 1973; Shirley *et al*., 1978; Branch *et al*., 1988; Speakman *et al*., 2004; Glazier, 2005; Seibel and Drazen, 2007; Killen *et al*., 2010; Burton *et al*., 2011).

Table 4.1. Three main factors that have effects on oxygen consumption and heart rate of different marine animals.

Taxa	Reference	Factors		
		Temperature	Oxygen	Salinity
Invertebrates	Newell, 1969	Mixed		
	Newell and Pye, 1973	Mixed		
	DeFur and Mangum, 1979	Mixed	Mixed	Mixed
	Herreid II, 1980		Mixed	
	Ferraris *et al.*, 1994	Mixed		Mixed
	Salvato *et al.*, 2001	Mixed	Mixed	Mixed
Molluscs	Bayne, 1971a, b		Mixed	
Littorinids	Sandison, 1967	Dependent		
	Newell and Pye, 1970a, b, 1971	Mixed		
	Pye and Newell, 1973	Independent		
	McMahon and Russell-Hunter, 1978		Mixed	
	Shirley *et al*., 1978	Mixed		
	Moore and Sander, 1984	Dependent		Dependent
	Innes and Houlihan, 1985	Dependent		
	McMahon *et al.*, 1995	Mixed		
	Sokolova and Pörtner, 2001, 2003	Mixed		
	Marshall and McQuaid, 2010	Independent		
	Marshall *et al.*, 2010	Independent		
	Melatunan *et al*., 2011	Dependent		
	Marsden *et al*., 2012		Mixed	
	Stenseng *et al.*, 2005a, b	Dependent		
Whelks	Dye and McGwynne, 1980	Mixed		
	Brown and Meredith, 1981			Dependent
	Stickle and Bayne, 1982	Mixed		Mixed
	Brown and Da Silva, 1979, 1984	Mixed		
	Wynberg and Brown, 1986		Mixed	
Limpets	Pickens, 1965	Dependent		
	Bannister, 1974	Dependent		
	McMahon and Russell-Hunter, 1978		Mixed	
	Houlihan, 1979	Independent		
	McMahon and Russell-Hunter, 1981	Mixed	Mixed	

Taxa	Reference	Factors		
		Temperature	Oxygen	Salinity
Crustaceans	McMahon, 2001a		Mixed	
	Whiteley *et al*., 2001	Dependent		
Crabs	Vernberg, 1959	Mixed		
	Vernberg and Vernberg, 1966; 1969	Mixed		
	Teal and Carey, 1967		Mixed	
	Newell *et al.*, 1972	Mixed		
	Wallace, 1972	Dependent		
	Sastry and McCarthy, 1973	Mixed		
	Breteler, 1975	Dependent		
	Hill and Koopowitz, 1975		Mixed	
	Laird and Haefner Jr., 1976	Mixed		Little
	Spaargaren, 1977		Mixed	
	Spaargaren and Achituv, 1977	Dependent		
	Findley *et al*., 1978			Mixed
	Stickle and Sabourin, 1979			Mixed
	Dye and van der Veen, 1980	Mixed		Mixed
	Burggren and McMahon, 1981	Dependent		
	Laughlin and Neff, 1979; 1980; 1981	Mixed		Mixed
	Moreira *et al.*, 1981	Mixed		
	Hawkins *et al*., 1982	Independent		
	Mickel and Childress, 1982	Dependent	Mixed	
	Du Preez, 1983	Dependent		
	Shumway, 1983			Dependent
	Wernick and Penteado, 1983		Mixed	
	Gutermuth and Armstrong, 1989	Dependent		
	Emmerson, 1990	Dependent		
	Sébert *et al.*, 1995	Dependent		
	De Wachter and McMahon, 1996	Dependent		
	Stillman and Somero, 1996	Mixed		
	Brown and Terwilliger, 1999	Dependent		Independent
	De Pirro *et al*., 1999	Dependent		
	Crear and Forteath, 2000	Dependent	Independent	

	Shumway, 1981		Independent	Independent
	Shumway and Marsden, 1982	Dependent	Independent	Dependent
	Dye, 1987	Dependent		
	Branch *et al*., 1988	Dependent		
	Marshall and McQuaid, 1991; 1992	Dependent	Mixed	
	Marshall and McQuaid, 1993a; 1994	Mixed	Dependent	Dependent
	De Pirro *et al.*, 1999a, 2001	Mixed		Independent
	Chelazzi *et al.*, 1999, 2001	Mixed		Mixed
	Santini *et al.*, 1999; 2000	Dependent		
	Morritt *et al.*, 2007			Dependent
	Dong and Williams, 2011	Dependent		
Bivalves	Bayne, 1973a		Independent	
	Lowe, 1974	Dependent		
	Stickle and Sabourin, 1979			Dependent
	McMahon and Wilson, 1981	Independent	Mixed	
	Jansen *et al*., 2007	Dependent		
Mussels	Pickens, 1965	Dependent		
	Moon and Pritchard, 1970		Mixed	
	Trueman and Lowe, 1971	Dependent		Dependent
	Coleman, 1973	Independent		
	Widdows, 1973	Dependent		
	Bayne *et al*., 1976	Independent		
	de Vooys, 1976	Dependent		
	Famme, 1980		Mixed	
	Wilbur and Hilbish, 1989	Dependent		
	Dahlhoff *et al.*, 1991	Mixed		
	Marshall and McQuaid, 1993a, b		Dependent	Mixed
	Rao and Khan, 2000	Dependent		
	Hicks and McMahon, 2002	Mixed	Regulate	
	Nicholson, 2002	Dependent	Decreases	Little
	Bakhmet *et al.*, 2005a, b			Dependent
	Braby and Somero, 2006	Dependent		Dependent
Clams	Anderson, 1978	Mixed		
	Williams, 1984	Dependent		Mixed
	Eshky and Ba-Akdhah, 1992	Independent		
	Tang *et al.*, 2005	Mixed		Mixed
Oysters	Findley *et al*., 1978			Mixed
	Shumway and Koehn, 1982	Dependent		Dependent
	Haure *et al.*, 1998	Dependent		
	Cherkasov *et al*., 2006	Dependent		
	Lannig *et al*., 2006; 2008; 2010	Dependent		
Anthozoa	Griffiths, 1977	Dependent		

	Frederich and Pörtner, 2000	Dependent		
	Robertson *et al*., 2001a, b; 2002	Dependent		
	Camus *et al.*, 2004	Dependent		
	Storch *et al*., 2009	Dependent		
	Walther *et al*., 2009	Dependent		
	Iftikar *et al*., 2010	Dependent		
	Lardies *et al*., 2011	Dependent		
Amphipods	Bulnheim, 1979	Mixed	Mixed	
	Marsden, 1984	Mixed		
	Van Senus, 1985	Dependent		
	Spicer and Taylor, 1987	Dependent		
	Tedengren *et al*., 1988			Dependent
	Einarson, 1993	Mixed		
	Rastrick and Whiteley, 2011	Mixed		
Isopods	Bally, 1983; 1987	Mixed		
	Vetter *et al.*, 1999		Mixed	
	Salomon and Buchholz, 2000	Dependent		
	Whiteley and Faulkner, 2005	Dependent		
Copepods	Vernberg and Moreira, 1974	Mixed		
	Gyllenberg and Lundqvist, 1979	Mixed		Mixed
	Teare and Price, 1979	Dependent		
	Gee, 1985	Mixed		
Mysids	Simmons and Knight, 1975	Dependent		Dependent
	Marshall *et al.*, 2003	Dependent		Independent
Octopods	Seibel and Childress, 2000		Mixed	
Shrimps	Anderson, 1978	Independent		
	Emmerson, 1985	Dependent		
	Tande, 1988	Mixed		
	Villarreal and Rivera, 1993	Mixed		Mixed
	Villarreal *et al.*, 1994	Mixed		Dependent
	Agard, 1999	Dependent		Dependent
	Rosas *et al.*, 1999		Mixed	Mixed
	Isla and Perissinotto, 2004	Dependent		None
	Tian et al., 2004	Mixed		
	Allan *et al*., 2006	Dependent		Dependent
Prawns	Nelson *et al*., 1977	Mixed		Mixed
	Morris and Taylor, 1984	Independent		
Lobsters	Crear and Forteath, 2000	Dependent	Mixed	
	Thomas *et al*., 2000	Dependent		
	Tully *et al.*, 2000	Dependent		
Ascidians	Jiang *et al.*, 2008	Dependent		Dependent
Brittle star	Christensen *et al*., 2011	Dependent		
Anemones	Griffiths, 1977	Mixed		

	Navarro *et al.*, 1981, 1987	Mixed				Ortega *et al*., 1984	Mixed		
Sea urchin	Brockington and Clarke, 2001	Dependent				Seibel and Drazen, 2011	Independent		
	Siikavuopio *et al*., 2008	Dependent							
Cockle	Newell and Bayne, 1980	Mixed							
Scallops	Pilditch and Grant, 1999	Dependent							
Trochids	Houlihan and Innes, 1982	Dependent							
Abalones	Dahlhoff and Somero, 1993	Dependent							
Fishes	Du Preez *et al*., 1986	Independent							
	Berschick *et al.*, 1987	Dependent	Mixed						
	Johnston *et al.*, 1991	Dependent							
	Weinstein and Somero, 1998	Dependent							
	Claireaux and Lagardére, 1999	Mixed	Independent	Dependent					
	Clarke and Johnston, 1999	Mixed							
	Mallekh and Lagardére, 2002	Dependent	Mixed						
	Meloni *et al*., 2002			Mixed					
	Mark *et al.*, 2002	Dependent							
	Zakhartsev *et al*., 2003	Dependent							
	Clark *et al.*, 2008	Dependent							
	Steinhausen *et al*., 2008	Dependent							
	Pirozzi and Booth, 2009	Dependent							
	Vinagre *et al*., 2012	Dependent							
Eels	Sébert *et al.*, 1995	Dependent							

These differences in response can be due to interactions with other environmental or biological factors. For example, carbon dioxide or ocean acidification, pollutants, size and activity are also known to influence the response of metabolic rates to changes in temperature, oxygen levels and salinity (Newell, 1973; Shumway and Marsden, 1982; Ferraris *et al.*, 1994; Camus *et al.*, 2004; Sokolova and Lannig, 2008; Killen *et al*., 2010; Christensen *et al*., 2011). This means that the level of response to one factor can be modified in a positive or negative way by other changes occurring simultaneously (Laird and Haefner Jr., 1976; Bulnheim, 1979; Moore and Sander, 1984; Claireaux and Lagardére, 1999; Sokolova *et al*., 2012). Differences in experimental design and protocols can also explain some of the differences observed (Findley *et al*., 1978; McMahon, 1990; 2001b; Sokolova and Pörtner, 2003; Bakhmet and Khalaman, 2006). For example, most studies have tested the effects of prolonged exposure to abiotic factors lasting for hours or days, without taking into account the possible effects of short-term exposure to sudden stress, which can be particularly frequent in the intertidal environment (Newell and Bayne, 1973; Dye and McGwynne, 1980; Haure *et al.*, 1998; Anestis *et al*., 2008).

Differences can also arise as a result of adaptation to conditions in the various habitats that marine animals exploit. For example, intertidal species that are frequently exposed to harsh and fluctuating conditions regulate better than their subtidal and open ocean counterparts (Moon and Pritchard, 1970; De Pirro *et al.*, 1999a; Sokolova and Pörtner, 2001b; 2003; Altieri, 2006). In addition, phylogenetic and ecological differences, physiological state and developmental stage can all affect the response of metabolic rate to abiotic stressors (Sastry and McCarthy, 1973; Vernberg and Moreira, 1974; Moreira *et al*., 1981; Aagaard, 1996; Brown and Terwilliger, 1999; Glazier, 2005; Seibel and Drazen, 2007). Although no study has looked at the effect of phylogeny or taxon on metabolic rates, phylogenetic differences in responses are evident (see Table 4.1; Clarke and Johnston, 1999; Glazier, 2005). Littorinids that inhabit the highest levels on intertidal shores seem to regulate metabolic rates better than bivalves and crustaceans which inhabit the mid and low shores.

Marine animals show a wide range of morphological, behavioural, physiological and biochemical mechanisms to survive changes in the above factors. Although rarely discussed, the usual first response is a drastic reduction in activity (see Herreid II, 1980; Little, 1981;

Shumway *et al.*, 1983) followed by inactivity (see Garrity, 1984; Kronberg, 1990; McMahon, 1990; Lee and Williams, 2002; Williams *et al.*, 2005). Once inactive, animals can enter dormancy (in this case we are concerned with aestivation) which is accompanied by metabolic rate depression (down regulation of cellular metabolism) and/or a switch to anaerobic metabolism (which is highly inefficient and accelerates the consumption of energy stores) to supplement the decline in energy production from aerobic metabolism (see Storey and Storey, 1990; 2004; 2007; Storey, 1998; Guderley and St-Pierre, 2002; Pörtner, 2010; Williams *et al.*, 2011). By suppressing metabolic rate to low levels, animals can enter a hypometabolic state that allows them to endure long-term exposure to stressful environmental conditions while saving energy. This is because most of the energy consuming processes, such as protein synthesis, are down-regulated (see Boutilier, 2001; Storey and Storey, 2004; 2007; Sokolova *et al*., 2012). Reducing metabolic rates also reduces the need for exposure of gas surfaces (e.g. mantle cavity wall) of high shore gastropods to the external atmosphere minimizing water loss (see McMahon, 1988b; Sokolova and Pörtner, 2001b). On the other hand, switching to anaerobic metabolism allows animals to close off (e.g. valve closure in mytilids) from the external environment in order to avoid deleterious conditions (see McMahon, 1988b; Anestis *et al*., 2007).

Marine invertebrates show the most diverse metabolic responses to changes in salinity, and this depends on the direction of change. For example, metabolic rate can increase or decrease (dependent) as salinity changes, but overall, they tend to decrease as salinity deviates from normal levels (see Table 4.1). In some species, however, metabolic rates remain constant (independent) as salinity changes while others show both (mixed) responses (see Table 4.1). The differences in responses to salinity change are related to many extrinsic and intrinsic factors and conditions, as well as the combinations and/or interactions between these factors. For example, temperature (Simmons and Knight, 1975; Gyllenberg and Lundqvist, 1979; Sherman and Eichrodt, 1982; Stickle and Bayne, 1982; Williams, 1984), oxygen tension (Shumway, 1981; Taylor, 1981; Salvato *et al*., 2001) and pollutants (Laughlin Jr. and Neff, 1979; 1980; 1981; Tedengren *et al.*, 1988) can significantly influence an animal‟s metabolic response to salinity change. Nelson *et al.* (1977) found a more pronounced depression of oxygen consumption with increasing salinity at higher temperatures than at low temperatures in the juvenile prawn *Macrobrachium rosenbergii*.

In general, high temperatures increase the dependence of metabolic rates on salinity, while low temperatures reduce it (see Shumway and Koehn, 1982; Moore and Sander, 1984; Brown and Terwilliger, 1999; Williams *et al*., 2011). In addition, the critical points (salinity at which metabolic depression was lost) were different, showing a decrease with increasing temperature. Animals that live in environments where they experience frequent fluctuations in salinity levels (e.g. estuarine and tide pools living species) are able to regulate better than those that experience constant salinity levels such as subtidal and open ocean species (De Pirro *et al.*, 1999a; Sokolova and Berger, 2000; Sokolova *et al*., 2012). Different metabolic responses may also be caused by the different experimental design adopted including the level of salinity tested and duration of exposure to salinity stress (see Findley *et al*., 1978; Villarreal and Rivera, 1993; Bakhmet and Khalaman, 2006).

As a result of exposure to fluctuations in salinity, marine animals, including intertidal invertebrates, have developed mechanisms to survive changes in salinity. At the behavioural level, snails withdraw into the shell and close the aperture with the operculum to isolate themselves from the surrounding environment (Todd, 1961; Little, 1981; Taylor and Andrews, 1988). This type of behavioural isolation response is evident in other intertidal molluscs including bivalves which close their valves and limpets, which clamp down on rocks in order to limit exposure to adverse environmental conditions (Bayne, 1973a; Marshall and McQuaid, 1993a; Cheung and Lam, 1995; Yaroslavtseva *et al.*, 2000). Mobile animals actively seek favourable environments or escape from unfavourable salinity conditions; this is particularly true for many motile estuarine species (Hendrix Jr. *et al.*, 1981; Berger and Kharazova, 1997; Dowd *et al*., 2010b).

Molluscs are well known to maintain hyperosmotic haemolyph or blood and decrease membrane permeability under hypoosmotic conditions (see Little, 1981; Moran and Pierce, 1984; Ferraris *et al.*, 1994). Animals in estuaries and intertidal zones survive fluctuations in salinity through changes in permeability, liberation of osmotic effectors to haemolyph, active ion uptake, breakdown of cellular proteins and excretion of excess amino acids (Spaargaren, 1974; 1975; Findley *et al*., 1978; Rosas *et al.*, 1999; Dowd *et al*., 2010b; Sokolova *et al*., 2012). Cellular mechanisms of adaptation such as reversible changes of protein and RNA synthesis, alteration of the pattern of multiple molecular forms of enzymes, regulation of

ionic or osmotic content and cell volume, production of heat shock proteins (Moran and Pierce, 1984; Ferraris *et al.*, 1994; Ji *et al*., 2008) are also used to tolerate and survive salinity changes. One of the most important and widely exhibited responses is the ability of animals to depress metabolic rates when exposed to changing salinity levels (Marshall and McQuaid, 1993a; Sokolova *et al*., 2000a, b; Morritt *et al.*, 2007; Williams *et al.*, 2011).

Despite some deviations, animal metabolic rates generally decrease as oxygen levels decrease (see Table 4.1). Thus, at low oxygen levels a typical response is bradycardia. In other species, metabolic rates are independent of change in oxygen levels down to critical levels below which they become dependent, while others show both responses (see Table 4.1). As with salinity, metabolic responses to decreased oxygen can be modified by other biotic and abiotic factors. For example, variation in temperature (Taylor, 1981; Hawkins *et al*., 1982; Berschick *et al.*, 1987; Vetter *et al*., 1999), salinity (Bayne, 1973; Shumway, 1981; Ferraris *et al.*, 1994; Rosas *et al.*, 1999; Salvato *et al*., 2001), size (Marsden *et al*., 2012) and activity (Brown, 1979) are known to affect oxygen uptake rate. For the mussel *Perna perna*, the ability to regulate oxygen consumption under hypoxia increased from poor regulation at 10°C to good regulation at 30°C (Hicks and McMahon, 2002a). Shumway and Koehn (1982) found that low salinity alone and/or in combination with high temperatures had the most adverse effect on oxygen consumption by the American oyster *Crassostrea virginica* during declining oxygen tension. Animals that live high in the intertidal (i.e. littoral zone and fringe species) are often able to respire in air and hence show different metabolic rate responses from subtidal species (Helm and Trueman, 1967; Widdows *et al*., 1979; Houlihan and Innes, 1982; Deaton, 1991; Marshall and McQuaid, 1993b; Vetter *et al*., 1999; Altieri, 2006).

To survive during periods of low oxygen availability, marine animals have developed a wide range of mechanisms (see below). Intertidal gastropod snails have a highly vascularized mantle cavity that functions as a diffusion lung when filled with air (Gutierrez, 1988; McMahon and Russell-Hunter, 1978). Mussels have the ability to trap water in the mantle cavity which can serve as a store of oxygen for respiration during anoxic conditions (Moon and Pritchard, 1970). Some bivalves periodically gape the shell valves to promote aerial gas exchange (Moon and Pritchard, 1970; Bayne, 1973a; Nicastro *et al*., 2010). Hermit crabs retreat into the shell and isolate themselves from the surrounding environment (Reese, 1969;

Wernick and Penteado, 1983). Some fish and other animals emerge to the surface and begin aerial respiration (emergence response) to enhance oxygenation of the gills (Herreid II, 1980; Hill *et al.*, 1991; Martin, 1995; Halpin and Martin, 1999; Richards, 2011).

Most marine animals are extremely efficient at extracting oxygen from water by slowing or increasing the ventilatory stream (Saint-Paul, 1984; deFur, 1988; Hourdez and Lallier, 2007), altering heart rate, cardiac stroke volume and cardiac output (Bayne, 1971a; McMahon, 1988a; Airriess and McMahon, 1994; Hourdez and Lallier, 2007), and having a relatively large gill surface area and high circulation rate (McMahon, 2001a; Mandic *et al.*, 2009). Other animals have respiratory proteins (i.e. hemocyanin, hemoglobin, hemerythrin, etc) with a particularly high affinity for oxygen (Herreid II, 1980; Saint-Paul, 1984; deFur, 1988; McMahon, 1988a; Wells, 1999; Richards, 2011).

Marine animals are also able to suppress their metabolic rate and/or switch to anaerobic metabolism (see above) in order to save energy and supplement the decline in energy production from aerobic metabolism (McMahon, 1988a, b; Stickle *et al*., 1989; Marshall and McQuaid, 1991; Childress and Seibel, 1998; Larade and Storey, 2007; Mandic *et al.*, 2009). Other adaptations include suppression of ATP-demand and ATP-supply pathways (Oeschger and Storey, 1993; Hochachka *et al.*, 1996; Hochachka and Lutz, 2001; Boutilier, 2001; Sokolova *et al*., 2012), a global decline in protein biosynthesis (Hochachka *et al.*, 1996; Storey and Storey, 2004), a generalized decrease in membrane permeability (Hochachka *et al.*, 1996), and regulation of key enzymes (Oeschger and Storey, 1993).

Although, oxygen level and salinity profoundly affect metabolic rates, temperature is the abiotic factor that exerts the greatest influence on heart rate and oxygen consumption, and considerable work has focused on this factor (see Table 4.1). deFur and Mangum (1979) suggested that the effect of temperature on heart rate is at least the same as and often greater than the effect of temperature on oxygen consumption. On the other hand, Spaargaren (1977) found the effect of temperature on oxygen consumption to be stronger than that on heart rate. Among ectotherms, particularly marine species, there is no general consensus on how an ectothermic animal‟s metabolic rate responds to temperature. Some authors suggest partial or total temperature-dependence, while others suggests temperature-independence of metabolic

rates. The dispute occurs across a wide range of ectothermic animals, including marine molluscs such as littorinids and species from other phyla (see Table 4.1).

Heart rate and oxygen consumption generally increase as temperature increases until a threshold is reached, after which they decrease (see Table 4.1). This is in agreement with the metabolic theory of ecology; thus the "Universal Temperature Dependence (UTD) of metabolism" (Clarke, 2004; 2006; Clarke and Fraser, 2004, 2009). This is because reaction rates are temperature dependent increasing with increasing temperature until declining above optimum or near lethal temperatures (Clarke, 1993b; Gillooly *et al.*, 2001, 2002, 2006; Brown *et al.*, 2004; Clarke and Fraser, 2004, 2009; O'Connor *et al*., 2007). Other species, however, are able to regulate metabolic rates as temperature increases (see Table 4.1), and this can occur across temperature ranges experienced in the field (Vernberg and Vernberg, 1966; 1969; Newell and Pye, 1970a, b; Branch *et al*., 1988; Cheung and Lam, 1995; Clarke, 2004; etc).

Some animals show mixed or both responses with a narrow zone of temperature independence bounded by zones of dependence on either side (see Table 4.1). For example, the metabolic rate of *Littorina saxatilis* is temperature-dependent at low and mid ambient temperature ranges, but becomes partially independent above normal ambient temperatures (see Sokolova and Pörtner, 2003). This is also true for the tropical eulittoral fringe *E. malaccana* (Marshall *et al.*, 2010; 2011), the clam *Meretrix meretrix* (Tang *et al.*, 2005), the intertidal bivalve *Cerastoderma edule* (McMahon and Wilson, 1981), the adult females of the copepod *Calanus glacialis* (Tande, 1988), the temperate intertidal isopod *Ligia oceanica* (Whiteley and Faulkner, 2005), the amphipods of the genus *Gammarus* (Bulnheim, 1979), the juveniles of the shrimp *Fenneropenaeus chinensis* (Tian *et al*., 2004), the adult females of the crab *Emerita brasiliensis* (Moreira *et al.*, 1981), and the sea bass *Dicentrarchus labrax* (Claireaux and Lagardére, 1999), etc.

Mixed responses suggest that animals are able to regulate metabolic rates, especially within the temperature ranges experienced in the field or when temperatures approach lethal limits. It is claimed that temperature independence at relatively high temperatures is adaptive

because it enables animals to thermoregulate while maintaining metabolic homeostasis (see Vernberg and Vernberg, 1966; Bulnheim, 1979; Hawkins, 1995; Tian *et al*., 2004). The maintenance of metabolic homeostasis is an important adaptation to littoral existence during exposure to air because there is little time to feed and food is scarce (de Zwaan and Wijsman, 1976; Hawkins *et al*., 1982; Spicer and Taylor, 1987; Branch *et al.*, 1988; Tully *et al*., 2000; etc). Thus, metabolic homeostasis is the predominant adaptive strategy of metabolic responses that allows an organism to survive environmental stress or disturbances (see Somero, 2004; Sokolova and Lannig, 2008; Lannig *et al*., 2010; Sokolova *et al*., 2012).

And as for both salinity and oxygen concentration, differences in metabolic responses to temperature can be related to many extrinsic and intrinsic factors and conditions, as well as the combinations and/or interactions between these factors (see below). For example, salinity, oxygen tension, carbon dioxide, and pollutants can significantly influence an animal‟s metabolic response to temperature (see below); however, this is not true to for all animals or species (see Nelson *et al.*, 1977; Brown and Terwilliger, 1999; Allan *et al*., 2006; Williams *et al*., 2011). Differences can also arise as a result of adaptation to conditions in the different habitats that animals exploit. For example, animals that experience frequent fluctuations and extremes of temperatures (e.g. eulittoral zones species) regulate better than those that experience constant and moderate temperatures (see Vernberg, 1969; Spaargaren and Achituv, 1977; McMahon and Wilson, 1981; Hawkins *et al*., 1982; Marsden, 1987; Dahlhoff *et al*., 1991; Somero, 2002; Isla and Perissinotto, 2004).

This is supported by studies which have shown that metabolic rates differ when measured in different respiratory medium (air or water) as a result of animals‟ adaptation to different habitats, thus conditions (McMahon and Russell-Hunter, 1981; Navarro *et al*., 1987; De Pirro *et al*., 1999b; Santini *et al*., 2000). The findings (increase and decrease as temperature increases) of field and laboratory measurements on heart rate of the tropical limpet *Cellana grata* led Chelazzi *et al*. (1999) to suggest that limpets (may be true for other animals) in some habitats may be able to regulate their metabolic rate when resting on hot substrates.

Marine animals have developed a wide range of mechanisms to survive heat stress. Depending on the level of stress, adaptations can be behavioural, physiological, biochemical

and/or a combination (see McMahon, 1990; Sinclair *et al*., 2004; Wang *et al*., 2007a; Miller and Denny, 2011; Sokolova *et al*., 2012). Below physiological limits, behavioural mechanisms such as choice of suitable microhabitats by mobile animals (Bally, 1983; Gutierrez, 1988; McMahon, 1990), and withdrawal into the shell by snails and other shelled gastropods (see Garrity, 1984; McMahon, 1990; Williams and Morritt, 1995) are used to limit exposure of the animal to adverse environment conditions. When heat stress increases, some animals, including some littorinids, use physiological mechanisms such as metabolic adjustments (see Shirley *et al*., 1978; McMahon, 1988a, b; 1990; Sokolova and Pörtner, 2001a, b), thermal regulation, tolerance and acclimation (see Huey and Bennett, 1990; McMahon, 1990; Horowitz, 2001; 2002), etc. Some animals synthesize heat shock proteins which are involved in thermal acclimation and tolerance (see Feder and Hofmann, 1999; Tomanek, 2002; Anestis *et al*., 2010; Marshall *et al.*, 2011). Other cellular or biochemical mechanisms such as down-regulation of protein synthesis, changes in protein (enzyme) structure, and increased enzyme stability and activity (see Somero, 1978; 1995; 2004; Dahlhoff and Somero, 1993a; Schmidt *et al*., 2007; Dong and Somero, 2009; Tattersall *et al*., 2012) are also important to survive heat stress.

This chapter will investigate if temperate littorinids employ metabolic adjustment (regulation), which is a survival mechanism used by tropical littorinids against thermal stress (see Marshall and McQuaid, 2010; Marshall *et al.*, 2010; 2011). This will help us to understand if metabolic regulation is a physiological response aimed at preserving energy and surviving environmental challenges during emersion in the littoral zone as seen in other tropical species.

Within the littorinid snails, there seem to be fundamental differences among genera and species in the relationship between temperature and both oxygen consumption and heart rate. There is a clear difference in metabolism between tropical and subtropical or temperate species, and likewise for eulittoral fringe and eulittoral species. Tropical species show good regulation of oxygen consumption and heart rate, rendering them independent of temperature across a range of temperatures (Marshall and McQuaid, 2010; Marshall *et al.*, 2010; 2011). In contrast, temperate species show mixed responses (strong regulation, partial regulation and non-regulation) of oxygen consumption and heart rate (Sokolova and Pörtner, 2003). At the

same time, eulittoral fringe species show better regulation of metabolic rates than lower shore species (Marshal *et al.*, 2010). These differences seem to relate to differences in biogeography, ecology, and phylogeny. The tropical littorinids fill a completely new niche in the eulittoral fringe and have different adaptive physiological attributes that allow them to cope with temperature stress better than eulittoral species (see McMahon, 1990). Thus, eulittoral fringe rocky species are better able to regulate heat uptake and cope with heat stress than their eulittoral and low shore counterparts and this is complemented by different metabolic responses (see above).

In comparing different studies, some of these trends may be obscured or unclear as a result of differences in the methods followed. For example, McMahon (1990) and others measured oxygen consumption and heart rate at discrete intervals of 5ºC, and this may have led to false conclusions concerning the effect of temperature on metabolism. Recently, Marshall and McQuaid (2010) and Marshall *et al.* (2010) measured oxygen consumption and heart rate continuously and this allowed the identification of distinct breakpoints in metabolism when temperature is continuously raised, that were not clear in other studies. They found clear indications of a thermally independent (thermoneutral) zone of metabolism, which was followed by a sharp increase (towards the species‟ upper limits temperature) and a decline (towards the critical maximum temperature) in heart rate and oxygen consumption. This indicates that these species can regulate their metabolism over a wide range of temperatures, before starting to increase their metabolism. Metabolic responses to temperature rise can depend on the rate of increase. For example, the time course of heat exposure was much faster in the study of Marshall *et al.* (2010) than in some previous investigations, but was chosen to mimic naturally encountered conditions. The rapid increase in temperature may have contributed to mixed responses observed in other species, especially for species that are not normally exposed to rapid and fluctuations temperatures.

The use of invasive techniques such drilling of the shell or surgical procedures in other studies (Bayne, 1973a; Butler *et al.*, 2004; Braby and Somero, 2006) may influence the findings by inducing additional stress. The development of non-invasive techniques such as the use of infrared sensors glued to the shell of animal (Depledge and Depledge, 1990, Aagaard *et al.*, 1991; McMahon, 1999; Chelazzi *et al.*, 1999; 2001) has allowed the

investigation of animal responses to heat stress with minimal treatment-induced stress. In some studies, animals were not allowed enough time to acclimate after handling or treatment (McMahon, 1999) and so may have shown different performance such as extremely high metabolic levels at the outset due to stress. Animals thus may have responded mainly to experimental stress, rather than heat stress. More recent experiments using non-invasive methods and more favourable experimental conditions indicate that a majority of animals are good regulators, often able to regulate metabolic rates at high temperatures (Sokolova and Pörtner, 2003; Marshall and McQuaid, 2010; Marshall *et al.*, 2010; 2011).

Together with a significant correlation between heart rate and metabolic rates in studied species (Marshall and McQuaid, 1992b; Santini *et al.*, 1999; 2000; Butler *et al.*, 2002; 2004), the development of non-invasive techniques has made heart rate monitoring more popular in ecophysiological studies of marine invertebrates such as molluscs, and crustaceans as well as fishes (Depledge and Depledge, 1990, Aagaard *et al.*, 1991; Briggs and Post, 1997; Calosi *et al*., 2003). The method involves the use of distant monitoring of cardiac muscle volume (plethysmogram) based on the infrared illumination of the heart region and recording reflected light using a computerised system (Depledge and Depledge, 1990; Calosi *et al*., 2003). In the current study, this approach was used to investigate the metabolic response of littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* to heat stress. Since the heart is the first organ to fail as temperature rises (see Somero, 2002; Pörtner and Knust, 2007; Iftikar *et al*., 2010), the heart rate method offers an additional and/or alternative approach to oxygen consumption for measuring metabolic rates or activity.

Several other factors may alter the response to temperature so that under certain conditions a regulating animal may respond with non-regulation or *vice versa*. Apart from environmental factors such as oxygen level (Shumway and Marsden, 1982; Berschick *et al.*, 1987), salinity (Laird and Haefner Jr., 1976; Gyllenberg and Lundqvist, 1979; Williams *et al*., 2011), chemicals (Camus *et al*., 2004; Lannig *et al*., 2006; 2008), carbon dioxide (Langenbuch and Pörtner, 2002; Christensen *et al*., 2011), etc. These include size or age (Newell *et al*., 1972; Dahlhoff *et al*., 2002), sex (Vernberg and Moreira, 1974; Crear and Forteath, 2000) as well as the animals‟ health (Anderson, 1975a, b; Thompson, 1983; Curtis, 2002), feeding or nutritional status (Newell and Bayne, 1973; Branch *et al.*, 1988; Shumway *et al*., 1993;

Robertson *et al*., 2002), developmental stage (de Vooys, 1976; Spaargaren and Achituv, 1977; Hatcher *et al*., 1997), and level of activity (Wallace, 1972b; Newell and Bayne, 1973; Steinhausen *et al*., 2008), etc.

Marsden *et al.* (1973) found the metabolism of small shore crab *Carcinus maenas* less temperature dependent than that of large crabs, suggesting temperature independence in the former. Nutritional status is known to influence not only the level of metabolism, but temperature relationships of metabolism in littorinids and other marine animals (see Lewis, 1971; Bayne *et al*., 1976). Animals infected or attacked by macroparasites such as trematodes are known to be less resistant to high temperature, as well as anoxia (Berger and Kharazova, 1997; Granovitch *et al.*, 2000; Huxham *et al*., 2001; Bates *et al*., 2011). Acclimation or previous thermal history (Vernberg, 1969; Newell and Pye, 1970b; McMahon *et al*., 1995) as well as season (Newell and Pye, 1970a; Simmons and Knight, 1975; Jansen *et al*., 2007) have effects on animals‟ metabolic responses (De Pirro *et al*., 1999b). Thus, multiple endogenous and extrinsic factors can influence the type of temperature metabolic rate responses observed, and of course these factors can interact (see Newell, 1973; Newell and Roy, 1973; Hawkins, 1995; Aagaard, 1996; McMahon, 1999).

Other studies have explicitly overlooked patterns of regulation or non-regulation because they were interested in metabolic rate differences linked to medium (i.e. water versus air; McMahon and Russell-Hunter, 1977; Houlihan, 1979; Dye, 1987; Marshall and McQuaid, 1992a; Halpin and Martin, 1999), season (i.e. summer versus winter; Newell and Pye, 1970a, b; Shirley *et al*., 1978; Marshall and McQuaid, 1994), activity (Newell *et al.*, 1979; Brown, 1979), biogeographic region (i.e. tropics versus temperate; Vernberg, 1959; 1969; Pickens, 1965; Innes and Houlihan, 1981; 1985; Lardies *et al*., 2011), shore level (i.e. eulittoral fringe versus low shore; Burggren and McMahon, 1981; Brown and Da Silva, 1984; Bally, 1987), habitat (i.e. coastal versus estuarine; Vinagre *et al*., 2012) or origin (i.e. invasive versus native species; Iftikar *et al*., 2010). Examination of the original data in such studies indicates that in some cases there was an overlooked and unreported degree of temperature independence in certain species, supporting the idea of regulation in wide range of animals. For example, looking at the results of Isla and Perissinotto (2004), it is clear that both sexes of the estuarine copepod *Pseudodiaptomus hessei* partially regulated their basal metabolic rates.

Missing from our understanding of metabolic responses to temperature are the patterns of metabolic physiology of warm temperate and subtropical species as most of studies have been done on tropical and cold temperate species (McMahon, 1990; Sokolova and Pörtner, 2001b, 2003; Marshal and McQuaid, 2010; Marshall *et al*., 2010; 2011). This chapter aims to address the metabolic physiology of *Afrolittorina* spp. that have distributions that overlap in the warm temperate region of South Africa. The objectives of this study were to compare the heart rate-temperature relationships of two co-existing southern African species of the genus *Afrolittorina*: *A. africana* and *A. knysnaensis*. In addition, the responses of these *Afrolittorina* species were compared to those of subtropical and tropical species of *Echinolittorina* (*E. natalensis*, *E. malaccana* and *E. vidua*) and *Littoraria glabrata*. This allowed me to investigate the effects of phylogeography, species identity and biogeographic distribution on heart rate responses to heat stress.

An approach followed by Marshall *et al*. (2010) was used, and the objectives included determining whether metabolic independence of temperature differed among regions and how the response of individuals found in warm temperate regions compared to that found in other tropical and cool temperate regions. It was hypothesized that the heart performance of conspecific individuals of *Afrolittorina* from different biogeographic regions would be similar, but different from that of *Echinolittorina* and *Littoraria* species from subtropical and tropical zones. The results of this study will help to shed light whether the ability to regulate metabolism under conditions of changing temperature is fixed phylogenetically or is an adaptive response differing among individuals from different biogeographic regions. It will also shed light on how these species (and other ectotherms) will respond to climate change (especially temperature change) under a scenario of global warming.

4.2. Materials and Methods

4.2.1. Study species

Six littorinid species of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* were used, namely: *A. knysnaensis*, *A. africana*, *E. natalensis*, *E. malaccana*, *E. vidua*, and *L. glabrata*. See Chapter 1 for species distribution ranges and patterns of vertical zonation as well as microhabitat use and aestivation behaviour.

4.2.2. Collection and transportation

Specimens of *A. africana*, *A. knysnaensis*, *E. natalensis* and *L. glabrata* were collected from natural rocks at different sites (see Fig. 4.1 and Table 4.2) along the South Africa coast from September 2010 to March 2011. While *E. malaccana* and *E. vidua*, were collected from an artificial seawall at Jerudong, Brunei Darussalam (4°32'N; 114°43'E) in November 2009. Individuals of each species were collected from the highest shore levels at which the species occurred; thus, the eulittoral fringe and upper eulittoral which ranges between 2 m Chart Datum to around 5 m above the mean high water level (2.5 m CD), depending on locality. Similar sized individuals that were feeding or had fed within 12 hours (assumed to have fed since they were collected immediately after or during high tides) were returned to the laboratory in labelled plastic bags placed inside an insulated cool box.

4.2.3. Handling and treatment conditions

On arrival at the laboratory, specimens were washed in seawater, allowed to emerge from their shells and to reattach to 8 cm lidded plastic Petri dishes or 2 L plastic containers before being exposed to air, when they exhibited behavioural emergence. Active animals were blotted dry with paper towel and dried using a fan at room temperature to induce

aestivation before use or treatment. Specimens were kept on dry paper towel at room temperature (18-22ºC) before immediate use, or kept in a fan blown incubator (Memmert UFE 500, Schwabach**,** Germany) set at 30ºC until later use, within 3 hours. These specimens were also used to investigate the effects of acclimation on heart performance.

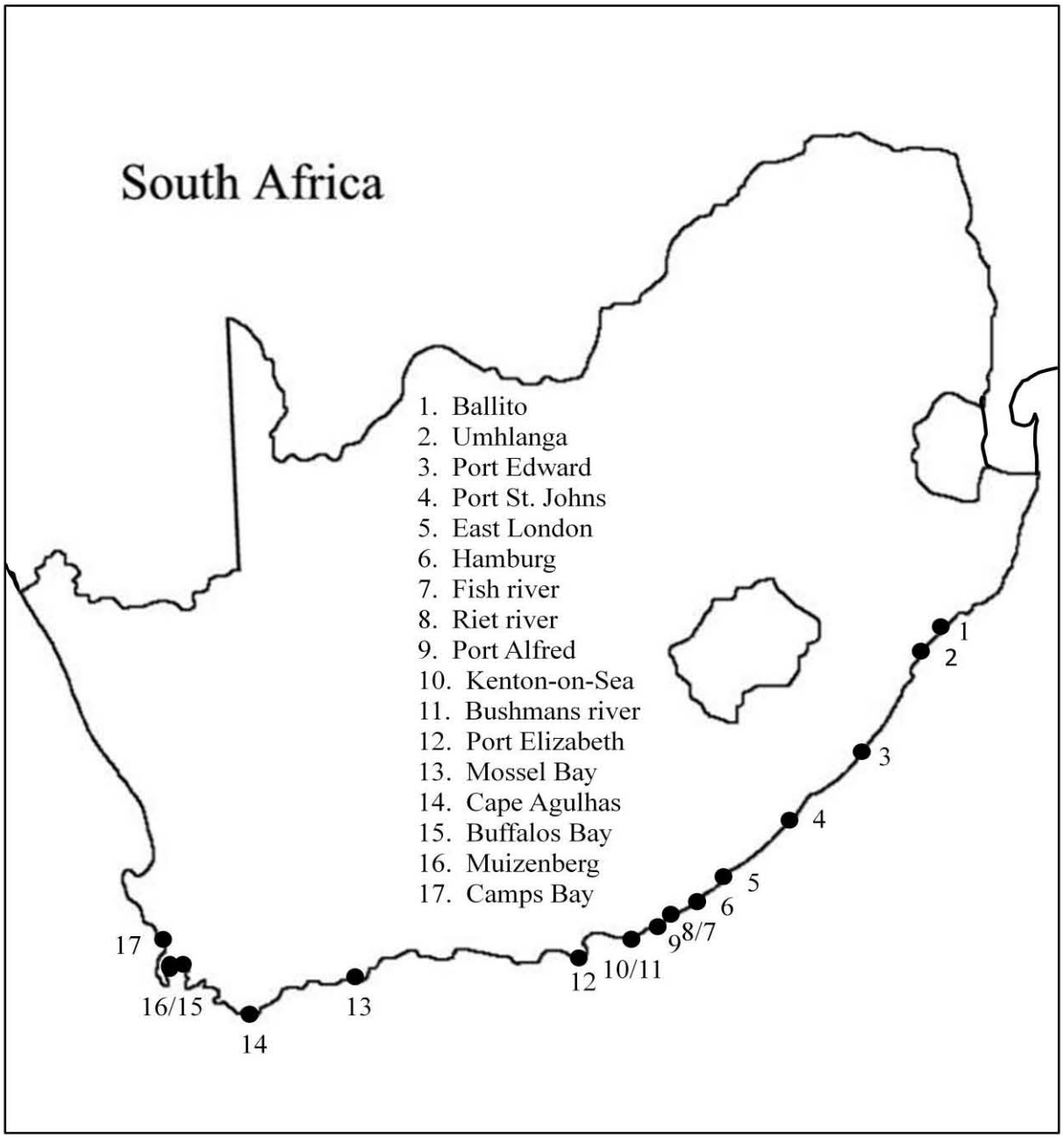

Figure 4.1. Map of South Africa showing sampling sites (see Table 4.2) for littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* used for heart function experiments.

Table 4.2. Sampling sites for littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from South Africa used for heart function experiments.

Bioregion	**Site (Abbreviation)**	**Species samples**
Subtropical	1. Ballito (BA)	*A. africana*; *E. natalensis*; *L. glabrata*
	2. Umhlanga (ML)	*A. africana*; *E. natalensis*; *L. glabrata*
	3. Port Edward (PE)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*
	4. Port St. Johns (PJ)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*
	5. East London (EL)	*A. knysnaensis*; *A. africana*; *E. natalensis*; *L. glabrata*
Warm temperate	6. Hamburg (HU)	*A. knysnaensis*; *A. africana*
	7. Fish river (FR)	*A. knysnaensis*; *A. africana*
	8. Riet river (RR)	*A. knysnaensis*; *A. africana*
	9. Port Alfred (PA)	*A. knysnaensis*; *A. africana*
	10. Kenton-on-Sea (KOS)	*A. knysnaensis*; *A. africana*
	11. Bushmans river (BU)	*A. knysnaensis*; *A. africana*
	12. Port Elizabeth (NN)	*A. knysnaensis*; *A. africana*
	13. Mossel Bay (MBB)	*A. knysnaensis*; *A. africana*
Cool temperate	14. Cape Agulhas (CA)	*A. knysnaensis*
	15. Buffalos Bay (BB)	*A. knysnaensis*
	16. Muizenberg (MU)	*A. knysnaensis*
	17. Camps Bay (CB)	*A. knysnaensis*

In an attempt to investigate the effect of starvation on heart performance, specimens were left (emersed) on dry paper towel at room temperature (18-22ºC) for 14 days or more without feeding. For heat shock experiments, 10 aestivating individuals of each species were placed in a 20 ml dry container that was immersed in a Grant programmable water bath (GP 200, Grant, Germany) set to 20ºC. Temperature was increased in 5°C increments over 10 minute intervals to reach 45°C and left for 1 hour. Temperature inside the container was monitored using T-type thermocouples (Cromega and ADInstruments, Australia). After 1 hour at 45°C, the container was removed from the water bath and allowed to cool for 2 hours prior to running the experiments.

An attempt was also made to investigate the effect of repeated exposure on heart performance using *Afrolittorina* species. A similar procedure as for the heat shock experiments (see above) was used, except that temperature was raised at 0.25°C min^{-1} between 20 and 45ºC after 20-30 min at 20ºC. Once at 45ºC, animals were taken out of the water bath and left in air at room temperature (approx. 20ºC) to recover before treatment. For the experimental treatment, animals where either left with sensors attached at (1) room temperature or (2) 30°C for 1-3 days (no feeding), or (3) "feeding" for 1-3 days at room temperature (no sensors attached) before final exposure.

4.2.4. Heart rate measurements

Heart rate measurements were based on aestivated snails held and treated in different ways (see above); and each replicate comprised a pair of two snails. Occasionally, behavioural emergence or aestivation were quickly induced prior to measurements. Heart rate was measured in dry air by wrapping animals in dry paper towel, and enclosing them in dry plastic bags to avoid wetting by water in the waterbath. At the start of an experiment, plastic bags containing snails were placed in a Grant programmable water bath (GP 200, Grant, Germany) set to 20ºC.

Heart performance was recorded using non-invasive plethysmography (see Depledge and Anderson, 1990). Optoelectronic (infrared) reflective-sensors (Vishay Semiconductors, V69 CNY70 732/735, Germany) were adhered to the shells of isolated snails near the mantle cavity with Blue-Tac or Prestick (Bostick Ltd, United Kingdom). Signals from the sensors were amplified and filtered with a custom-built preamplifier, and then digitally-logged/recorded with a computerised recording system (PowerLab/4SP and 4/30, Chart version 5 and 7, ADInstruments, Australia). Sampling rate was set at 40 Hz and the amplitude varied between 40 and 1000 mV. An additional smoothed trace (Triangular-Bartlett smoothing) was derived on a separate channel, and this was used in further analyses.

Except for few cases when *Afrolittorina* spp. were exposed to a slower rate of 0.5°C min^{-1}, experimental temperature was raised at 0.25°C min^{-1} between 20 and 55ºC, after 20-30 min at 20ºC by the GP 200 programmable water bath. Temperature was monitored and recorded every 1 min using the same PowerLab recording system and a thermocouple pod (T-type pod, ADInstruments, Australia) fitted with a fine K-type thermocouple (Cromega and ADInstruments, Australia), and a Fluke 54II Thermometer (Fluke Corporation, USA) fitted with a T-type thermocouple (Fluke Corporation and Cromega) inserted into the plastic bags with the animals. Heart rate, in beats per minutes (bpm), was logged every 1 minute for snails under constant heating (0.25 or 0.50°C min^{-1}) between 20 and 55°C, after an initial 20-30 min at 20°C. Temperatures used in experiments were within the broad range of actual body temperatures measured in field (unpub. data). Once an animals‟ heart had failed, it was taken out of the water bath and left in air at room temperature to assess recovery (for repeated exposure experiments only) of cardiac function. Recovery and mortality rates were determined 12-24 hours after exposure. At the end of each run, snail shell length in millimeters (mm) was determined using Vernier Callipers to the nearest of 0.02 mm.

4.2.5. Data analysis

Sample sizes for the various experiments and treatments are presented in Table 4.3. Heart rates (bpm) were plotted against temperature using Sigma Plot version 10.0 (SPSS Inc.). The temperature ranges used for determining the first breakpoint (the temperatures at which thermoneutrality was lost) were 30 to 38°C and 38 to 46°C, and the second breakpoint (temperatures at which heart rate show a sharp decrease; Arrhenius Breakpoint) were 40 to 48°C for *Afrolittorina* species and 46 to 57°C for *Echinolittorina* and *Littoraria*. Temperature ranges used to determine breakpoints were based on the results of preliminary analyses where breakpoints were found to lie within these ranges. Pairwise linear regression with breakpoints (Statistica 10, Statsoft) was used to determine the breakpoints using recorded heart beat (independent variable) and temperature (dependent variable), endpoints were determined manually from edited heart rate data, and figures were drawn using Sigma Plot version 10.0.

Table 4.3. Proportions of regulating and non-regulating littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from tropical, subtropical and temperate regions of Brunei Darussalam and South Africa.

Taxa	Bioregion	Experiment or Treatment	Proportion in number and percentage (%)		
			Regulating	Non-regulating	Total
A. knysnaensis	All	Normal	142 (67.9)	67 (32.1)	209
	Cool temperate	Normal	42 (76.4)	13 (23.6)	55
	Warm temperate	Normal	100 (64.9)	54 (35.1)	154
	All	Starved	6 (75.0)	2 (25.0)	8
	All	Shocked	6 (50.0)	6 (50.0)	12
	Warm	Chronic	15 (83.3)	3 (17.7)	18
	All	20°C acclimated	10 (52.6)	9 (47.4)	19
	All	30°C acclimated	11 (55.0)	9 (45.0)	20
A. africana	All	Normal	88 (53.0)	78 (47.0)	166
	Subtropical	Normal	18 (50.0)	18 (50.0)	36
	Warm	Normal	70 (53.8)	60 (46.2)	130
	All	Starved	2 (18.2)	9 (81.8)	11
	All	Shocked	5 (41.6)	7 (58.4)	12
	Warm	Chronic	7 (63.6)	4 (46.4)	11
	All	20°C acclimated	9 (47.4)	10 (52.6)	19
	All	30°C acclimated	11 (55.0)	9 (45.0)	20
L. glabrata	Subtropical	Normal	10 (62.5)	6 (37.5)	16
	Subtropical	Starved	4 (66.7)	2 (33.3)	6
	Subtropical	20°C acclimated	6 (75.0)	2 (25.0)	8
	Subtropical	30°C acclimated	7 (70.0)	3 (30.0)	10
E. natalensis	Subtropical	Normal	33 (73.3)	12 (26.7)	45
	Subtropical	Starved	5 (62.5)	3 (37.5)	8
	Subtropical	Shocked	1 (100)	-	1
	Subtropical	20°C acclimated	9 (56.3)	7 (43.7)	16
	Subtropical	30°C acclimated	10 (66.7)	5 (33.3)	15
E. vidua	Tropical	Normal	18 (90.0)	2 (10.0)	20
E. malaccana	Tropical	Normal	19 (95.0)	1 (5.0)	20

4.3. Results

4.3.1. Effect of region, phylogeny and ecology on heart performance

Results of different experiments show that region, phylogeny and ecology all affect heart performance of the species investigated (see below).

4.3.1.1. Are there regional or phylogenetic differences in stress response patterns?

There were patterns of both thermal independence from temperature and dependence of heart rate on temperature in the species investigated. The tropical *Echinolittorina* species showed thermal independence while the subtropical and temperate *Echinolittorina*, *Littoraria* and *Afrolittorina* species showed both thermal dependence and independence of heart rate on temperature (Fig. 4.2). Overall, the subtropical *Echinolittorina* and *Littoraria* species tended to show thermal independence overall, but there were exceptions. The *Afrolittorina* spp. showed high individual variability, some individuals exhibiting thermal independence, while others did not.

The point at which the heart rate became independent of temperature was higher for tropical and subtropical (approximately for 21-23°C) than for the temperate (approximately 14ºC) species. There was also a difference in the proportions of individuals that showed thermal independence and dependence in the studied species (see Fig. 4.3 and Table 4.3). For thermal independence, the trend was for higher values (approximately 90-95%) for tropical species, followed by the subtropical (approximately 62-73%) and temperate (approximately 53-67%) species, respectively. On the other hand, for thermal dependence, the trend was reversed: temperate species (approximately 33-47%), followed by subtropical (approximately 27-37%) and tropical (approximately 5-10%) species, respectively.

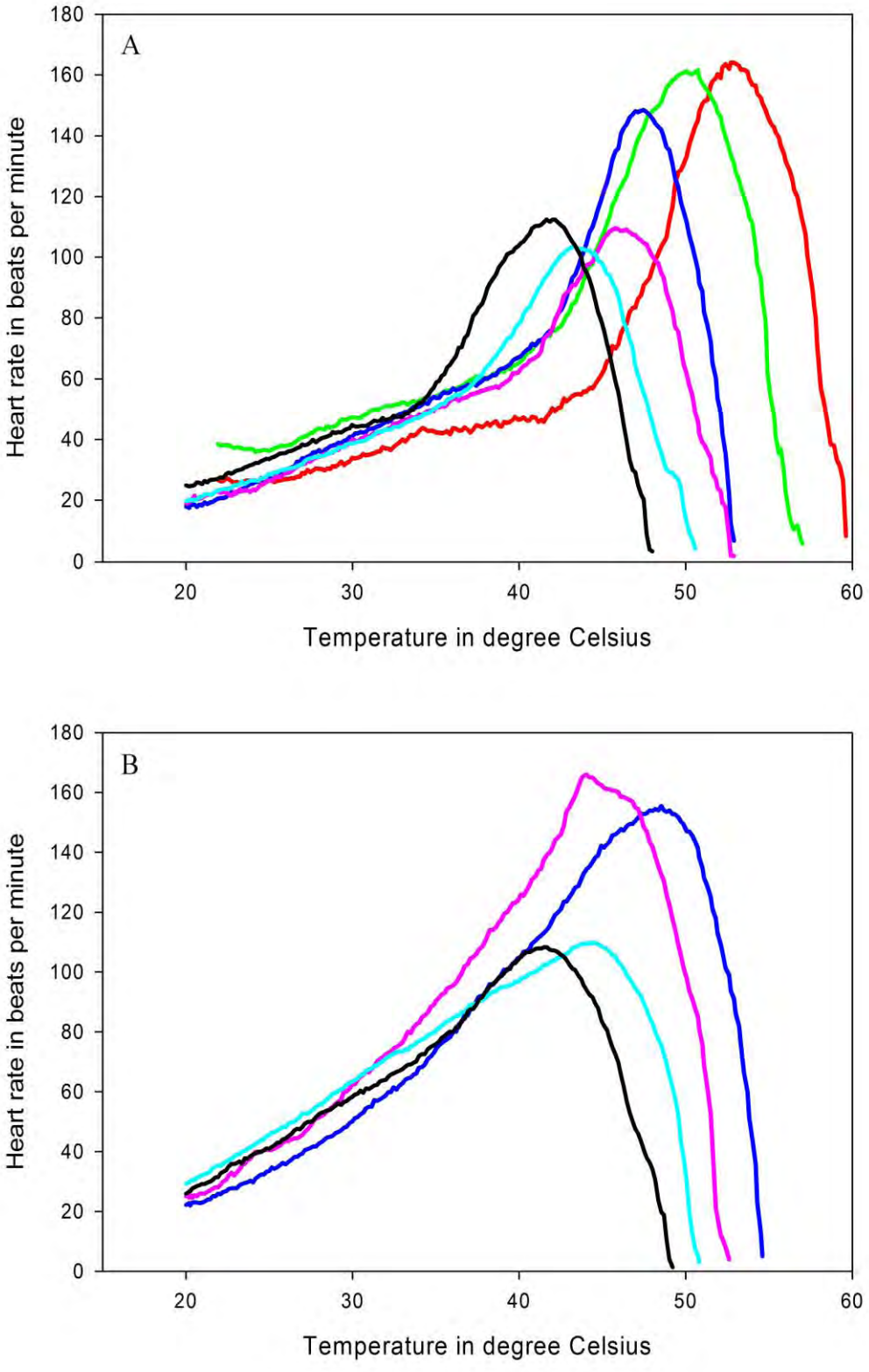

Figure 4.2. Heart patterns of regulating (A) and non-regulating (B) *E. malaccana* (red), *E. vidua* (green), *E. natalensis* (blue), *L. glabrata* (pink), *A. africana* (cyan) and *A. knysnaensis* (black). Traces are means of the best five selected individuals‟ traces.

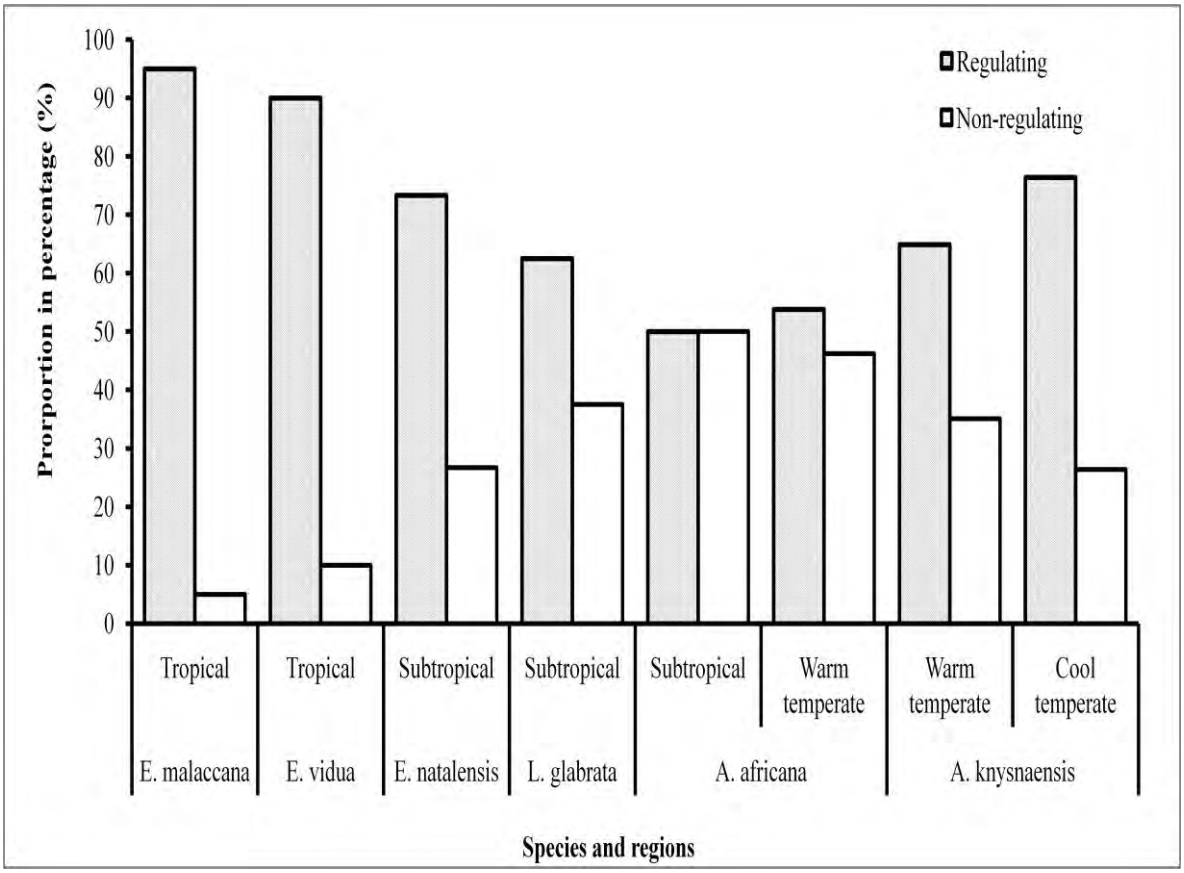

Figure 4.3. Proportions in percentage (%) of regulating and non-regulating members of *E. malaccana*, *E. vidua*, *E. natalensis*, *L. glabrata*, *A. africana* and *A. knysnaensis* from tropical, subtropical and temperate regions of Brunei Darussalam and South Africa.

Heart rate varied during constant increase in temperature and showed distinct breakpoints and endpoints. The first breakpoint, the Thermoneutral Breakpoint Temperature (TBP) at which heart function became independent of temperature change occurred at 43 and 41ºC and the second, the Arrhenius Breakpoint Temperature (ABT), the temperatures at which heart rate showed a sharp decrease occurred at 54 and 50ºC (see Table 4.4) for tropical *E. malaccana* and *E. vidua* respectively; while the subtropical *E. natalensis* and *L. glabrata* both had TBPs at 41ºC and ABTs at 49ºC. The two *Afrolittorina* species had similar TBPs and ABTs at 34ºC and 44ºC (see Table 4.4). The Endpoint Temperature (EPT), the temperature at which heart function ceased, occurred at 59.6, 57.0, 57.0, 53, 51 and 49°C for *E. malaccana*, *E. vidua*, *E. natalensis*, *L. glabrata*, *A. africana* and *A. knysnaensis*, respectively, showing a clear ranking of tropical > subtropical > temperate species.

Table 4.4. Breakpoints and Endpoints temperatures of littorinid snails of the genera *Afrolittorina*, *Echinolittorina* and *Littoraria* from tropical, subtropical and temperate regions of Brunei Darussalam and South Africa.

Species	**Region**	**Treatment**	**Response**	**Break and Endpoints**		
				TBTs	**ABTs**	**EPTs**
A. knysnaensis	All	Not treated	Regulating	34.10	44.10	47.99
	All	Not treated	Non-Regulating	-	44.10	49.20
	Cool temperate	Not treated	Regulating	34.10	43.11	47.32
	Cool temperate	Not treated	Non-Regulating	-	44.14	47.70
	Warm temperate	Not treated	Regulating	34.10	43.11	48.00
	Warm temperate	Not treated	Non-Regulating	-	44.14	49.2
	All	Starved	Regulating	33.99	44.05	49.01
	All	Starved	Non-Regulating	-	44.05	49.71
	All	Shocked	Regulating	34.5	44.5	49.87
	All	Shocked	Non-Regulating	-	44.5	48.84
	Warm temperate	Chronic	Regulating	38.5	45.5	50.50
	Warm temperate	Chronic	Non-Regulating	-	45.5	50.90
A. africana	All	Not treated	Regulating	34.10	44.10	50.56
	All	Not treated	Non-Regulating	-	44.10	50.80
	Subtropical	Not treated	Regulating	34.10	44.05	50.52
	Subtropical	Not treated	Non-Regulating	-	44.05	50.80
	Warm temperate	Not treated	Regulating	34.10	44.05	50.57
	Warm temperate	Not treated	Non-Regulating	-	44.05	50.80
	All	Starved	Regulating	33.99	44.05	50.75
	All	Starved	Non-Regulating	-	44.05	50.51
	All	Shocked	Regulating	34.5	44.5	51.80
	All	Shocked	Non-Regulating	-	44.5	50.04
	Warm temperate	Chronic	Regulating	38.5	45.5	51.30
	Warm temperate	Chronic	Non-Regulating	-	45.5	50.50
L. glabrata	Subtropical	Not treated	Regulating	41.05	49.06	52.9
	Subtropical	Not treated	Non-Regulating	-	49.06	52.9
	Subtropical	Starved	Regulating	41.98	49.06	52.70
	Subtropical	Starved	Non-Regulating	-	49.06	52.60
E. natalensis	Subtropical	Not treated	Regulating	41.05	48.5	52.9
	Subtropical	Not treated	Non-Regulating	-	49.14	54.60
	Subtropical	Starved	Regulating	41.98	49.11	56.5
	Subtropical	Starved	Non-Regulating	-	49.11	52.9
	Subtropical	Shocked	Regulating	42.6	49.11	54.5
E. vidua	Tropical	Not treated	Regulating	41.00	50.6	57.00
E. malaccana	Tropical	Not treated	Regulating	43.02	54.10	59.6

TBTs = Thermoneutral Breakpoint Temperatures; ABTs = Arrhenius Breakpoint Temperatures; EPTs = Endpoint Temperatures.

The heart beat (expressed as beats per minutes; bpm), measured within each species‟ thermal limits, showed a trend of higher values of maximum heart rate at the ABT (mean of approximately 170 bpm) for tropical species, followed by the subtropical (mean of approximately 160 bpm) and temperate (mean of approximately 110 bpm) species, respectively (Fig. 4.2).

4.3.1.2. Do species from same region show the same response patterns?

There were differences between species within all three regions. In the tropics, the eulittoral fringe *E. malaccana* showed stronger regulation (i.e. this was based on the slope of heart rate over the thermoneutral zone; weak regulators had a slope that was more different from zero) of heart rate than the upper eulittoral zone to lower eulittoral fringe *E. vidua*. The temperatures at which the heart rate was independent of temperature were also higher (approximately 23°C) for *E. malaccan*a than for *E. vidua* (approximately 21°C) and *E. malaccana* had higher breakpoints and endpoints (see Fig 4.4A). In the subtropics, the eulittoral fringe to eulittoral zone *E. natalensis* and *L. glabrata* showed better regulation of heart than the eulittoral to *A. africana*, which showed mixed responses. The temperatures at which the heart rate was independent of temperature were higher (approximately 21°C) for *E. natalensis* and *L. glabrata* than for *A. africana* (approximately 14°C). Although *E. natalensis* and *L. glabrata* had the same TBTs, *E. natalensis* had higher EPTs than *L. glabrata* while *A. africana* showed lower TBTs and EPTs (Fig 4.4B).

In all these comparisons there is a confounding of species identity with height on shore. In contrast, members of the two *Afrolittorina* species from the warm temperate region co-exist at similar heights on the shore and in similar habitats. The two species showed similar response patterns, including similar thermoneutral zones (starting at approximately 14°C) and breakpoints, except for the EPTs which were marginally higher in *A. africana* than *A. knysnaensis* (Fig. 4.4C).

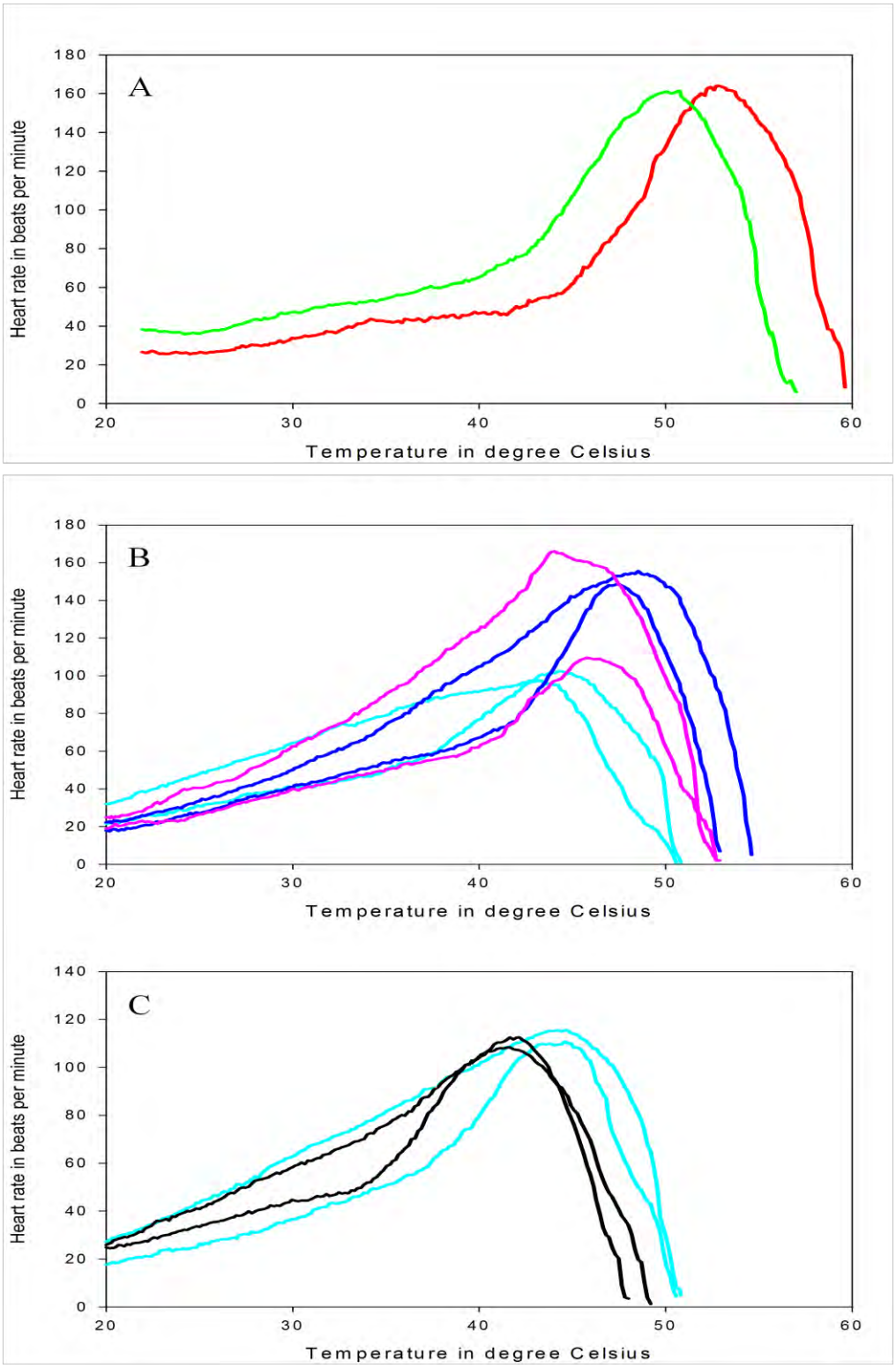

Figure 4.4. Heart patterns of regulating and non-regulating (A) tropical *E. malaccana* (red) and *E. vidua* (green), (B) subtropical *E. natalensis* (blue), *L. glabrata* (pink) and *A. africana* (cyan) and (C) warm temperate *A. africana* (cyan) and *A. knysnaensis* (black) species. Traces are means of best the five selected individuals‟ traces.

4.3.1.3. Do species from the same genus show the same response patterns?

When comparing species of the same genera, it was clear that the tropical *Echinolittorina* species showed better regulation of heart rate than the subtropical *E. natalensis*, which showed both non-regulation and regulation depending on the individual. *E. malaccana* had higher TBTs, ABTs and EPTs than *E. vidua* and *E. natalensis*, which had similar TBTs, but different ABTs and EPTs to one another (see Fig. 4.5A). On the other hand, *Afrolittorina* species showed the same responses, i.e. the same heart patterns, TBTs and ABTs, except for EPTs, which were higher for *A. africana* than for *A. knysnaensis* (Fig. 4.5B).

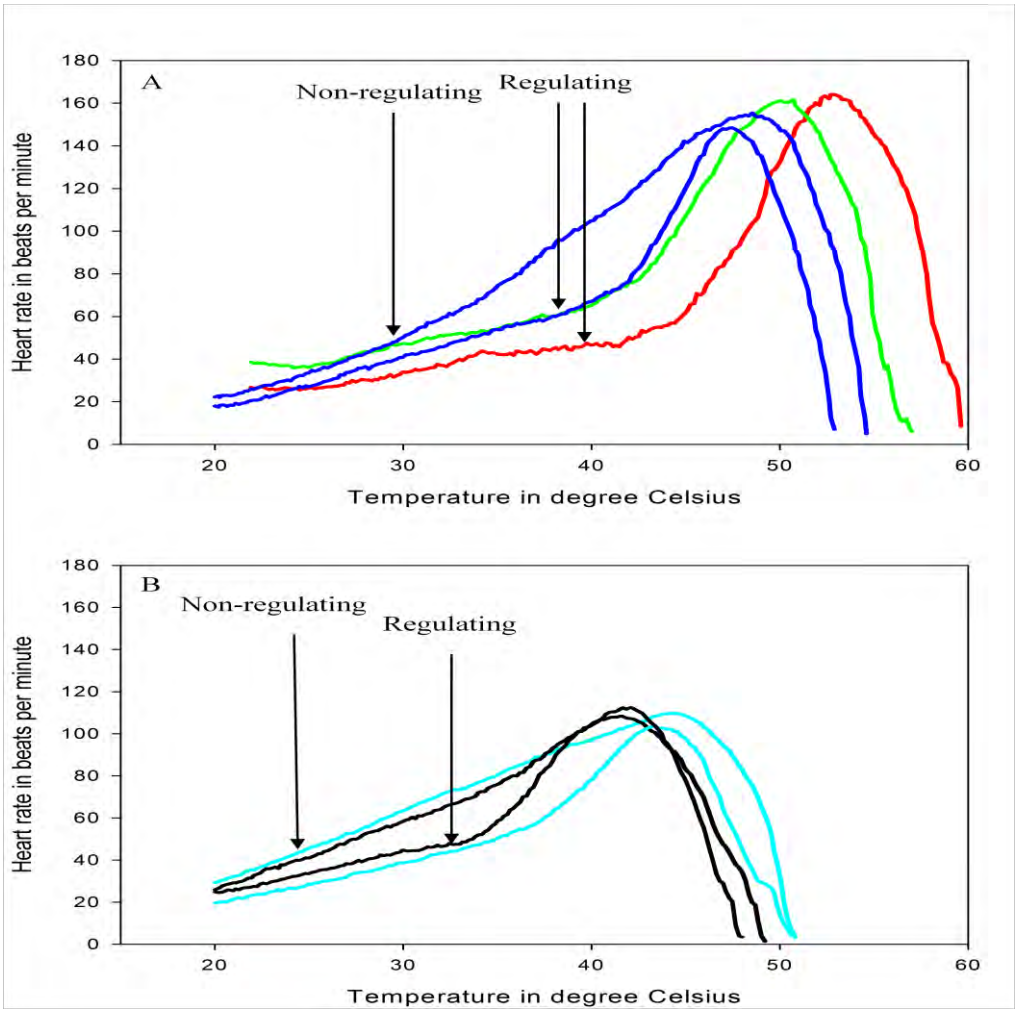

Figure 4.5. Heart patterns of regulating and non-regulating (A) *Echinolittorina* species; *E. malaccana* (red), *E. vidua* (green) and *E. natalensis* (blue) and (B) *Afrolittorina* species; *A. africana* (cyan) and *A. knysnaensis* (black). Traces are means of the best five selected individuals‟ traces.

4.3.1.4. Do species show the same responses in different regions?

Comparing the heart rates of *A. africana* from subtropical and warm temperate regions and of *A. knysnaensis* from cool and warm temperate regions revealed that there were no major differences in the response of conspecifics from different regions (Fig. 4.6). However, non-regulating *A. knysnaensis* from the warm temperate region showed slightly higher EPTs than the cool temperate populations. In addition, individuals of *A. knysnaensis* from the cool temperate region were more likely to show regulation than those from the warm temperate region (see Table 4.3). This was unexpected.

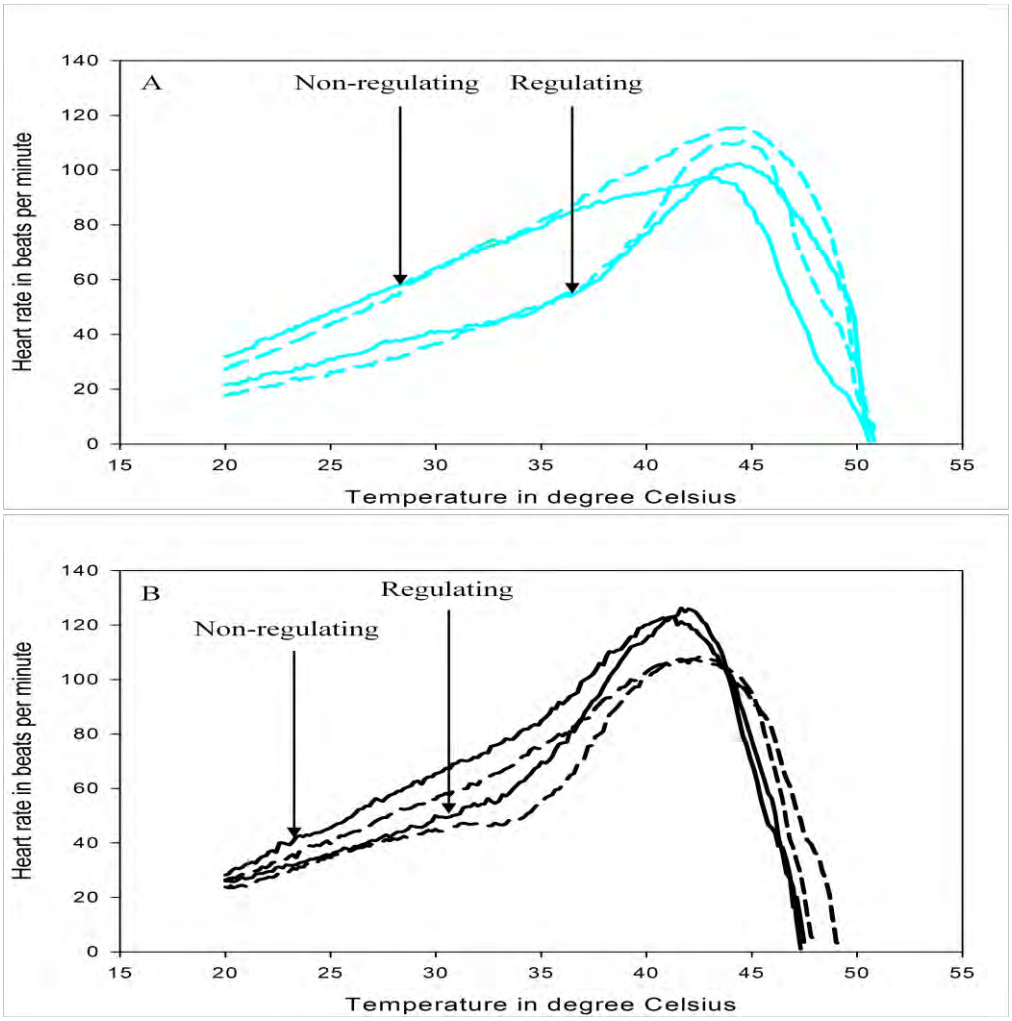

Figure 4.6. Heart patterns of regulating and non-regulating (A) *A. africana* from subtropical (solid lines) and warm temperate (dashed lines) regions and (B) *A. knysnaensis* from warm temperate (dashed lines) and cool temperate (solid lines) regions. Traces are means of the best five selected individuals‟ traces.

4.3.1.5. Does the same individual show the same responses over repeated exposures?

With a few exceptions, most animals showed the same heart patterns when repeatedly exposed to heat stress, irrespective of pre-exposure treatment (Fig. 4.7.1 and 4.7.2). This suggests that patterns of heart rate response are characteristic of individuals, rather than being caused by the conditions to which that individual is subjected.

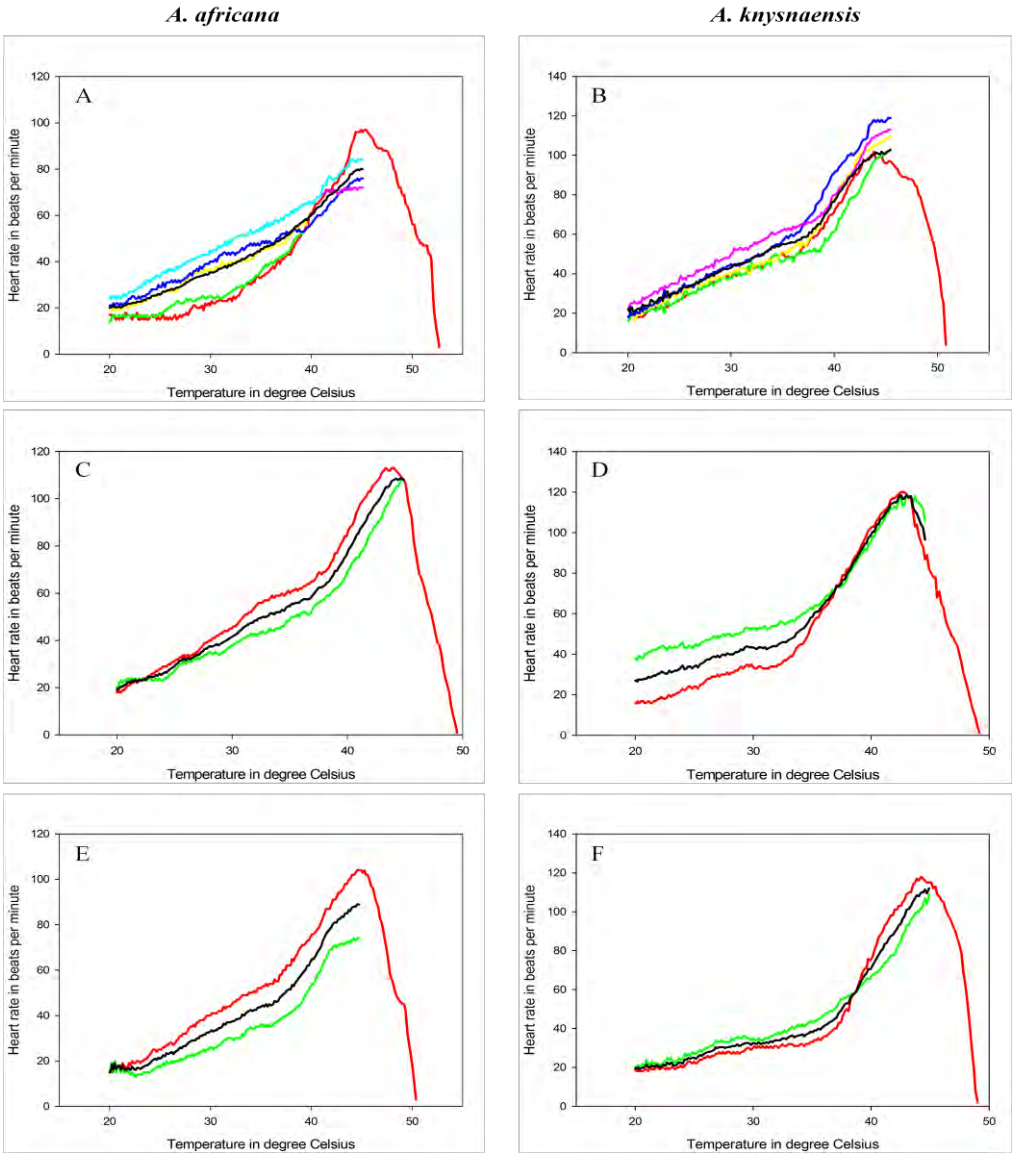

Figure 4.7.1. Heart patterns of animals of *A. africana* (right panel) and *A. knysnaensis* (left panel) (A, B) left on sensors; (C, D) allowed to "feed" for one day; and (E, F) allowed to "feed" for two days at room temperature (approximately 20°C) before final exposure. Each trace is from one individual of each species.

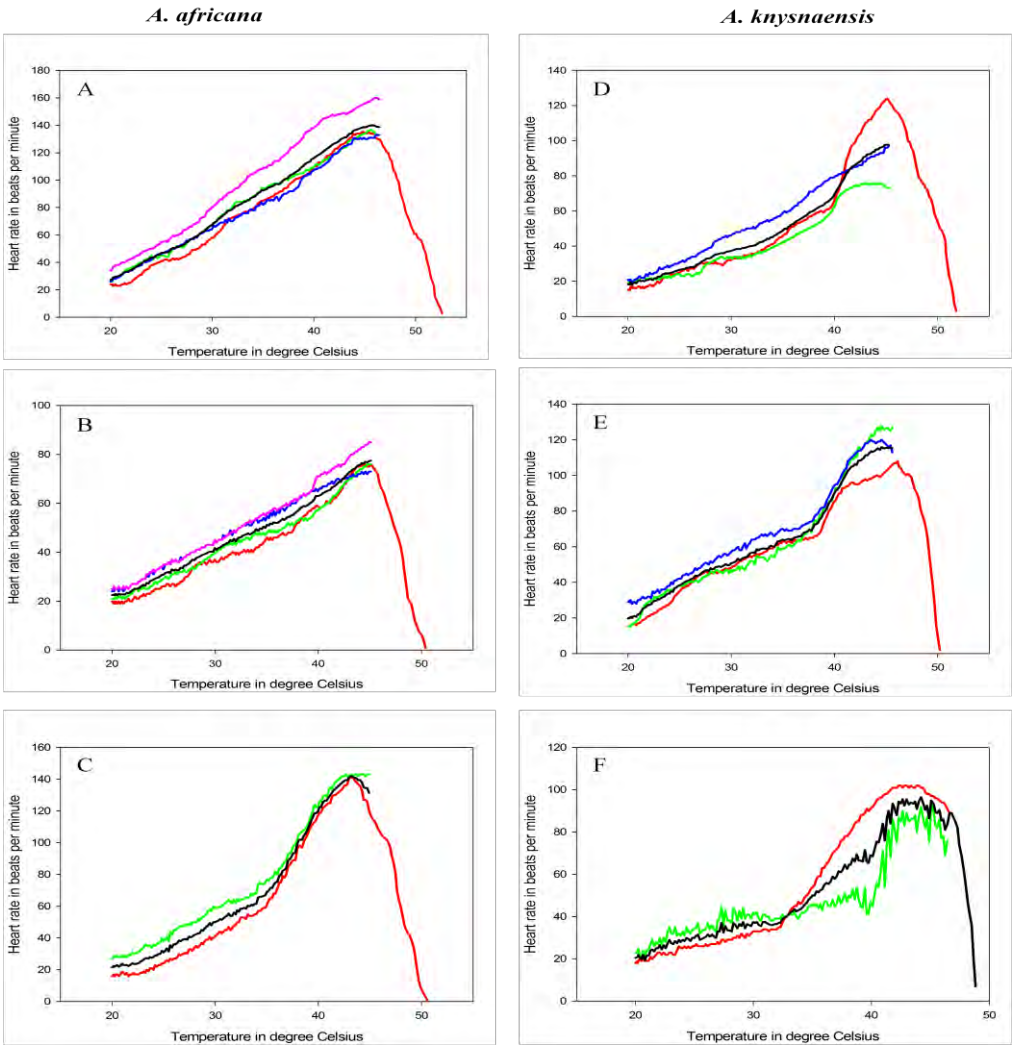

Figure 4.7.2. Heart patterns of animals of *A. africana* (right panel) and *A. knysnaensis* (left panel) (A, B) left on sensors at 30°C for 3 days; (D, E) left on sensors at room temperature for 2 days; and (C, F) allowed to "feed" for 3 days at room temperature (approximately 20°C). Each trace is from one individual of each species.

4.3.2. Effect of conditions on heart performance, particularly critical and threshold temperatures (breakpoints and endpoints).

Various experiments on *Afrolittorina* spp., *L. glabrata* and *E. natalensis* were used to investigate the effects of both environmental conditions and physiological state on heart performance. The data will also help in understanding the cause of high individual variability by eliminating various confounding factors.

4.3.2.1. Fast (Acute) versus slow (chronic) increase in temperature

When comparing methods of exposure, it was clear that a slow increase in temperature (chronic exposure rate) induced stronger thermal independence than a rapid increase in temperature (acute exposure rate) (Fig. 4.8). In addition, the chronic-exposed individuals regulated across a range of approximately 18°C, compared to approximately 14°C for acute-exposed individuals (Fig. 4.8). There were also noticeable shifts in breakpoints; the TBPs shifted by approximately 4.5°C from 34.0 to 38.5°C, and the ABTs by 1.3°C from 44.5 to 46.0°C in both *Afrolittorina* spp. (see Table 4.4). But the EPTs were the same for acute- and chronic-exposed (see Table 4.4).

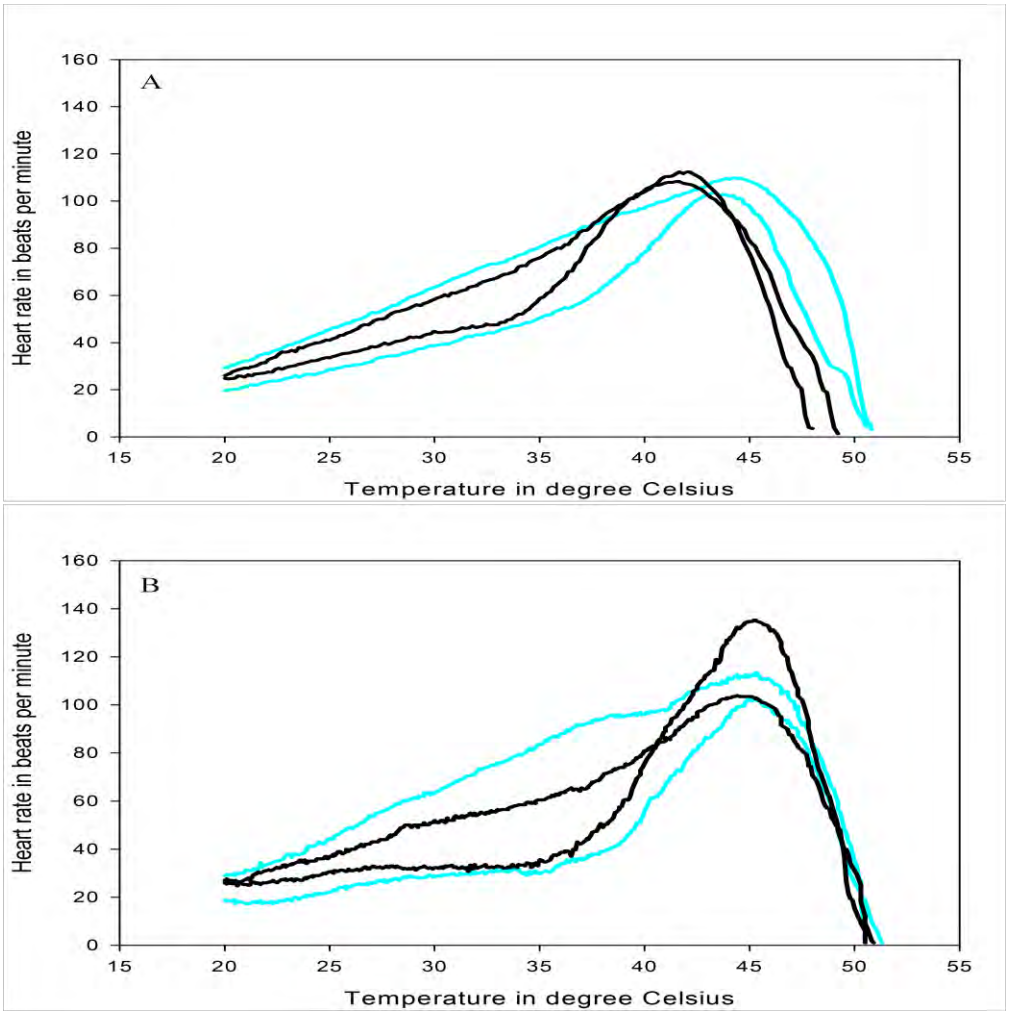

Figure 4.8. Heart patterns of regulating and non-regulating *A. africana* (cyan) and *A. knysnaensis* (black) after (A) fast and (B) slow exposure rate. Traces are means of the best five selected individuals‟ traces.

4.3.2.2. Acclimation temperature

The temperatures at which animals were acclimated for 7-14 days or longer had no effect on heart rate patterns; thus animals acclimated at 20°C showed the same responses (thermal dependence or independence, similar breakpoints and endpoints) to those acclimated at 30°C (see Fig 4.9 and Table 4.4).

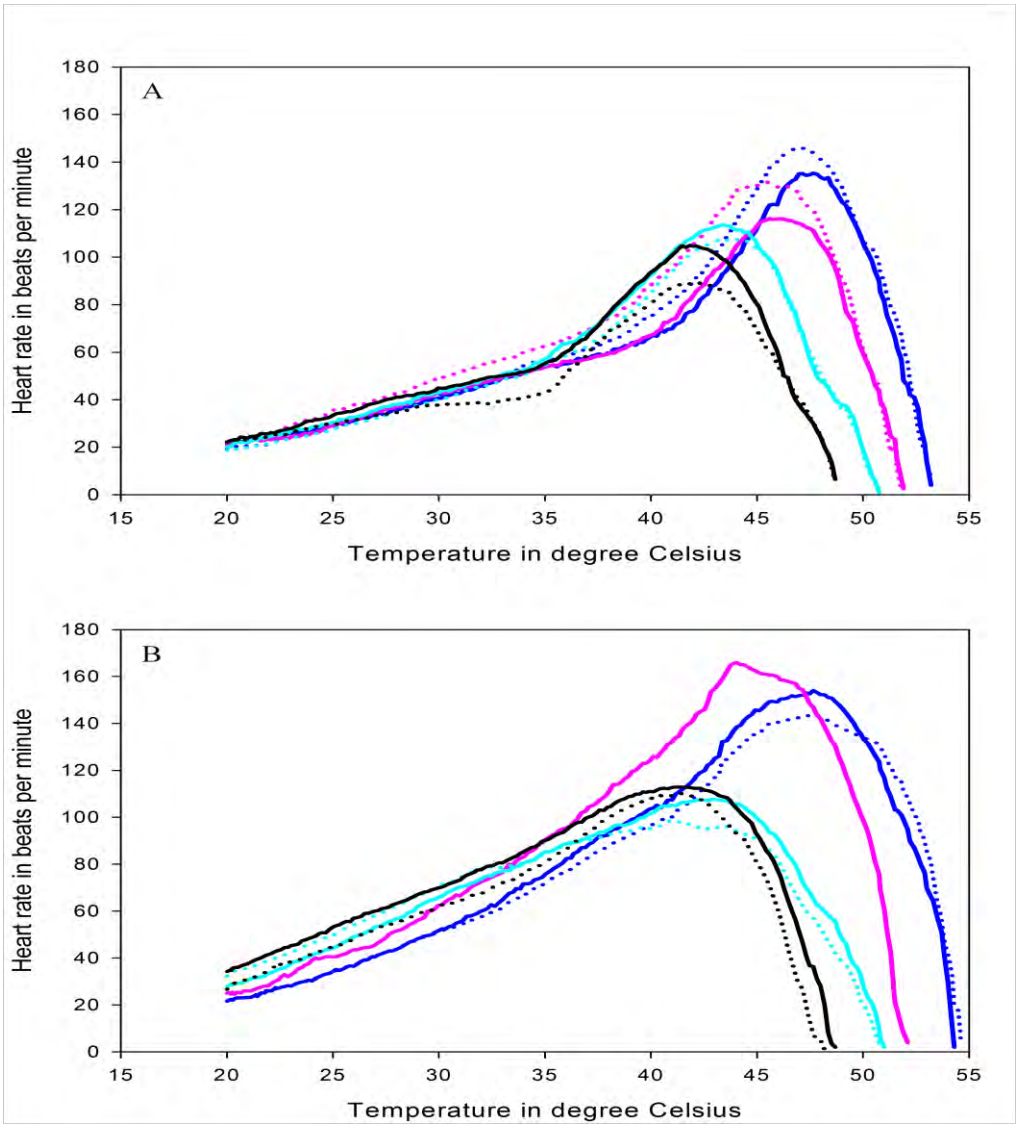

Figure 4.9. Heart patterns of (A) regulating and (B) non-regulating laboratory acclimated individuals of *E. natalensis* (blue), *L. glabrata* (pink), *A. africana* (cyan) and *A. knysnaensis* (black). Traces are means of the best five selected individuals‟ traces. Animals were acclimated at room (approximately 20 °C; dotted lines) and 30 °C (solid lines) for at least 14 days before use.

4.3.2.3. Heat shock

Exposure to abrupt heat stress (heat shock) had no effect on heart patterns; thus animals exposed to heat shock showed the same responses (thermal dependence or independence, similar breakpoints and endpoints) as non-shocked conspecifics (see Fig 4.10 and Table 4.4). The sole exception was that the ABT for *E. natalensis* increased by 1°C when it was heat shocked, but this was recorded for a single individual.

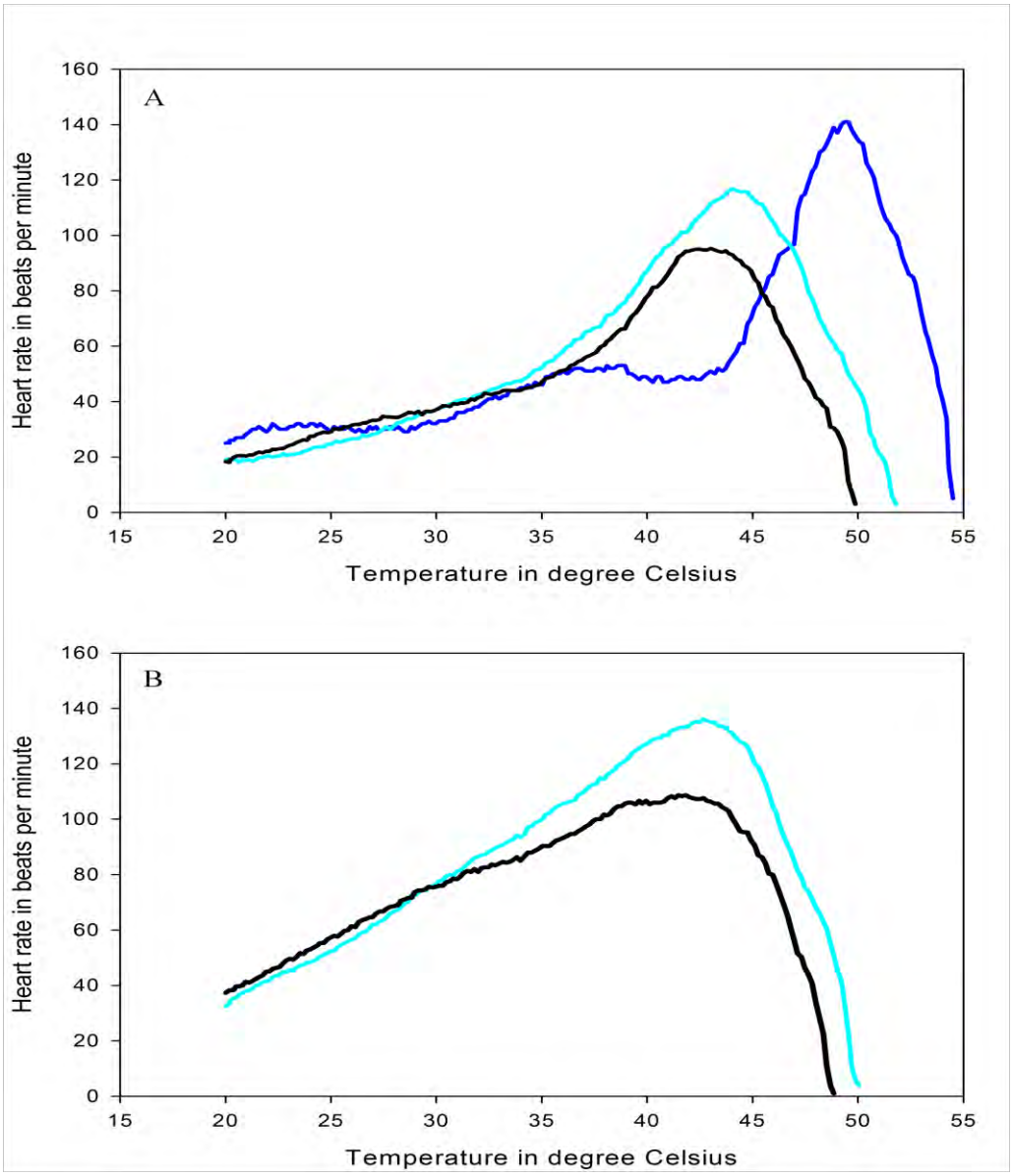

Figure 4.10. Heart patterns of (A) regulating and (B) non-regulating heat shocked *A. africana* (cyan), *A. knysnaensis* (black), and *E. natalensis* (blue). Traces are means of the best five selected individuals‟ traces, except for *E. natalensis* where one individual was used.

4.3.2.4. Starvation

Starvation for 14 days or more had no effect on heart rate patterns, breakpoint and endpoint temperatures when compared to non-starved animals (see Fig 4.11 and Table 4.4), indicating that the ability to regulate was not under the influence of nutritional status.

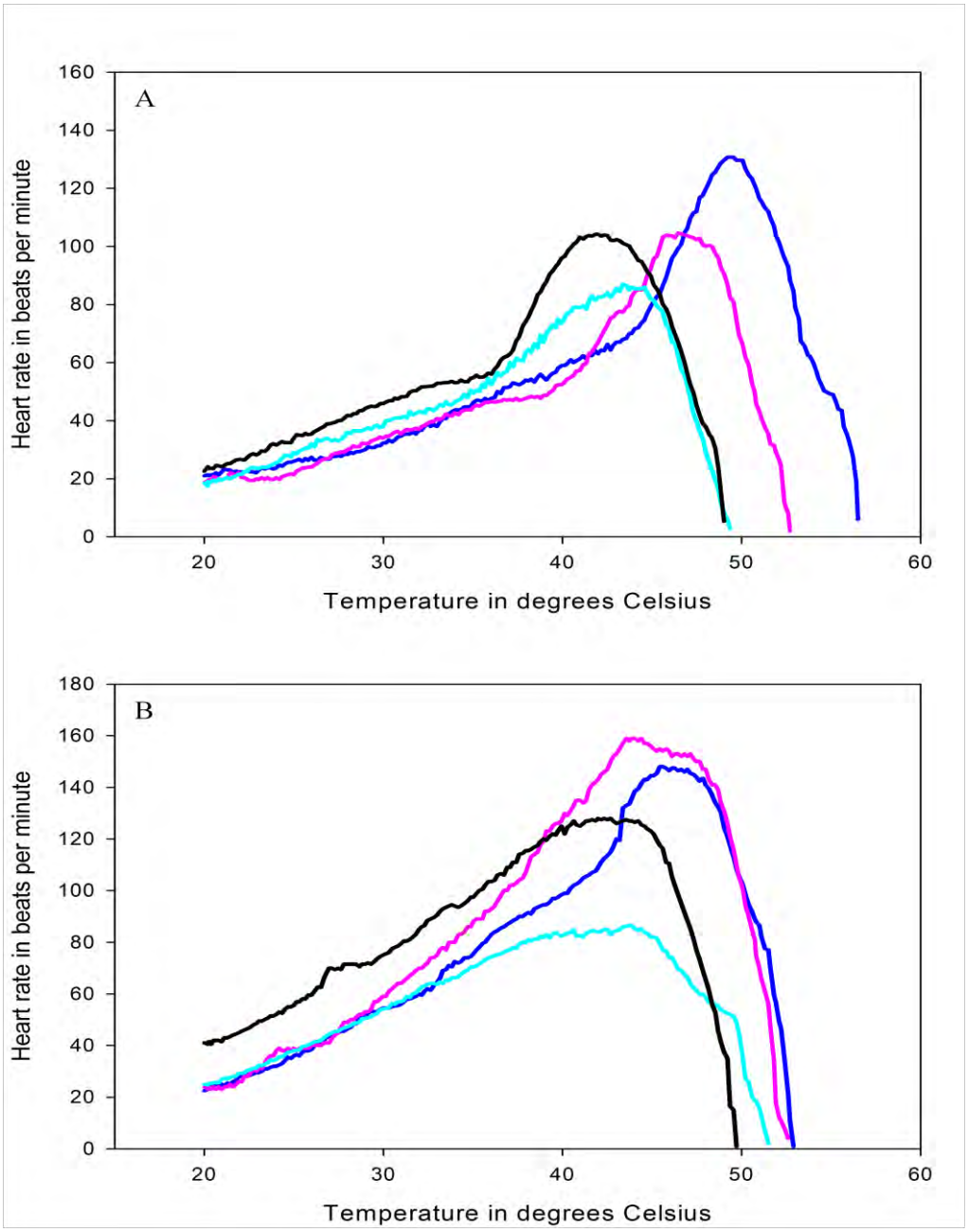

Figure 4.11. Heart patterns of (A) regulating and (B) non-regulating starved *A. africana* (cyan), *A. knysnaensis* (black), *E. natalensis* (blue) and *L. glabrata* (pink). Traces are means of the best five selected individuals‟ traces.

4.4. Discussion and conclusions

Environmental temperature change that might be of ecological significance influences the metabolic rates of intertidal animals, including littorinid snails (see Table 4.1). The influence of temperature on metabolic rates is of particular relevance to the animals in intertidal zones, especially those from temperate regions where there is strong seasonal variation in temperatures compared to the tropics and polar regions (see Clarke, 1993a; Brockington and Clarke, 2001; Stillman, 2003; Clarke and Gaston, 2006; Lannig *et al*., 2010; Pörtner, 2010). In addition, temperate regions are expected to experience a much greater magnitude of global warming than other regions (see Lozano *et al*., 2004; Caddy-Retalic *et al.*, 2011; Christensen *et al*., 2011).

Thus, in the light of rapid global warming where the mean air and sea surface temperatures as well as solar radiation have risen and are predicted to rise in the coming years, the question arises as to how intertidal animals will deal with temperature change in their habitats. This may be especially problematic for intertidal organisms such as littorinids as they live in harsh and fluctuating environments (see McMahon, 1990; 2001b; Emson *et al*., 2002; Muñoz *et al.*, 2008; Judge *et al*., 2011). Increased temperatures will have impacts on physiological processes such as metabolism, particularly for ectotherms as their performance is strongly under the influence of environmental temperature (see Hawkins, 1995; Sagarin *et al.*, 1999; McCarty, 2001; Pörtner and Knust, 2007; Helmuth *et al*., 2010).

Littorinid snails are ectothermic but, as for other marine species, there is no general consensus on how littorinid metabolic rates respond to temperature. Some studies suggest partial or total temperature dependence, while others suggest temperature-independence of metabolic rates. Marshall *et al.* (2010; 2011) studied the metabolic rates of the tropical eulittoral fringe *E. malaccana* and concluded that it regulates both heart rate and oxygen consumption across a range of temperatures. On the other hand, Sokolova and Pörtner (2003) showed that even populations of *Littorina saxatilis* from sub-Arctic White Sea shows some degree of metabolic regulation (although they did not make anything of it) while those from the cold temperate North Sea did not. Both regulation and non-regulation of metabolic rate have been shown in other littorinid snails and intertidal animals (see Table 4.1).

Preliminary results for heart rates of *E. malaccana* and *E. vidua* from Brunei Darussalam showed thermal independence from 20°C up to the thermoneutral breakpoint (41 or 43°C, respectively). In contrast, *Afrolittorina* spp. showed mixed responses, which included thermal dependence, partial-independence and independence. It seems that the capacity to depress metabolism and save energy through metabolic depression and thermal independence is fundamental to the physiology of many littorinid species, and is presumably linked to the energetic capacity to withstand long periods of emersion and inactivity. Not only is the capacity for depression of metabolism important in this respect, so too is the capacity to maintain depressed metabolic rates across a range of temperatures, the thermoneutral zone. Marshall *et al.* (2011) have highlighted the ecological and evolutionary significances of the thermoneutral zone to *Echinolittorina*; and this may apply to other littorinids and ectotherms.

Although *Afrolittorina* spp. showed mixed responses, there was some consistency in the limits of thermoneutrality and other threshold temperatures, as for tropical species. Tropical *E. malaccana* and *E. vidua* showed higher limits and threshold temperatures than the temperate *Afrolittorina* spp. However, there is no work to show how the limits and threshold temperatures generally vary among littorinids species or other marine ectotherms. The Arrhenius Breakpoint temperature has been shown to vary among porcelain crabs of the genus *Petroliathes* (Stillman and Somero, 1996; 1999) and among closely-related snails of the genus *Tegula* (Stenseng, 2005; Stenseng *et al*, 2005); the differences between species coinciding with the different thermal regimes under which they live. Such comparisons of closely related species (in my case *Afrolittorina* or *Echinolittorina* spp.) from different environments or temperature regimes separates differences in environmental adaptations from differences in phylogenetic history because more closely related species tend to have more similar ecologies than do more distantly related species.

The littorinid snails studied here have distinct geographical and vertical distribution patterns that were hypothesized to be reflected in differences in metabolic response to heat stress. Although genus and biogeography are to some extent confounded in the case of *Afrolittorina* and *Echinolittorina*, as expected, the six studied species demonstrated different types of response to heat stress, suggesting differences in heart performance in species from different regions. The tropical species showed good regulation of heart rate while the subtropical and

temperate species showed mixed responses characterized by high individual variability. Interesting was the difference in times taken to induce good regulation and complete regulation.

The tropical species took minutes to a few hours to induce good regulation with complete regulation within 3 days of acclimation at 30°C, while the subtropical and temperate species took days to induce good regulation, some failing to show complete regulation even after days of acclimation at 30°C. In addition, the heart rates of the six species examined showed distinct limits and threshold temperatures, with a trend of thresholds and limits decreasing in the order of tropics to the subtropics to temperate regions. Thus, my heart rate data show fundamentally different metabolic strategies for temperate species compared to subtropical and tropical species, and this is due to differences in physiological adaptation to high temperatures exposure. However, *A. africana* is an exception, explained by its restriction to lower levels on the shore in the subtropics (pers. obs.).

Similar patterns of regulation and non-regulation or mixed responses, and shifts in limits and thresholds temperatures due to adaptations occur in other members of the family Littorinidae and other marine animals (see below). Few studies have looked at the metabolic rates of conspecific animals from different regions or latitudes (Vernberg, 1959; 1969; Pickens, 1965; Vernberg and Moreira, 1974; Pulgar *et al*., 2006; 2007; Rastrick and Whiteley, 2011). In most cases they explicitly overlooked patterns of regulation/non-regulation because they were interested in: 1) the metabolic differences (i.e. magnitude) between regions (e.g. tropics versus temperate; Vernberg, 1959; 1969; Innes and Houlihan, 1985; Johnston *et al*., 1991; Whiteley *et al*., 1997; Braby and Somero, 2006) or 2) the difference in threshold (e.g. ABTs) and limit (e.g. EPTs) temperatures (Dahlhoff *et al*., 1991; Dahlhoff and Somero, 1993b; Weinstein and Somero, 1998; Stillman, 2004).

The main findings are that animals from cold environments (e.g. temperate and low shore species) show higher metabolic rates, and lower thresholds and limits than those from warm environments (e.g. tropical and high shore species) when measured at the same temperatures (Vernberg, 1959; 1969; Pickens, 1965; Vernberg and Vernberg, 1966; Anderson, 1978;

Emmerson, 1990; Calosi *et al*., 2007; Whiteley *et al*., 2011). For example, Pulgar *et al*. (2006) found that juveniles of the fish *Girella laevifrons* from the southern populations showed higher oxygen consumption than those from northern populations. Hilbish *et al*. (1994) found a slightly higher metabolic rate for the warm water mussel *Mytilus galloprovincialis* than for the cold water *M. edulis*. Stenseng (2005) and Stenseng *et al*. (2005) found that the high intertidal species *Tegula funebralis* showed higher threshold (ABT) and limit (EPT) temperatures than the low to mid intertidal congeners, *T. brunnea* and *T. montereyi*.

Sokolova and Pörtner (2003) compared *Littorina saxatilis* from different regions and found the ability to regulate differed. Where other comparisons were made, the results were confounded by species identity since species are rarely found in more than one region. A shortcoming of most physiological studies has been the lack of a broad comparative analysis of a large number of species that, while phylogenetically closely related, are adapted to a broad range of temperatures (see Sokolova and Pörtner, 2001a; Whiteley *et al*., 2011). Most studies have been done on animals from different shore levels or habitats (e.g. eulittoral fringes versus low shores; Hawkins *et al*., 1978; Stillman and Somero, 1996; Somero, 2002; Dong and Williams, 2011), or focused on a single species from one region (Newell and Pye, 1971a, b; Marshall and McQuaid, 2010; Marshall *et al.*, 2010; 2011). Where regulation occurs, animals tend to regulate over the temperature ranges experienced in their habitats, with a trend of low for polar to high for tropical species (see Vernberg and Vernberg, 1966; 1969; McMahon and Russell-Hunter, 1977; McMahon, 1990; Eshky and Ba-Akdhah, 1992; Jansen *et al*., 2007). However, confounding species identity with geography or region makes comparison between different studies difficult or impossible.

The differences in temperature responses might be related to the effect of many factors and conditions (see below). For example, Sokolova and Pörtner (2003) found that populations of *L. saxatilis* from sub-Arctic White Sea that experience larger fluctuations in conditions regulated their oxygen consumption, but the cold temperature North Sea populations that experience lesser fluctuation conditions did not. In Zakhartsev *et al*. (2003), the common eelpout *Zoarces viviparus* from Baltic Sea and North Sea populations partially regulated oxygen consumption, while those from Norwegian Sea populations did not. Different

developmental stages of crab species and other crustaceans showed different temperature metabolic rate responses, which is explained by differences in the habitats exploited by different stages (see Sastry and McCarthy, 1973; Moreira *et al*., 1981; Gutermuth and Armstrong, 1989; Agard, 1999; Brown and Terwilliger, 1999). Bulnheim (1979) found differences in the metabolic rate responses in the amphipods of the genus *Gammarus* from different habitats.

In addition, most studies have investigated the effects of a single environmental factor (e.g. temperature; see Sokolova and Pörtner, 2003; Stenseng *et al.*, 2005; Marshall *et al*., 2010; 2011), overlooking the effects of multiple factors which might be of importance in nature (see Newell, 1973; Marsden, 1984; Hawkins, 1995; Tully *et al*., 2000; Chelazzi *et al*., 2001; Melatunan *et al*., 2011). Cheung and Lam (1995) found that salinity, either alone or in combination with temperature, affects temperature metabolic responses. Brown and Terwilliger (1999) found that oxygen uptake by the megalopa of the crab *Cancer magister* increased at 20°C under low compared to normal salinities, while salinity levels had no effect on oxygen uptake of other developmental stages at either 10 or 20°C. This was also true for the American oyster *Crassostrea virginica*, where low salinity increased the effect of temperature on oxygen consumption (Shumway and Koehn, 1982).

On the other hand, Nelson *et al.* (1977) found that the temperature metabolic response of juveniles of the prawn *M. rosenbergii* was unaffected by salinity. Although most studies of the effects of CO_2 or hypercapnia on temperature metabolic responses show different results, there is a general trend of reducing of limits and thresholds (see Walther *et al*., 2009; Lannig *et al*., 2010; Christensen *et al*., 2011*)*. This is also true for the effects of pollutants or chemicals (see Rao and Khan, 2000; Cherkasov *et al*., 2006; Sokolova and Lannig, 2008). For example, Lannig *et al*. (2008) found that the standard metabolic rate and heart rate of cadmium-exposed eastern oysters, *Crassostrea virginica*, was strongly temperature dependent as compared to the control group during acute warming from 20 to 28°C.

Differences in methods and treatments used are also problematic in comparing studies. For example, Marshall *et al.* (2010 and 2011) raised temperature continuously from 20 to 60°C (the approach taken here), while in other studies it was raised in increments of 5°C every 5 or

10 minutes (see Bulnheim, 1979; McMahon, 1990; Sokolova and Pörtner, 2003; Tian *et al*., 2004). In addition, some of the studies used the Q_{10} (the factor by which a physiological process changes with a 10°C rise in temperature) value method, which results in further confusion (see Vernberg and Vernberg, 1966; McMahon and Russell-Hunter, 1977; Innes and Houlihan, 1985; Eshky and Ba-Akdhah, 1992; Clarke and Johnston, 1999; Iftikar *et al*., 2010). Newell and Pye (1971a) found that the active metabolism (measured as oxygen consumption) of the winkle *Littorina littorea* was temperature dependent, while the standard metabolism was independent of temperature (see below). In a different study however, this same species showed temperature independence of active metabolism (see Newell, 1969; Newell and Pye, 1970a, b). Other explanations for differences in responses might relate to the effect of previous thermal history (acclimation or acclimatization), experimental conditions, and nutritional status as well as the animal‟s health, developmental stage and physiological condition (see below).

Differences between species were also seen within regions. In the tropics, the eulittoral fringe *E. malaccana* showed stronger regulation with a wider thermoneutral zone and higher thresholds and limits than *E. vidua*, which occurs rather lower on the shore. The time course for induction of regulation and complete regulation was faster in *E. malaccana* than *E. vidua*, and this can also be related to their position on the shore. Animals that occupy the higher shore show more rapid compensation of metabolic rates than those found at lower levels (see Burggren and McMahon, 1981), and this was true for the induction of regulation in the above species. This is also supported by the results of studies on lethal temperature limits of the two species which found higher tolerances limits for *E. malaccana* than for *E. vidua* (see Cleland and McMahon, 1986; Mak, 1996; Lee and Lim, 2009; Marshall *et al*., 2011). In addition to their differences in position on the shore, the two species also show different preferences for humidity. For example, although both species are found on open rock surfaces, *E. vidua* seems to prefer humid surfaces, making it less exposed to heat stress.

Likewise among the subtropical species, *E. natalensis* and *Littoraria glabrata* extend higher on the shore and show better regulation of heart rate than *A. africana*. The ability of *E. natalensis* and *L. glabrata* to show better regulation suggests that the two species are of tropical origin (see Reid, 1989; 1996; 2007; Inness-Campbell *et al*., 2003; Williams and Reid,

2004, Torres *et al*., 2008; Reid *et al*., 2010; 2012), and thus adapted to higher temperatures. *E. natalensis* induced regulation faster than *L. glabrata* and *A. africana*, and this might be explained by their ability to use different microhabitats. For instance, *L. glabrata* prefers shaded and humid environments whereas *E. natalensis* can live on open rock as well as in cervices (pers. obs.). Thus, *L. glabrata* minimizes the effects of extreme temperature variation during daylight emersion by remaining in cooler, more thermally stable microhabitats under exposed rock surfaces and crevices.

On the other hand *A. africana* is frequently found around the margins of shallow and temporary pools in the subtropics (pers. obs.) which might be cooler and more stable. The difference in responses was also noticed in the limits and threshold temperatures which were higher for the two eulittoral species than for *A. africana*. Although the two eulittoral species had the same thresholds, *E. natalensis*, which occurs in more exposed habitats, had higher lethal limits than *L. glabrata*, which prefers shaded and humid microhabitats.

In the warm temperate region, the two *Afrolittorina* spp. showed similar responses characterized by high individual variability. Both species took days to induce good regulation and not all individuals regulated after acclimation at 30°C. Similarity in response patterns is expected since the two species co-exist in the warm temperate region where they occupy the same levels and microhabitats on the shore (pers. obs.). Although *Afrolittorina* spp. showed similar thresholds, lethal limits were 2°C higher in *A. africana*, and this correlates with their geographical distributions (see below). McQuaid and Scherman (1988) and my results on thermal tolerance (see Chapter 3) found a difference of 1-2°C in lethal (LT_{50}) limits between the two species, with higher tolerance in *A. africana*.

Unexpected was the higher number of individuals of *A. knysnaensis* than of *A. africana* that showed regulation. Although the sample sizes were rather different between *A. africana* and *A. knysnaensis*, the number for *A. africana* was still reasonable (ca. 100), lending confidence in this unexpected result. Colour differences might have contributed to the high proportion of regulating specimens of *A. knysnaensis*. In the field, the dark-coloured *A. knysnaensis* is expected to absorb more solar radiation and heat up to a greater degree than the light-coloured *A. africana* (see Marshall and Scherman; 1988; McQuaid, 1992; 1996a), resulting in

different metabolic responses. Thus, *A. knysnaensis* is expected to show better response than the blue-grey *A. africana*.

It must be noted that eventhough black bodies are known to absorb larger fraction of solar radiation (see Wilkens and Fingerman, 1965; Markel, 1971; Phifer-Rixey *et al*., 2008; Miller and Denny, 2011), it remains near the surface and is easily removed by either re-radiation or convection or air cooling (see Britton and Morton, 2003; Miller and Denny, 2011; Marshall and Chua, 2012). This might have been the case in *A. knysnaensis* since the body temperature of both species did not differ irrespective of colour differences (unpub. data). An alternative explanation might be that the results for *A. knysnaensis* include specimens from cool temperate region which experience more widely fluctuating conditions. Thus, intense upwelling resulting in rapid and marked fluctuations in water temperatures in the cool temperate region might have resulted in regulation in a high proportion of individuals from that region.

The problem of course is to separate the confounded effects of species identity and the biogeographic regions where the species occur. As expected, the two tropical *Echinolittorina* spp. showed better regulation than the subtropical *E. natalensis*, which showed mixed patterns. *E. malaccana* had higher limits and threshold temperatures than *E. vidua* and *E. natalensis* which had similar thermoneutral and Arrhenius Breakpoint limits, but different lethal limits. Most papers show little difference in thermal tolerances of species found between 0 and 30 degrees latitudes (see McMahon, 1990), with the latitudinal effect being more significant between 30 degrees and higher latitudes. So, one might not expect a major difference in thresholds and/or lethal limits between tropical and subtropical species and this was generally the case in this study.

E. vidua and *E. natalensis* showed the same thermoneutral and Arrhenius Breakpoint limits (the genus *Echinolittorina* is of tropical origin; Williams and Reid, 2004; Reid, 2007; Reid *et al*., 2012), though lethal limits were higher for the tropical *E. vidua* than subtropical *E. natalensis*. The difference in lethal limits (EPTs) was expected given the geographical distributions of the two species, and the fact that the eulittoral zone and fringe are extended

upwards in tropical shores compared to subtropical shores (see Hartnoll, 1976). *Afrolittorina* spp. showed the same pattern; heart patterns, thermoneutral zones and breakpoints were similar, but lethal limits were 2°C higher for *A. africana* than for *A. knysnaensis* from the same region. As for *E. vidua* and *E. natalensis*, the difference in lethal limits (supported by differences in LT_{50}; see Chapter 3), was expected given the geographical distribution of these species (McQuaid and Scherman, 1988; McQuaid, 1992; Reid and Williams, 2004; d'Errico *et al*., 2008; Reid *et al*., 2012).

Although species identity and regions are usually confounded, I could compare populations of *Afrolittorina* spp., each of which occurs in two regions. In case of *A. africana*, I predicted that populations from the subtropics would show better regulation than warm temperate populations, and for *A. knysnaensis* that cool temperate populations would show less regulation than warm temperate populations. In fact, there were no major effects of region on patterns of heart rate response for either species, and this may reflect the fact that these species are exposed to terrestrial conditions for most of time, while the regions are identified mainly on the basis of sea surface temperatures (SST) (see Maree *et al*., 2000; Harrison, 2002; 2004; Sinclair *et al*., 2004).

Along the southern African coast, air and substratum temperatures can rise well above the SST, and rock temperature is often much higher than that of the surrounding air (pers. obs.). Therefore organisms such as littorinid snails that live highest on the shore experience most stressful temperatures as a result of long periods of exposure as compared to subtidal species. In addition, it is possible that conditions (i.e. temperature) experienced during emersion are less dissimilar than expected among regions. If conditions were different as expected based on SST (decrease gradually from subtropics towards warm and cool temperate regions; see Isaac, 1937; Maree *et al*., 2000; Harrison, 2004; Sinclair *et al*., 2004; Harrison and Whitfield, 2006), one would have expected a difference in responses of animals from different regions and populations as found by Sokolova and Pörtner (2003).

Situations where there were no geographical or latitudinal changes in metabolic rates over thermal gradients have been found in other intertidal ectotherms (see Monaco *et al*., 2010;

Rastrick and Whiteley, 2011). However, other studies have found differences or adaptive changes in metabolic rates of geographically separated populations (see Vernberg, 1969; Vernberg and Vernberg, 1966; Vernberg and Moreira, 1974; Jansen *et al*., 2007; Rastrick and Whiteley, 2011; Whiteley *et al*., 2011) as was expected in this study. The absence of regional difference in *Afrolittorina spp*. may also be explained by the lack of genetic diversity among populations (see Chapter 2; Grant and Lang, 1991), suggesting that the ability to regulate is also genetically controlled.

Equally unexpected was the high proportion of individuals of cool temperate *A. knysnaensis* showing regulating compared to warm temperate populations. This was not observed for *A. africana* populations from different regions. The unexpected result for *A. knysnaensis* may be explained by the fact that studies were done in spring and summer for the warm populations as compared to only summer for the cool populations. Individuals from the cool region might have been collected after exposure to hot events or already acclimatized to hot summer days. On the other hand, data for warm temperate individuals might have been confounded by the mixture of spring and summer acclimated individuals. This is also supported by the fact that such differences were not found in *A. africana* where one would expect the subtropical population to regulate more than the warm temperate population.

Although the sample sizes were rather different between *A. knysnaensis* in cool and warm temperate regions, the number for cool temperate was still reasonable (ca. 100), lending confidence in this unexpected result. However, it cannot be ignored that the high proportion of regulation by the cool temperate population might be related to the conditions in that region. For example, conditions in the cool region might be highly fluctuating as a result of upwelling than for warm region. Although not discussed in Sokolova and Pörtner (2003), their data for populations of *Littorina saxatilis* from the White Sea would have shown a higher proportion of regulation than the North Sea population. The differences in performance between North and White Sea populations of *Littorina saxatilis* also occur for enzymes involved in metabolic process (Sokolova and Pörtner, 2001a). Of the five enzyme studied, there was a constitutive difference in the activity of three enzymes between snails from North Sea and White Sea populations of *Littorina saxatilis* and its congener *L. obtusata*. Panova and Johannesson (2004) also found differences in the activity of aspartate

aminotransferase between upper and lower shore populations of *Littorina saxatilis* from Sweden.

In conclusion, the results show clearly that the six studied species have the ability to regulate metabolic rates, with a gradient in this ability declining from the tropical species to the subtropical and then to the temperate species. The ability of these species to regulate is related to their highest position on the shore where they experience extremes and rapidly fluctuating conditions. It is well known that animals that live high on rocky shores show stronger metabolic adjustment to changes in temperature than those from the low shore (see Spaargaren and Achituv, 1977; Bulnheim, 1979; Burggren and McMahon, 1981; Wilbur and Hilbish, 1989; Marshall and McQuaid, 1991; Somero, 2002).

During low tides the studied species experience long periods of exposure high on the shore, where there is little food, limited time when feeding is possible (see Branch *et al*., 1988 Norton *et al*., 1990; Marshall and McQuaid, 1991; Bates and Hicks, 2005; Menge *et al*., 2007) and both heat and desiccation stresses are high (see Barnes *et al*., 1963; Shick *et al*., 1988; McMahon, 1990; Muñoz *et al.*, 2008). These stresses are more pronounced in the tropics than the subtropics and temperate regions (see Hartnoll, 1976; Garrity, 1984; Little, 1989; Lesser and Kruse, 2004). Thus, high temperature and low food and/or feeding opportunities work together to reduce metabolic rates for animals on exposed shores.

Although all species regulated their heart rate in response to heat stress, there was a difference in responses between tropical, subtropical and temperate species. The tropical species experience the most energetically constrained conditions and showed good and quick regulation of heart rate. In contrast, the temperate and subtropical species, that are subjected to slightly less severe conditions showed mixed responses and high individual variability, and slow regulation of heart rate (see below). Thus, while extremes of temperatures are expected to occur in the microhabitats of South African species, local climate conditions, timing of low tides, and the position of the species in the intertidal make them unlikely to experience the same conditions as tropical species. For example, South African species, especially *Afrolittorina* spp., might benefit from wave splashes which tend to keep temperatures low

even at high shore levels; thus providing some protection during midday exposure and allowing animals to maximize feeding time (i.e. energy assimilation). On the other hand, the tropical species, particularly *E. malaccana*, spend most of their time on exposed rock surfaces and so have limited feeding opportunities.

Apart from differences in temperature regime, there are also differences in emersion periods between regions, as a result of different tidal regimes with longer exposure periods in the tropics (dominated by platform-like rocky shores) than subtropical and temperate (dominated by narrow rocky shores) regions (see Hartnoll, 1976; Aagaard, 1996; Pulgar *et al*., 2006; Finke *et al*., 2007). This is supported by differences in aestivation periods between subtropical/temperate (approximately 14 days; see McQuaid and Scherman, 1988; McQuaid, 1992; Sinclair *et al*., 2004) and tropical (approximately 60 days; see Marshall and McQuaid, 2010) species. In addition, Finke *et al*. (2007) found the South African coast to experience the lowest exposure times at all tidal levels.

Due to longer exposure periods, tropical intertidal zones are subjected to intense solar radiation which may raise surface temperatures as high as 50°C during summer hot days (Lewis, 1963; Williams and Morritt, 1995; Chan *et al.*, 2006; Marshall and McQuaid, 2010), while temperatures might rarely exceed 45°C in the subtropics and temperate regions (pers. obs.; Morley *et al*., 2009; Zardi *et al*., 2011; but see Whiteley *et al*., 1997). In addition, there are tidal, diurnal and seasonal variations in temperatures that differ depending on geographical location (see Aagaard, 1996; Finke *et al*., 2007). Some regions experience moderate to constant temperature changes while others experience more pronounced changes. For example, most tropical regions are known to be characterized by moderate to constant conditions while most of temperate regions are dominated by fluctuating conditions (see Huey and Bennett, 1990; Sommer *et al.*, 1997; Tomanek and Somero, 1999; Sokolova and Pörtner, 2003; Christensen *et al*., 2011).

Rapid changes in and extremes of temperature make specific demands on an animal, such as high energy costs to support or maintain metabolic rates and/or the synthesis of heat shock proteins for deference or repair (Burggren and McMahon, 1981; Branch *et al.*, 1988; Parsons,

1990; Somero, 2002; Lannig *et al*., 2010; Marshall *et al.*, 2011; Miller and Denny, 2011). Thus, the costs of living are higher for animals living in the tropics than for those living in subtropical, temperate and polar regions (see Somero, 2002; 2010; Whiteley *et al*., 2011). This means that subtropical and temperate species have less need to conserve energy (resulting in mixed responses and high individual variability) than tropical species which strongly regulate or depress metabolic rates to save energy (see Newell, 1973; Clarke, 1993a, Pörtner *et al*., 2005; Dong *et al*., 2011; Whiteley *et al*., 2011). Thus, metabolic regulation acts as a time-limited adaptation strategy to survive unfavourable conditions such as extremes of temperatures and low feeding opportunities.

On the other hand, the differences in thresholds and limits observed in this study can be viewed as physiological adaptations of the studied species to their environments, or as the physiological mechanisms (e.g. acclimation) that allow them to occupy those environments. For example, all three *Echinolittorina* species as well as *L. glabrata* occupy the eulittoral fringes in the tropics and subtropics, and show high thresholds and limits. In contrast, *Afrolittorina* spp. are dominant in the eulittoral zones in the subtropics/temperate, though they can be found in the eulittoral fringe, and show lower thresholds and limits. Thus, although the eulittoral zone is frequently exposed to air for several hours, exposure times of more than one day occur occasionally, while in the eulittoral fringe, aerial exposure of more than one week is common (see Kronberg, 1990), explaining the differences in thresholds and limits found in this study.

Interestingly the tropical *E. vidua* and the two subtropical species *E. natalensis* and *L. glabrata*, showed similar thermoneutral breakpoints, suggesting that the latter two may be of tropical origins. Indeed, the genera *Echinolittorina* and *Littoraria* are mainly tropical with few representatives occurring in the subtropics and temperate (see Reid, 1989; 1996, 2007; Inness-Campbell *et al*., 2003; Williams and Reid, 2004; Torres *et al*., 2008; Reid *et al*., 2010; 2012). The thresholds determine the thermal limit to energy conservation (TBTs) and the temperature above which time dependent mortality sets in (ABTs), for aestivating animals. For example, South African *Echinolittorina* and *Afrolittorina* spp. probably do not experience the same energetic constraints as the tropical *Echinolittorina* spp. and showed lower

breakpoints. On the other hand, the lethal limits (EPTs), which showed high intraspecific variability, represent temperatures at which mortality or death occurs.

High individual variability and mixed responses in temperate and subtropical species

The main finding and maybe the most unexpected was the high individual variability in *Afrolittorina* spp., and the mixed responses in subtropical *E. natalensis* and *L. glabrata*. This was also noticed in tropical species where some individuals showed partial regulation a few hours after collection (see Marshall *et al.*, 2011; pers. obs.). Collecting animals from nature without disturbing their natural physiological state is very difficult, especially when collecting specimens from multiple sites at different times. In addition, individuals or populations living in close proximity may experience different environmental conditions (Helmuth, 1998; Sinclair *et al*., 2006; Denny *et al*., 2011), which can lead to different responses and hence high individual variability (Dahlhoff *et al*., 2002). Another challenge is that natural systems, including the intertidal, are themselves characterized by variability (Hofmann and Somero, 1995; Tian *et al*., 2004; Helmuth *et al*., 2005; Tomanek, 2010; Pincebourde *et al*., 2012), making the resultant data often complex and difficult to interpret (Sørensen, 2010). Therefore, care must be taken when investigating or comparing performance of animals from different locations and shore levels.

Efforts were made to reduce the effect of inequalities (i.e. acclimation history, nutritional state, size and level on the shore) by comparing individuals of a standard size from similar habitats and levels, collected in the same season, and acclimated under the same conditions. In addition, experiments such as repeated exposure of the same individuals, a slow increase in temperature, heat shock and starvation were conducted to eliminate as many confounding factors as possible (see below). Large numbers of specimens were used for each species per experiment or treatment (see Table 4.4) in order to compensate for individual variability. As such, high individual variability (as observed on thermal tolerance and proteomics results) found in this study was a true response which might be explained by the effect of other factors or conditions which need to be investigated (see below).

High individual variability in *Afrolittorina* spp. and other South African species could relate to the need to generate energy in some individuals (especially those that have just fed) but not others (those that have had limited time to feed or have not fed at all). For example, snails that have just fed generally did not show the same degree of thermal independence of heart function and metabolism as those that had been quiescent either in the field or the laboratory for a few days before experimentation (pers. obs.). This individual variability was also noticed in data for freshly collected *Echinolittorina* snails from Brunei, but after three days of acclimation at 30°C the patterns became clear (especially in *E. vidua*). Marshall *et al.* (2011) also found that some individuals of *E. malaccana* showed less thermal independence than others, suggesting individual variability in this species as well. Therefore, it would be best to measure metabolic rates in starved individuals rather than prescribe a fixed level of food intake which could be functionally different for different individuals (Agard, 1999; Speakman *et al*., 2004).

The obligatory increase in metabolic rates, also called the „Specific Dynamic Action" (SDA), that occurs after feeding represents the energy used for ingestion, digestion, absorption and assimilation of a meal and the increased synthesis of proteins and lipids associated with growth (see Vahl, 1984; Peak and Veal, 2001; Mallekh and Lagardére, 2002; Pulgar *et al*., 2006; Jansen *et al*., 2007; Pirozzi and Booth, 2009). This has been reported in marine animals including littorinids. For example, Shumway *et al*. (1993) found an increase in oxygen consumption of starved marine periwinkles, *Littorina littorea* and *L. obtusata*, after being fed on algal food. This is also true for other marine animals such as molluscs (see Lilly, 1979; Peck, 1996; Clarke and Prothero-Thomas, 1997; Peak and Veal, 2001), crustaceans (see Carefoot, 1990; Houlihan *et al*., 1990; Chu *et al*., 1994; Crear and Forteath, 2000; Robertson *et al*., 2001a, b; 2002; Whiteley *et al*., 2001; Kemp *et al*., 2009) and fishes (Du Preez *et al*., 1986; Chakraborty *et al*., 1992; Ross *et al*., 1992). In addition, individual differences in body composition could potentially result in variation in metabolic rates and thus high individual variability among similarly sized individuals (see Regnault, 1981; Carefoot, 1990; Chakraborty *et al*., 1992; Ross *et al*., 1992; Speakman *et al*., 2004).

Littorinids are often attacked by macroparasites, especially trematodes (Berger and Kharazova, 1997; Williams and Brailsford, 1998; Granovitch *et al.*, 2000; Arakelova *et al.*,

2004), and one possibility not explored here is that parasitism may affect the physiological response to temperature stress. In fact, many studies fail to mention if test animals were healthy or infested due to the general belief that larval trematodes do little if any harm to the host as well as notorious ignorance of diseases and parasite problems (see Lauckner, 1987). Studies have shown greater variability in temperature metabolic rates of infected animals, with infected individuals increasing or depressing their rates (see Lee and Cheng, 1971; Anderson, 1975a, b; Lauckner, 1987; Meißner and Schaarschmidt, 2000; Shinagawa *et al.*, 2001). This is because the resulting metabolic stress (costs in terms of energy) of combined infection and temperature stresses can be tremendous, leading to temperature dependence. However, in other species infections lead to depressed or low metabolic rates (see Thompson, 1983; Huxham *et al*., 2001) as a strategy to save energy. Anderson (1975b) found that infested shrimps of *Palaemonetes pugio* showed lower oxygen consumption (with the effect being more pronounced in the smallest hosts) than the non-infested shrimps of equal size. In addition, the degree of metabolic temperature independence was not significantly altered as a result of infestation.

Metabolic rate can show changes during development, including embryonic development (see Sastry and McCarthy, 1973; Vernberg and Moreira, 1974; Gutermuth and Armstrong, 1989; Hatcher *et al*., 1997; Brown and Terwilliger, 1999; Glazier, 2005), and this can lead to different responses and high individual variability, especially when size is used to estimate the age of an animal. For example, small animals were assumed to be juveniles and were expected to show higher metabolic rates and greater temperature dependence to reflect their greater metabolism, since they need more energy for growth as compared to adults (see Shumway *et al*., 1993; Chelazzi *et al*., 1999; Dahlhoff *et al*., 2001; O'Connor *et al*., 2007; Pörtner and Knust, 2007). In the case of *Afrolittorina* spp. different sizes were also expected to show different response patterns, with small animals showing some degree of regulation than adults as they tend to occupy different heights on the shore (McQuaid, 1981a, b; McQuaid, 1992). Therefore, it would be important to investigate the effect of size and zonation as well as ontogenetic stage on the metabolic rates of *Afrolittorina* spp. and other littorinids. In addition, reproduction in females may influence individual differences in thermal sensitivity of metabolism as there may be a need for continual energy supply (leading to an obligatory increase in metabolic rates) to facilitate egg development (Baeza and Fernández, 2002). Future work should probably account for the effect of sex and/or

reproductive stage as well as ontogenetic stages on metabolic rates as reported in other studies (see Anderson, 1975b; Dawirs, 1983; Quetin and Ross, 1989; Chu *et al*., 1994; Marsh *et al*., 1999; Baeza and Fernández, 2002; Cook, 2004), something not investigated in this study.

When the physiological limits or thresholds have been passed, some animals are known to synthesize heat shock proteins as a defense mechanism (Feder and Hofmann, 1999; Whiteley and Faulkner, 2005; Pörtner and Knust, 2007; Sørensen, 2010) and this can lead to increases in metabolism as more energy is needed for protein biosynthesis (see Anderson, 1975a; Hawkins and Bayne, 1991; Hawkins, 1995; Sokolova and Lannig, 2008; Marshall *et al.*, 2011). Few studies have, however, investigated the relationship between metabolic rates and rates of protein (Hsps) synthesis (see Whiteley *et al*., 1997; 2001; Dahlhoff, 2004; Dong *et al*., 2011). Houlihan *et al*. (1990) found protein synthesis to account for 20-37% of total oxygen consumption of the shore crab *Carcinus maenas*. Whiteley *et al*. (1997) suspected that protein turnover was the factor that might have amplified metabolic response upon transfer of specimens of the mussel *Mytilus edulis* from 10 to 20°C (see Hawkins *et al*., 1987; Hawkins, 1995). An increase in temperature from 20 to 55°C in this study triggered an increase of heart rate of certain individuals as compared to others, probably in response to an increase in metabolic demand to meet the high energy requirements of protein synthesis. Thus, one possible explanation for the high individual variability in *Afrolittorina* spp., *E. natalensis* and *L. glabrata* may lie in individual differences in energy requirements for protein biosynthesis.

Increase in metabolic rates can result from differences in behavioural ecology (e.g. activity) or physiological responses to elevated temperatures (see Newell, 1973; Santini *et al*., 1999; Salomon and Buchholz, 2000; Crear and Forteath, 2000; Dong *et al*., 2006; O'Connor *et al*., 2007; Gracey *et al*., 2008). Because present day temperatures (air) on the South Africa coastline rarely rise above 45°C (pers. obs.), there is no apparent thermal related impact of temperature on littorinid populations. Therefore, an increase in heart rates of *Afrolittorina* spp. and other subtropical species between 20 and 50°C indicates that aerobic metabolism is in the range corresponding to that expected and is not temperature limited. Although the adaptive significance of temperature dependent metabolism is not clear, it can be suggested

that *Afrolittorina* spp. and other temperate species that are not often exposed to very hot conditions must expend more energy to maintain basal metabolism while not feeding. It can also be suggested that high individual variability of *Afrolittorina* spp. might indicate a high degree of adaptability as they exploit different levels of the shore from the eulittoral zone to eulittoral fringes and microhabitats (pers. obs.).

Effects of conditions on heart performance, including breakpoints and endpoints temperatures

Slow increase in temperature

Little or no work has compared the effects of different methods of increasing temperate on metabolic responses (see Hawkins, 1995). However, it can be speculated that a slower rate of increase in temperature can lead to better regulation of metabolic rates since animals have time to adjust, and indeed that is what the results showed. This is supported by studies on thermal tolerance that show that a slow increase in temperature results in higher tolerances and shifts in thresholds and limits compared to a fast increase (see Ospina and Mora, 2004; Mora and Maya; 2006; Angilletta Jr., 2009). Thus, a slow increase in temperature had a significant impact on heart performance of both *Afrolittorina* spp. with the heart independent of temperature across a range of approximately 18°C compared to 14°C of fast increase. In addition, there was a noticeable shift in limits and thresholds; TBPs shifted by approximately 4.5°C and the ABTs by 1.3°C in both species. Nevertheless, there was as much individual variability with slow as with fast temperature rises, suggesting that temperature is not the only factor that determines regulation, but that other factors such as food availability might be involved (see below).

The marked ability to regulate and shifts in breakpoints after a slow increase in temperature raises questions about the field conditions experienced by South African species as it implies that they may not be well adapted to cope with rapid temperature rises though measurements of body temperature in the field (unpub. data) showed that these do rise rapidly, especially

between morning and afternoon. Body temperatures then remain roughly constant during the afternoon but decrease rapidly with submersion or as the sun drops. Extreme temperature regimes lasting for hours (1-6 hrs) are highly characteristic of the intertidal zone (see Burggren and McMahon, 1981) as a result of long exposure periods. So the ability to show good regulation, and shifts in limits and thresholds with a slower rate of increase in temperature indicates that this species can tolerate high temperatures during the long hot summer afternoons.

Acclimation or acclimatization

Temperature acclimation leading to some degree of temperature independence as well as shifts in limits and thresholds among ectotherms is more common in animals (including marine animals) that experience fluctuations in conditions than those that experience moderate to constant conditions (see Segal, 1961; Bulnheim, 1979; Bennett, 1990; Dahlhoff and Somero, 1993b; Somero, 2004; Clarke and Gaston, 2006). The lack of an acclimation response by tropical or polar organisms presumably relates to the absence of any significant seasonal temperature variation in these regions (see Vernberg and Vernberg, 1966; 1969; Brockington and Clarke, 2001; Pörtner, 2001; Peck *et al*., 2009a, b; Nguyen *et al*., 2011).

This explanation may well apply to some molluscs such as gastropods and bivalves, particularly those inhabiting intertidal zones that only experience slight or moderate seasonal fluctuations in temperature (see Vernberg and Vernberg, 1969; Somero, 2010; Tomanek, 2008; Christensen *et al*., 2011). Littorinids and other high intertidal ectotherms have limited or no ability to acclimate metabolic rates as a result of their position on the shore. Thus, by living highest (i.e. eulittoral fringes) on the shore, these animals live close to their upper thermal limits, and have narrower thermal windows for further acclimation (see Stillman and Somero, 1999; Stillman, 2003; 2004; Somero, 2005; 2010).

Studies on acclimation show conflicting outcomes, some suggesting that acclimation occurs, while others suggest no acclimation in metabolic rates (see below), and this is further

complicated by the methods or approaches used (see Vernberg and Moreira, 1974; Laughlin and Neff, 1980; Emmerson, 1985; Van Senus, 1985; 1990; Tian *et al*., 2004). In general, animals from cool environments (e.g. temperate and subtidal species) show acclimation, while those from warm environments (e.g. tropical and high intertidal species) do not (see Anderson, 1978; McMahon and Wilson, 1981; De Pirro *et al*., 1999b; Stillman, 2002; 2003; Sinclair *et al*., 2006). However, most of the studies were interested in the differences in magnitude and/or breakpoints (see Vernberg, 1969; de Vooys, 1976; Van Senus, 1985; Navarro *et al*., 1987; Stillman, 2004; Whiteley and Faulkner, 2005), but not whether there is regulation or not. Examples of non-acclimation among littorinids (Innes and Houlihan, 1985; McMahon *et al*., 1995) and other marine invertebrates from different phyla are known (Pickens, 1965; Cheung and Lam, 1995; Pilditch and Grant, 1999; Whiteley and Faulkner, 2005). For example, McMahon *et al*. (1995) found that acclimation of the intertidal snails of the genus *Littorina*, *L. saxatilis* and *L. obtusata*, at 4 or 21°C did not affect metabolic rates. Hicks and McMahon (2002a) found that acclimation of the brown mussel *Perna perna* from the subtropics at 15, 20 and 25°C did not affect regulation of oxygen consumption.

In contrast, other studies have shown that acclimation can lead to good regulation of metabolic rates with noticeable shifts in limits and threshold temperatures (see Segal, 1956; 1961; Widdows, 1973; Markel, 1974; Ortega *et al*., 1984; Gee, 1985; Einarson, 1993; etc). In addition to partial regulation of standard oxygen consumption, Newell and Pye (1970a, b) found that *Littorina littorea* and the mussel *Mytilus edulis* showed shifts in limits and thresholds with acclimation temperature and season. In addition, the ABTs changed from 6-10°C for *Littorina littorea* acclimated at 5°C to as high as 30-35°C in animals acclimated at 25°C (Newell and Pye, 1970b). Pye and Newell (1973) found that season and thermal acclimation led to temperature independence of the standard oxygen consumption of quiescent intact and cell-free homogenates isolated from mitochondria of *L. littorea*. Although not interested in regulation, Stenseng *et al*. (2005) found a shift of ABT and Flat-line Temperatures (FLT) of snails of the genus *Tegula* acclimated to 14°C and 22°C, with the largest changes in the low-intertidal to subtidal species, *T. brunnea* and *T. montereyi*. In addition, the mid- to low-intertidal *T. funebralis* showed the smallest change in ABT, suggesting limited ability to acclimate in this species.

McMahon and Russell-Hunter (1981) found that specimens of the salt-marsh snail *Melampus bidentitas* acclimated at 20°C regulated oxygen consumption as compared to those acclimated at 10°C, which did not. Zakhartsev *et al*. (2003) found that individuals of the common eelpout *Zoarces viviparus* from Baltic Sea showed a change from non-regulation at 3°C to partial regulation at 12°C. On the other hand, Fangue *et al*. (2009) found that, in addition to the ability to regulate metabolic rate, fishes acclimated at different temperatures (when acclimated at 15, 20 and 25°C) showed a noticeable 5°C shift in thermoneutral breakpoints. Most of these studies have shown that limits and thresholds increase with increasing acclimation temperatures, and this differs for species from different temperature regimes. Thus, acclimation leads to widening of the thermoneutral zone (which is important to save energy) and shifts in limits and thresholds in other species (see Horowitz, 2001).

In this study, animals acclimated at 30°C were expected to show good regulation and higher limits and threshold temperatures compared to those acclimated at 20°C. However, the results showed no difference between the two groups; thus, no sign of acclimation. The lack of acclimation in South African littorinids was unexpected and raises questions as to what causes such inability to acclimate. One possibility is that the acclimation period (7-14 days) used in this study was too short to induce acclimation though in other studies, 14 days of acclimation was sufficient (see Newell and Bayne, 1980; Hawkins *et al*., 1987; McMahon and Russell-Hunter, 1981). Shumway and Koehn (1982) found that the oxygen consumption of the American oyster *Crassostrea virginica* did not acclimate after 3 weeks. This shows that time to acclimation, which can vary from species to species, is an important factor (see Anderson, 1978). For example, Bulnheim (1979) found that *Gammarus locusta* required longer periods to acclimate as compared to other *Gammarus* species which required shorter periods to acclimate.

In addition, the acclimation method (constant temperatures) used in this study might have been inappropriate to induce acclimation in the study species which experience fluctuating conditions higher on the shore (see above). In fact, studies have shown that animals acclimated to fluctuating temperatures (typical of conditions normally encountered in nature) depress metabolic rates at certain temperate ranges compared to those acclimated at constant temperatures (see Tian *et al*., 2004 and references herein). Alternatively, since the study was

done during spring and summer, it might be that animals were already acclimatized to high temperatures as temperature acclimation may not occur during summer (see Pickens, 1965; Griffith, 1977).

Although not investigated in this study, seasonal acclimatization is expected to result in reduced temperature dependence or lead to temperature independence of metabolic rates as seen in littorinids and other intertidal animals (see Newell, 1973; Newell and Roy, 1973; Shirley *et al*., 1978; McMahon *et al*., 1995). It must be noted however that seasonal acclimatization might be due to acclimation to other factors (e.g. food availability) in the field that can affect responses to temperature (see Griffiths, 1977; Navarro *et al*., 1987; Marsden, 1984; Navarro and Torrijos, 1994; Hummel *et al*., 2000; Tully *et al*., 2000; Walther *et al*., 2009). For example, Brockington and Clarke (2001) claimed that 80-85% of the increase in summer metabolism of the sea urchin *Sterechinus neumayeri* was caused by physiological activities associated with feeding, growth and spawning, while temperature increase caused only 15-20% of the increase.

Other possibilities include the fact that the eastern seaboard of South Africa is semi-tropical and has reasonably constant or reduced fluctuations in conditions (see Kibirige *et al*., 2002; Isla and Perissinotto, 2004; Marshall *et al*., 2003), perhaps making it similar to the tropics where species are known to have limited capacities to acclimate to temperature. Second, although the acclimation temperatures used, 20 and 30°C, are regularly experienced in the field, they may have been too high to allow acclimation as these *Afrolittorina* spp. show high (50-80%) mortality in less than five days when acclimated at 35 and 40°C, respectively (unpub. data). An alternative explanation for the lack of acclimation in this study may be the cost which comes with acclimation. As other investigators have emphasized (see Hawkins *et al*., 1987; Hofmann and Somero, 1995; Jansen *et al*., 2007; Tomanek, 2008; 2010), that acclimation comes at a cost because heat shock protein synthesis requires more energy (see Stillman, 2002; Whiteley and Faulkner, 2005; Sørensen, 2010; Fitzgerald-Dehoog *et al*., 2012). For example, stress proteins can represent up to 7% of total protein pool with increasing turnover during stressful conditions (see Kültz, 2003; Sokolova and Lannig, 2008; Sokolova *et al*., 2012).

As for thermal tolerance (see Stillman and Somero, 1999; Stillman, 2002; 2003; Tomanek and Helmuth, 2000), the inability to acclimate metabolic rates including limits and thresholds could mean that these species and other littorinids are likely to be the most vulnerable to rising temperature in hot summer months and during global warming (see Stillman, 2004; Tomanek, 2010; Somero, 2010). Therefore, there is a need to investigate the effect of acclimation or season on metabolic rates of South African species and other littorinids. This is further supported by the results with slower rates of increase in temperature, when animals showed better regulation of heart rates as well as shift in limits and thresholds.

Heat shock

No studies have looked at the effect of heat shock or sudden exposure on the heart performance of littorinids or other intertidal animals. A brief exposure to sublethal temperature is known to increase thermal tolerance on second or subsequent exposure (see Stillman and Somero, 1999; Hopkin *et al*., 2006; Madeira *et al*., 2012b, c), and this can be true for metabolic responses. This study showed that heat shock (thermal history) had little or no effect on the heart performance of *Afrolittorina* spp. and *E. natalensis*. This is further supported by the results of repeated exposures of individuals of *Afrolittorina* spp. which showed the same heart patterns irrespective of previous heat stress encounter or thermal history.

The only exception was the thermoneutral breakpoint for *E. natalensis*, which increased by 1°C, but these data came from a single individual. The findings for *E. natalensis* relative to those for *E. malaccana* indicated that the later species displayed a noticeable shift in its thermoneutral breakpoint after heat shock (Marshall unpub. data) suggesting that it is a tropical species physiologically adapted to high temperatures. Marshall *et al.* (2011) showed that the tropical *E. malaccana* produces heat shock proteins (i.e. hsps70) once temperatures pass their thermoneutral limit. Proteins, particularly heat shock proteins are known to be involved in thermal acclimation and thermal tolerances of animals (see Buchner, 1996; Krebs and Bettencourt, 1999; Somero, 2004; Pörtner and Knust, 2007; Dong and Williams, 2011; etc) and thus shifts in limits and threshold temperatures (see Hopkin *et al*., 2006).

Lack of effects of heat shock in this study might be explained by the cost associated with protein (heat shock) synthesis (see Feder, 1999; Feder and Hofmann, 1999; Tomanek, 2010). Thus, animals which rarely experience stressful conditions, like South African species, do not need to spend energy on heat shock protein production. The results of this study show that thermal history has no effect on heart performance of littorinids, but this requires further investigation using various protocols, including repeated exposure. For example, repeated exposure has been shown to have an effect (a decrease in the daily mean oxygen consumption) on metabolic rate of individuals of the juvenile Chinese shrimp *Fenneropenaeus chinensis* acclimated under diel temperature fluctuation (Tian *et al*., 2004). Therefore, future studies to investigate the effect of heat shock and/or repeated exposure (i.e. thermal history) on metabolic performance would be valuable, especially with the possibility of unpredicted heat events resulting from climate change.

Effect of starvation or nutritional status

The effect of starvation or nutritional status on metabolic rates has also received little attention. Indeed many authors fail to mention when the animals studied were last fed, if at all, prior to the experimentation. This is surprising since the availability of food and nutritional status are amongst the most important factors influencing the metabolism of animals (Wallace, 1973; Branch *et al.*, 1988; Tully *et al*., 2000; Hatcher *et al*., 1997; Brockington and Clarke, 2001; Dahlhoff *et al*., 2002; Isla and Perissinotto, 2004; Pörtner and Knust, 2007; etc). This is important for animals that live in areas such as the eulittoral fringe, particularly in summer, where food availability is low and time for feeding is limited (Newell and Roy, 1973; Branch *et al.*, 1988; Little, 1989; Norton *et al*., 1990; Bates and Hicks, 2005). It is well known that there are differences in metabolic rates between starved and non-starved animals, with higher rates for fed than starved animals (Wallace, 1973; Aldrich, 1975; Hiller-Adams and Childress, 1983a; Hawkins, 1995).

Where the effect of starvation has been investigated, most authors were interested in the differences (i.e. magnitude) between fed and starved animals, and not whether this affected metabolic regulation (Vernberg, 1959; Wallace, 1973; Hiller-Adams and Childress, 1983a,

b). In most animals, metabolic rate generally declines following starvation (Pickens, 1965; Bayne *et al*., 1976; Du Preez, 1983; Höjesjö *et al.*, 1999; Santini *et al*., 2002; Tian *et al.*, 2010) as the result of reduction in SDA and gradual exhaustion of metabolic reserves (see Bayne, 1973b; Kristensen, 1989; Chakraborty *et al*., 1992; Percy, 1993). For example, Ansell (1973) and Marsden *et al.* (1973) found a progressive decrease in daily oxygen consumption in the starved crabs *Cancer pogurus* and *C. maenas*, respectively. It is interesting that both fed and starved animals regulated oxygen consumption in the resting state (Ansell, 1973), showing that the ability to regulate was not under the influence of nutritional status. Newell and Bayne (1973) found that crabs of the genus *Carcinus* starved for 3 weeks not only showed a decline in oxygen consumption, but temperature independence of oxygen consumption, with the effect being more pronounced in small crabs than large ones.

These studies show that starvation not only affects the magnitude of metabolism of animals, but also its relationship with temperature. Low metabolic expenditure or costs as a result of reduced or depressed metabolic rates is one of the mechanisms employed by animals to survive periods of starvation or poor feeding conditions and during rapid temperature change and extremes (see Newell and Bayne, 1973; Parsons, 1990; Peck, 1996; Höjesjö *et al.*, 1999; Harper and Peck, 2003; Tian *et al.*, 2010). However, some species show an increase in metabolic rates (Marsden *et al.*, 1973; Carefoot *et al*., 1993; Brockington and Clarke, 2001); while others do not show a change of their metabolic rates following starvation (Roberts, 1957; Aldrich, 1975; Carefoot *et al*., 1993; Percy, 1993). For example, Pickens (1965) found that the metabolic rates of mussels did not change during the first week and remained constant for two weeks of starvation, after which they declined, possibly as a result of starvation.

In this study, there was no difference in heart rate between animals that were „starved" and „non-starved", showing that nutritional status had little or no influence on the metabolic rate response to temperature. This was supported by the results of repeated exposure of the same individuals, where there was no difference or change in heart rate of animals fed and those which were not fed after the first exposure. This requires further investigation as one individual of *Afrolittorina africana* changed from non-regulation to partial regulation after repeated exposure without food (see A in Fig. 4.7.1). However, it is possible that the period

(14 – 30 days) used in this study was not long enough to induce starvation. Littorinids are known to aestivate for long periods (i.e. at least 14 days for temperate and subtropical species and 60 days for tropical species) when they depress metabolic rates to save energy (McMahon, 1990; Sokolova and Pörtner, 2001b; Judge *et al*., 2011; Marshall *et al*., 2010; 2011). Our species might have benefitted from metabolic depression during aestivation.

Studies on animals from other phyla have found that a week to two weeks (14 days) period does not induce starvation (see Pickens, 1965; Nicholson, 2002). In other studies however, a period of at least one to two weeks has been found to induce starvation as shown by reduction or decrease metabolic rates of studied species (see Wallace, 1973; Newell and Bayne, 1973; Regnault, 1981; Kristensen, 1989; Tian *et al.*, 2010). This shows that time to starvation differs for different animals or species, and can also depend on season (i.e. food availability) plus reproductive, developmental or physiological state (see Bayne, 1973b; Hiller-Adams and Childress, 1983a, b; Branch *et al.*, 1988; Norton *et al*., 1990; Bates and Hicks, 2005).

The lack of difference in heart rate of „starved" and „non-starved" animals in this study was irrespective of acclimation temperature. In other studies (Marsden *et al.*, 1973; Wallace, 1973; Pilditch and Grant, 1999) however, high temperatures have been linked to the accelerated decline in metabolic rate of starved animals, probably as a result of high metabolic costs at high temperatures. For example, Marsden *et al.* (1973) claimed that the effect which occurred after two weeks at 15°C may occur earlier at higher acclimation temperatures. But Siikavuopio *et al*. (2008) reported a negligible (as indicated by similar Q_{10} values between fed and starved animals) effect of temperature on oxygen consumption of the green urchin *Strongylocentrotus droebachiensis*. If my results about the lack of reduction in metabolic rate during starvation are correct, this suggests that these species are capable of enduring prolonged aerial exposure partially because they can save energy through metabolic depression during aestivation. Thus, in view of their ability to withstand a long period of starvation (i.e. 14-30 days) as seen in this study, it would be interesting to know if the studied species and other littorinids starve for such periods in nature.

In summary, the heart rate data indicate that these littorinid snails show intra- and interspecific differences in their physiological responses to temperature, and these seem to relate to differences in biogeography, species ecology and phylogeny. The ability to regulate is phylogenetically determined with little adaptation, while thresholds and lethal limits correspond to biogeography and species ecology. Phylogenetic differences do not lie in whether or not a species shows regulation, but in how quickly it can induce a depressed, thermally independent metabolic state. The speed of this response appears to be linked to the need to conserve energy. It is presumed that in snail species that feed regularly, even if rarely, like the studied South African species, there is less need for rapid induction of metabolic depression, compared to snails that feed unpredictably like supra-littoral tropical species such as *E. malaccana*.

The present study revealed that both the stresses found and mechanisms utilized in physiological adaptation to high temperature exposure by subtropical and temperate littorinids are more or less similar to those utilized by the littorinids from the tropics. The data also indicate that these littorinids can regulate their metabolism within the sub-lethal temperature range experienced under natural conditions and in this respect they are well suited to life in habitats where there are fluctuations in temperature and other environmental conditions. Therefore, like high thermal and desiccation tolerances, metabolic depression and/or regulation as temperature increases is a physiological adaptation of marine animals for life high in the intertidal zone.

CHAPTER 5: Proteomics of co-existing *Afrolittorina* species from the warm temperate region of South Africa

5.1. Introduction

It is well established that environmental temperatures (changes and extremes) affect the physiology as well as the distribution and abundance of marine animals, especially intertidal ectotherms (see Somero, 1995; 2002; 2005; 2010; Hofmann, 1999; 2005; Hofmann *et al*., 2002; Tomanek, 2002; 2008; 2010a, b; 2011; etc). This is because the body temperature of ectotherms is largely under the control of environmental temperature (see Tomanek and Somero, 1999; Dahlhoff *et al*., 2001; Helmuth *et al*., 2002; Broitman *et al*., 2009). In turn the physiological and biochemical processes of animals are under the control of body temperature (see Huey and Bennett, 1990; Huey and Berrigan, 2001; Peck *et al*., 2007; Ivanina *et al*., 2009). As such, the distribution and abundance of marine intertidal ectotherms is under the influence of environmental temperature as manifested in body temperatures (see Huey and Stevenson, 1979; Bertness *et al.*, 1999; Muñoz *et al.*, 2005; Menge *et al*., 2007).

In intertidal habitats, marine ectotherms face very extreme temperatures and abrupt changes in temperature and desiccation that occur during the tidal cycle (see Hofmann, 1999; Helmuth, 1998; 2000; Helmuth *et al*., 2002; 2006a, b; Somero, 2002; 2010; Stillman, 2002). This is because intertidal habitats are affected by both atmospheric and oceanic changes. Thus, intertidal animals live in rapidly fluctuating environments on a daily basis. Day time air temperatures can reach as high as 50-55°C in the tropics (see Lewis, 1963; Garrity, 1984; Marshall and McQuaid, 2010; Cartwright and Williams, 2012) and 30-45°C in the subtropics and temperate regions (pers. obs.; Morley *et al*., 2009). On rocky intertidal shores, substratum temperatures can increase from that of sea water temperature, e.g. 10°C, to over 40°C on temperate shores (see Dahlhoff *et al*., 2001; Harley and Helmuth 2003) and exceed 50°C on tropical shores (see Williams and Morritt, 1995; Marshall and McQuaid, 2010; Judge *et al*., 2011; Cartwright and Williams, 2012) in a matter of hours during a single low tide.

Consequently, animals on rocky intertidal shores can experience changes of up to 20°C and above in body temperature during summer midday low tides (see Hofmann and Somero, 1995; 1996; Tomanek and Somero, 1999; Fitzhenry *et al*., 2004; Judge *et al*., 2011). This means that rocky intertidal animals may experience body temperatures that exceed that of the surrounding air and sea water temperature, and regularly approach the animals‟ thermal limits (see Helmuth, 1998; 1999; Helmuth and Hofmann, 2001; Tomanek and Sanford, 2003; Helmuth *et al*., 2009; Miller and Denny, 2011). This can be true for animals such as littorinid snails that live in the intertidal since they are more likely to experience prolonged thermal and desiccation stress than lower intertidal and subtidal animals (see McMahon, 1990; Hofmann and Somero, 1995; Hofmann, 1999; Halpin *et al*., 2002). At higher intertidal heights, the intensity of thermal and desiccation stress is in part a function of the change in temperature intensity multiplied by the duration of exposure (see McMahon, 1990; Jones and Boulding, 1999; Muñoz *et al.*, 2008; Lee and Boulding, 2010).

As a result, intertidal animals have developed strategies to cope with and survive stressful conditions. Many physiological (e.g. increased thermal tolerance and metabolic adjustments), behavioural (e.g. active microhabitat selection and body orientation) and morphological (e.g. shell shape and colour) adaptations are used (see Huey and Bennett, 1990; Huey and Berrigan, 2001; Hickey and Singer, 2004; Wang *et al*., 2007a; Gracey *et al*., 2008; Harley *et al*., 2009; Evans and Somero, 2010). At the cellular level, in order to increase tolerance of heat stress, animals use adaptive mechanisms such as enhanced production of heat shock proteins (Feder and Hofmann, 1999; Tomanek, 2002; Tomanek and Sanford, 2003; Finke *et al*., 2009; Sørensen, 2010), increased heat stability of key metabolic enzymes (Jaenicke, 1991; Somero, 1995; 2004; Stillman and Somero, 2001; Zippay *et al*., 2004) and modification of enzymes (Somero, 1978; 1995; 2004; Schmidt *et al*., 2007; Dong and Somero, 2009), amongst others.

When environmental temperatures start to approach an animal‟s thermal limits or are intolerable, denaturation of proteins (enzymes) takes place (see Somero, 1995; Hofmann and Somero, 1996; Tomanek, 2002; Sørensen *et al*., 2003; González-Riopedre *et al*., 2007). This results in an increase in thermally damaged proteins, sometimes called “conjugated ubiquitin” (see Hofmann and Somero, 1995; Buckley *et al*., 2001; Buckley and Hofmann, 2002;

Somero, 2002), and in turn disruption of the protein pool and protein homeostasis. Following thermal perturbations, an animal‟s survival depends on its capacity to effectively maintain or restore the integrity of protein (cellular) homeostasis or homeodynamics (see Chapple *et al*., 1997; Kültz, 2003; Sørensen *et al*., 2003; Botton *et al*., 2006; Sørensen, 2010). This is achieved by employing cellular defence mechanisms such as synthesis of stress proteins, including molecular chaperons, antioxidases, proteases and DNA repair systems (see Feder, 1999; Feder and Hofmann, 1999; Pörtner, 2002b; Tomanek, 2002; Kültz, 2003; Sørensen, 2010; etc).

Although various techniques including Western blot, SD-PAGE and antibody detection methods have been and are useful to detect and quantify proteins (i.e. stress protein response) in animals, they have a limitation in that they target a certain group of proteins, the heat shock proteins (Hsps). Recently, techniques such as proteomics (study of the whole protein profile of a cell or animal) have been developed to analyze large numbers of proteins simultaneously to discern subtle changes in protein expression (see below). This will increase our understanding of the role of Hsps as molecular chaperons and other proteins in stress response. In addition, studying the proteome of organisms can help to identify the more universal adaptations underlying protein (enzymes) stability at high temperature and other stresses (see Hickey and Singer, 2004; Somero, 2004; Ulrich and Marsh, 2008; Dilly *et al*., 2012).

Thus, by establishing the proteome for animals exposed to various stressors, proteomics can potentially be used to study the response of animals at the molecular (protein) level (see Kültz *et al*., 2007; Nesatyy and Suter, 2007; Serafini *et al*., 2011; Dowd, 2012; etc) and to complement Hsps methods which are already widely used (see Kültz and Somero, 1996; Williams, 1999; Aebersold and Mann, 2003; Storey, 2006; Jonsson *et al*., 2006; McLean *et al*., 2007; Jurgen *et al*., 2011; etc). In this respect, proteomics enables the testing of hypotheses surrounding the molecular or biochemical basis (adaptation or acclimation) for stress responses in animals.

In addition, proteomics provides a link between physiology, ecology and genetics (see Williams, 1999; Zivy and Vienne, 2000; Naaby-Hansen *et al*., 2001; Jackson *et al*., 2002; Volckaert *et al*., 2008; etc). This is because unlike genomics (genome) and transcriptomics (transcriptome), which provide information about a cell‟s genetics and potential regulatory mechanisms, proteomics (proteome) provide relevant information about an animals‟ biological or physiological state (see Görg *et al*., 2004; Nunn and Timperman, 2007; Wang *et al*., 2007a; Martyniuk and Denslow, 2010; Chapman *et al*., 2011; etc). Thus, proteomics is a mutually complementary technique to genomics or transcriptomics (see Piñeiro *et al*., 2001; Storey, 2006; Rees *et al*., 2010; Lockwood and Somero, 2011; Diz *et al*., 2012a; etc), and as such has increasingly been used to study and understand biological systems and their dynamics under different conditions (see below). In addition, the proteome (i.e. proteins) is closer to the organisms‟ phenotype, the direct target of natural selection, and as such more useful in inferring the molecular basis of an adaptive process or evolution (see Feder and Walser, 2005; Piñeiro *et al*., 2010; Silvestre *et al*., 2012; Tomanek, 2010; 2012b).

Proteomics (qualitative or quantitative) is defined as the study of proteins expressed by a genome, tissue or cell (see Beranova-Giorgianni, 2003; Feder and Walser, 2005; Nunn and Timperman, 2007; Karr, 2008; Dow, 2012). Thus, the goal of proteomics is to study the whole protein profile (i.e. the proteome), including quantification, identification, possible modifications and tissue localizations of a cell, tissue or whole organism at a particular time under different conditions (see below; Fiévet *et al*., 2004; Karp and Lilley, 2007; Rees *et al*., 2010; Wright *et al*., 2012). This is because the analysis of an animal‟s proteome allows the detection of subtle changes in the levels of individual proteins in response to stimuli or conditions (see Monteoliva and Albar, 2004; Nesatyy and Suter, 2007; Sheehan and McDonagh, 2008; Enyu and Shu-Chien, 2011).

Although proteomics is a new and young approach, it is still based on a relatively old technique of protein separation, the two-dimensional gel electrophoresis (2-DE) developed by O‟Farrell and Klose in 1975 (see Zivy and Vienne, 2000; Monteoliva and Albar, 2004; Kim *et al*., 2008; Rabilloud *et al*., 2010; Rodrigues *et al*., 2012) first described by Kenrick and Margolis in 1970 (see Wang *et al*., 2007a). Two-dimensional gel electrophoresis is a very powerful and sensitive technique designed to separate complex protein mixtures (see

Witzmann and Li, 2002; Görg *et al*., 2004; López, 2007b; Rabilloud *et al*., 2010; Zhou *et al*., 2012). However, other techniques (e.g. liquid chromatographic) are also used for separation of proteins (see Williams, 1999; Aebersold and Mann, 2003; Monteoliva and Albar, 2004; Storey, 2006; Kültz *et al*., 2007; Forné *et al*., 2010; Wright *et al*., 2012).

The separation of proteins using 2-DE technique involves a combination of isoelectric focusing (IEF) where proteins are first separated based on their charge and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) where proteins are finally separated on the basis of their molecular weight or size (see Witzmann and Li, 2002; Marengo *et al*., 2005; Wang *et al*., 2007a; Rabilloud *et al*., 2010; Wright *et al*., 2012; etc). Together with mass spectrometry (MS; used for the identification of proteins), two-dimensional gel electrophoresis (2-DE; used for separation of proteins) is the most widely used tool in proteomic studies (see below; Shevchenko *et al*., 1996; Beranova-Giorgianni, 2003; Wittmann-Liebold *et al*., 2006; Rabilloud *et al*., 2010; etc). This is because mass spectrometry allows the identification of known and unknown proteins in the proteome without prior knowledge of the protein structures (see Shevchenko *et al*., 1996; Aebersold and Mann, 2003; Nunn and Timperman, 2007; Piñeiro *et al*., 2010; Abbaraju *et al*., 2012).

The proteomic (2-DE based and others) approach is applied in numerous fields, including many areas of marine animals‟ biology, physiology, ecology, taxonomy, toxicology and health (see Monteoliva and Albar, 2004; Biron *et al*., 2006; López, 2007a; De Souza *et al*., 2009; Forné *et al*., 2010; Thiyagarajan, 2010; Martyniuk *et al*., 2011; Rodrigues *et al*., 2012; etc), amongst others. This is because proteomics provide relevant information about biological events such as developmental stage, disease or physiological state as well as responses to environmental and other conditions (see below).

For example, Cordeiro *et al*. (2012) used 2-DE proteomics to characterize and identify potential molecular makers (i.e. proteins) of physiological response to stress (handling) in captivity in the Senegalese sole *Solea senegalensis*. Alves *et al*. (2010) also used 2-DE proteomics to identify possible metabolic molecular indicators of chronic stress (repeated handling and crowding) in the gilthead seabream *Sparus aurata*. Dowd *et al*. (2008) used 2-DE proteomics to investigate the effect of nutritional status (natural feeding) on protein

expression in the dogfish shark *Squalus acanthias*; and Enyu and Shu-Chien (2011) used 2-DE proteomics to show that starvation-related changes in protein expression led to a reduction in glycolysis and an increase in gluconeogenesis (which returned to normal after feeding was resumed) in the female zebrafish *Danio rerio*. In addition, the expression of proteins related to fatty acid and amino acid metabolism suggested the utilization of these reserves as an energy source during starvation.

In taxonomy or genetics, proteomics have been used to investigate species identity, phylogenetic relationships, population genetics and the genetic variability of marine animals (see Piñeiro *et al*. 2001; López and Alvarez, 2003; Chen *et al*., 2004; Blank *et al*., 2005; 2012; Martinez *et al*., 2007; Kim *et al*., 2008; Diz *et al*., 2009; etc). For example, Backeljau *et al*. (2001) used proteomics to investigate the relationships between *Littorina saxatilis*, *L. arcana* and *L. compressa* from the same and different regions. López *et al.* (2002b) used proteomics to characterize species-specific peptides (which showed minor differences) to identify the three European marine mussel species, *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus*. Mosquera *et al*. (2003) also used proteomics to investigate genetic polymorphism in polypeptides from the foot samples of individuals of the mussel *M. galloprovincialis*. López *et al*. (2005) found differences in protein spots (6 out of 18 protein spots which were exclusive to *M. galloprovincialis*) between the gels of bivalve larvae from the same region.

In disease or health studies, proteomics have been used to analyse the protein profiles of marine invertebrates and fishes in response to or to test for host immunity against infection (see Chongsatja *et al*., 2007; Bourchookarn *et al*., 2008; Zhang *et al*., 2010a; Chen *et al*., 2011; Dheilly *et al*., 2011; Huan *et al*. 2011; Peng, 2012; etc). For example, Cao *et al*. (2009) used 2-DE based proteomics to analyse the proteins (the number of which substantially decreased) in the haemolyph of the susceptible oyster *Ostrea edulis* and the resistant species *Crassostrea gigas* infected with the protozoan *Bonamia ostreae*. Similarly, Simonian *et al*. (2009) used 2-DE approach to identify markers (proteins) of QX disease resistance in the Sydney rock oyster *Saccostrea glomerata*. Wang *et al*. (2007b) also used 2-DE to analyse protein expression profiles (75% of which showed marked change) from stomach samples of the shrimp *Litopenaeus* (*Penaeus*) *vannamei* infected with white spot syndrome virus.

Proteomics have also been applied to investigate changes in proteome during larval development, attachment and metamorphosis in marine animals such as barnacles (see Thiyagarajan and Qian, 2008; Thiyagarajan *et al*., 2009; Zhang *et al*., 2010b), bryozoans (Thiyagarajan *et al*., 2009), polychaetes (Mok *et al*., 2009), ascidians (Nomura *et al*., 2009), fishes (Tay *et al*., 2006), and corals (deBoer *et al*., 2007). These studies show that different developmental stages have distinct proteomes, with differential expression of several proteins (most of which were associated with stress, protein degradation, energy metabolism, cell division and juvenile hormone binding) by different stages as well as before and after metamorphosis. For example, Chandramouli *et al*. (2011) found that proteins related to cell migration, cell division, energy storage and oxidative stress were abundant in competent larvae of the polychaete *Capitella sp. I*, while proteins involved in oxidative metabolism and transport regulation were abundant in the juveniles. Wong *et al*. (2010) found that the mitochondrial processing peptidase beta subunit and severin were abundant in the larval stage, but down regulated during metamorphosis in the marine bryozoan *Bugula neritina*. Sveinsdóttir *et al*. (2008) found that although the pattern of abundant proteins was largely conserved in two age groups of the Atlantic cod *Cadus morhua*; type II keratins were dominant in 6 day old larvae while the type I keratins were dominant in the 24 day old larvae.

Of interest is that proteomics not only identify relationships and differences among populations and species, but they also help to explain the relationships from biochemical, physiological and ecological points of view (see López *et al*., 2001; 2002; López, 2005, 2007a, b; Blank *et al*., 2005; 2012). This is because differences in protein expression patterns among species or populations are due to adaptation (i.e. acclimation), thus the conditions animals experience in their respective environments or habitats (see below). For example, Martínez-Fernández *et al.* (2008; 2010b) found a difference of about 7-16% in the protein profiles of two ecotypes of the marine snail *Littorina saxatilis* as a result of adaptations to different habitats. The smooth and unbanded (SU) ecotype which lives in wave-exposed (mussel belt) habitat showed regulation of proteins associated with energy metabolism (fructose-bisphosphate aldolase and arginine kinase), while the ridged and banded (RB) ecotype which lives at higher levels (barnacle belt) did not.

In addition, Diz *et al*. (2012b) found that the proteomes of these two ecotypes show ontogenetic differentiation. Differentiation was higher for the RB ecotype than for the SU

ecotype, although the level of protein expression differences was nearly constant from the late embryonic stage to adulthood in both ecotypes. López *et al*. (2002a) found significant differences in protein spots (15 were higher in *Mytilus edulis*, and 22 were higher in *M. galloprovincialis*) between the two mussels found in different geographical habitats. Diz and Skibinski (2007) found significant differences in protein expression between and within species of *M. edulis*, *M. galloprovincialis* and intermediate phenotypes in the mussel hybrid zone, suggesting adaptation to different habitats. In addition to Hsp70, which was more highly expressed in intertidal than in cultured mussels, López *et al.* (2001) found a higher number of protein spots in cultured compared to intertidal individuals of *M. galloprovincialis*, suggesting responses to different ecological conditions.

More importantly, the field of proteomics, specifically 2-DE MS based proteomics, has been increasingly applied to study proteome responses in model (organisms with genome sequence data) and non-model (organisms without genome sequence data) animals including marine species subject to various environmental stresses (see below). This is important given the anticipated effects of climate change when temperature (changes and extremes) and other environmental factors (e.g. pollutants) as well as ocean acidification will threaten marine biodiversity (see Warwick and Turk, 2002; Tomanek, 2008; 2010; 2011; 2012a, b; Helmuth *et al*., 2010; Piñeiro *et al*., 2010; etc).

For example, proteomics have been used to screen or analyse changes in protein expression profiles of various marine animals exposed to pollutants or chemicals (see Apraiz *et al*., 2006; Letendre *et al*., 2011; Ralston-Hooper *et al*., 2011; Sanchez *et al*. 2011; Martyniuk and Denslow, 2010; 2012; Campos *et al*., 2012; 2013; etc). Jonsson *et al*. (2006) found that exposure to diallylphthalate and crude oil affected several microsomal proteins in individuals of the blue mussel *Mytilus edulis*. Leung *et al*. (2011) found that a total of 15 protein spots were differentially expressed in the hepatopancreas and adductor muscles of the green-lipped mussel *Perna viridis* exposed to cadmium and hydrogen peroxide. Rodríguez-Ortega *et al*. (2003) found that 1-2% of the visible proteome of the clam *Chamaelea gallina* was affected by exposure to four pollutants. Amaral *et al*. (2012) found that approximately 5% of the proteome was differentially expressed between individuals of the oyster *Saccostrea glomerata* from acidified and reference sites, with five protein spots being more abundant and one less abundant at the acidified site. Wang *et al*. (2010) found that the protein profiles from

the brains of the zebrafish *Danio rerio* were remarkably altered by exposure to chronic microcystin-LR.

Using a proteomic approach to study the response of barnacle crypid larvae to ocean acidification, Wong *et al.* (2011) found that there was differential expression of proteins which were associated with molecular chaperones, respiration and energy metabolism. This suggests a potential strategy that the barnacle larvae could employ to tolerate ocean acidification stress. Tomanek *et al*. (2011) found that 12% of proteins (including cytoskeleton and oxidative stress proteins) were differentially expressed in the mantle tissues of the eastern oyster *Crassostrea virginica* exposed to hypercapnia. On the other hand, Dineshram *et al*. (2012) found a marked reduction of protein expression (loss of 18% of expressed proteins in control) in the larvae of the pacific oyster *Crassostrea gigas* after exposure to ocean acidification. Laura *et al*. (2011) found that a number of proteins (which were sometimes switched on in the selectively bred lines, but not in the wild lines) were differentially expressed in wild and selectively bred larvae of the Sydney oyster *Saccostrea glomerata* exposed to elevated carbon dioxide. Martin *et al*. (2011) found that the larval stages of the sea urchin *Paracentrotus lividus* showed up-regulation of candidate genes involved in development and biomineralization to simulated ocean acidification.

As for other environmental factors (e.g. pollutants and ocean acidification), studies using proteomics have shown that marine animals show changes in protein expression profiles following exposure to acute or chronic temperature (heat or cold) (see below). For example, Tomanek and Zuzow (2010) found changes in the expression patterns of several proteins (e.g. molecular chaperones, cytoskeletons, etc) in two congener mussels, *Mytilus trossulus* and *M. galloprovincialis*, exposed to acute and chronic heat stress. The cold adapted *M. trossulus* showed more pronounced expression patterns (clearer at the highest temperature) than the warm adapted *M. galloprovincialis* as reflected in thermal tolerances. On the other hand, Fields *et al*. (2012a) also found upregulation of proteins associated with energy metabolism, oxidative stress, chaperoning and cytoskeleton in the same two congeners after acclimation for 4 weeks at cold (7°C) and warm (13 and 19°C) temperatures. Ibarz *et al*. (2010) found that a total of 57 proteins significantly changed (many being down-regulated) in the gilthead seabream *Sparus aurata* exposed to cold (8°C) following acclimation to warm (22°C)

temperatures. Similar changes in protein expression profiles in response to exposure to temperature (acute or chronic) have been found in other gastropods (see Tomanek; 2005; Joyner-Matos *et al*., 2009; Fields *et al*., 2012b), crustaceans (see Wang *et al*., 2007a; Serafini *et al*., 2011; Dilly *et al*., 2012), and fishes (see Kültz and Somero, 1996; McLean *et al*., 2007; Silvestre *et al*., 2012).

This is also true for other environmental factors such as salinity (Shepard *et al*., 2000; Lee *et al*., 2006; Cheng *et al*., 2009; Tomanek *et al*., 2012), oxygen (Oehlers *et al*., 2007; Jiang *et al*., 2009; Dowd *et al*., 2010b; Mary *et al*., 2010), and ultraviolet radiation (Adams *et al*., 2012; Zubrzycki *et al*., 2012). For example, Campanale *et al*. (2011) found that exposure to ultraviolet radiation (UV) resulted in 14% change in proteins of the embryos of the purple sea urchin *Strongylocentrotus purpuratus*. Chen *et al*. (2013) found a 9.4% change in proteins (including metabolic enzymes, cytoskeleton and oxygen-binding proteins) in the skeletal muscle of the zebrafish *Danio rerio* exposed to low oxygen. On the other hand, Bosworth *et al*. (2005) found that hypoxia did not affect the general pattern of protein expression in the skeletal muscle of the above fish species, but affected the amounts of six low abundance proteins.

Dowd *et al*. (2010a) found changes in proteins associated with amino acid and inositol metabolism, energy metabolism, protein degradation and cytoskeleton in the gills and rectal gland of the leopard shark *Triakis semifasciata* exposed to low salinity. Chen *et al*. (2009) found differential expression of proteins involved in energy metabolism, biosynthesis, DNA methylation and cell differentiation, etc. in the trunk kidney of the juvenile ayu *Plecoglossus altivelis* exposed to brackish water. Ky *et al*. (2007) found that 362 protein spots were differentially expressed in the gills and intestines of the European sea bass *Dicentrarchus labrax* reared in seawater compared to those from freshwater, with five cytoskeleton and one aromatase cytochrome P450 being over expressed in gills of animals exposed to seawater.

In addition, changes in protein expression profiles to one factor can also be influenced by combination and/or interaction with other factors (see below). This is because multiple factors, rather than single factors (e.g. temperature) are encountered in the natural environment (see Backeljau *et al*., 2001; Roelofs *et al*., 2008; Joyner-Matos *et al*., 2009;

Nicastro *et al*., 2010; Chapman *et al*., 2011). For example, Gardeström *et al*. (2007) showed that increased oxygen availability affected the protein profiles of the dogwhelks *Nucella lapillus* when exposed to increased water temperature alone by increasing the similarity between heat shocked and control animals. Pineda *et al*. (2012) found an increase in hsp70 gene expression in the ascidian *Styela plicata* exposed to periodic high temperatures coupled with low salinities.

Shepard *et al*. (2000) reported specific induction and repression of several protein spots in the mussel *Mytilus edulis* exposed to Aroclor 1248, copper and lowered salinity. Kimmel and Bradley (2001) found that temperature-salinity combinations and their extremes resulted in increased differential expression of proteins in the calanoid copepod *Eurytemora affinis*. Kültz and Somero (1996) found that the gill epithelial cells of the fish *Gillichthys mirabilis* showed upregulation of certain proteins after exposure to both temperature [low (10°C) and high (20°C)] and salinity (diluted seawater). Silvestre *et al*. (2010) found that Hsp90, creatine kinase and other proteins or enzymes of green and white sturgeon *Acipenser medirostris* larvae were affected by temperature-selenium combinations, in addition to either factor.

In addition, reactive oxygen species (ROS) and/or oxidative stress which are generated during exposure to a variety of insults or stressors (e.g. change in environmental conditions) have effects (as a co-stressor) on animal proteome responses to environmental factors (see McDonagh and Sheehan, 2006; 2007; Sheehan and McDonagh, 2008; Kassahn *et al*., 2009; Tomanek, 2011; 2012a; Ibarz *et al*., 2012; Tomanek *et al*., 2011; etc).

In summary, marine animals including intertidal ectotherms show different and/or diverse (species and/or tissue-specific) proteome responses in terms of expression and quantity, as a result of adaptation to particular conditions, and the biological functions of different tissues (see above; De Souza *et al*., 2009; Piñeiro *et al*., 2010; Abbaraju *et al*., 2012). Of more interest is that organisms, including marine ectotherms, show changes (up- and down-regulation) in protein expression in response to exposure or adaptation to various environmental conditions or factors. Thus, proteins can either be made in large or small quantities in response to a stimulus, suggesting a potential molecular strategy that animals employ to tolerate environmental stress. The exclusive identification of cytoskeleton,

chaperones, antioxidant, energy production and stress proteins under various conditions or treatments could reflect their relative abundance or their role as major targets of environmental stress or conditions (see above; Rodríguez-Ortega *et al*., 2003; Kültz *et al*., 2007; Petrak *et al*., 2008; Wang *et al*., 2009; Campos *et al*., 2013).

Most proteome based studies on marine animals responding to environmental stressors have focused on environmental pollutants and/or ocean acidification, while few have looked at environmental temperatures (see above). Therefore, there is a need to understand the protein response of marine animals, especially intertidal ectotherms such as littorinids, to environmental temperature changes and extremes. This is more important now with the anticipated effects of climate change where the mean global temperatures (including extreme events) have risen and are predicted to continue to rise in the coming years (see above).

Using a proteomic approach, I measured proteins which are expressed by the two co-existing *Afrolittorina* spp. This experiment was intended to find out if there were differences in the total protein profile between (i.e. species) and within (i.e. sizes) of *Afrolittorina* spp. under non-stressed and heat stressed conditions. To accomplish my objectives, non-stressed or heat stressed large and small individuals of *Afrolittorina* spp. were analyzed by 2-DE and their protein profiles compared. This will shed light on how the two species deal with heat stress in their microhabitats (i.e. eulittoral fringe), and their likely molecular responses to climate change. Together with other physiological and molecular techniques, proteomics will help to resolve whether the two southern African *Afrolittorina* spp. respond in different ways to heat stress.

5.2. Materials and methods

5.2.1. Study species

Two *Afrolittorina* spp., namely: *A. knysnaensis* and *A. africana* were used. See Chapter 1 for species distribution ranges and patterns of vertical zonation as well as microhabitat use and aestivation behaviour.

5.2.2. Collection and transportation

Specimens of *A. africana* and *A. knysnaensis* were collected from the eulittoral zone at Fish River mouth (4°32'N; 114°43'E) on the south coast of South Africa in June 2009. About 50-100 each of large and small individuals of each species that were feeding or had fed within 12 hours (see Chapter 3) were returned to the laboratory in plastic bags placed inside an insulated cool box, treated (see below) and later taken (in an aestivation state) to Hong Kong for proteomic determinations (see below). For transportation to the Swire Institute of Marine Science (SWIMS) at Hong Kong University, Hong Kong, aestivating animals were wrapped in dry paper towels and kept in cabin luggage.

5.2.3. Handling and treatment conditions

On arrival at the laboratory (Rhodes University or SWIMS), specimens were washed in seawater, allowed to emerge from their shells and to reattach to 2L lidded plastic containers before being exposed to air, when they exhibited behavioural emergence. Active animals were blotted dry with paper towel and dried using a fan at room temperature (approximately 20ºC). Specimens were kept on dry paper towel at room temperature (18-20ºC) overnight or for up to five days to induce aestivation.

At the beginning, 15 x 5 small and 5 x 5 large individual snails of each species were taken out (controls or non-stressed), washed in double-distilled water (ddH_2O), the shell was crushed to remove the soft tissues, which were immediately frozen at -80°C until further use. For heat treatments, 15 x 5 small and 5 x 5 large aestivating individual snails (see above) of each species were placed in 20 ml dry lidded vials that were then placed in an oven set to 20ºC. Oven temperature was increased in 5°C increments over 10 minute intervals to reach 45°C, and left for 1 hour at this temperature. Temperature inside the vial was monitored using T-type thermocouples (Cromega and ADInstruments, Australia). After 1 hour at 45°C, the vials were removed from the oven and allowed to cool for 2 hours at room temperature (20°C) prior to processing (see below).

5.2.4. Two-dimensional (2-DE) gel electrophoresis

Two-dimension gel preparation and the subsequent separation were performed according to the optimized larval proteomic protocol of Thiyagarajan and Qian (2008) with minor modification.

5.2.4.1. Sample preparation

For each treatment, 15 small and 5 large individual snails of each species were washed in ddH_2O and blotted dry with a paper towel. The shells were then crushed with a small hammer on a dry paper towel to remove the soft tissues. The digestive gland was removed using forceps and fine scissors and discarded. The remaining tissues were rinsed in Milli-Q water to remove excess digestive contents, salts and shell fragments before freezing at -80°C for further use.

Thawed soft tissues were washed with Milli-Q water, blotted dry with a paper towel and then lysed in a 2-DE buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM dithiothreitol (DTT), and 2% Bio-Lyte 3/10 ampholyte. The contents were then solubilised

with a sonicator (Branson Sonifier, 150) on ice to prevent protein denaturation. The homogenates were centrifuged for 20 minutes at 16 000 g and the supernatants were collected into a new labelled Eppendorf tube and immediately quantified or stored at -80°C until use. The soluble protein concentration was quantified with the 2-D quant kit (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer‟s instructions, and immediately used for 2-DE separation or stored at -80°C until use.

5.2.4.2. Separations

In order to run the first-dimension separation (Isoelectrofocusing, IEF), 500 µg of protein was dissolved in rehydration buffer consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte, 3/10 ampholyte and 1% Bromophenol blue. Sample buffer (200 µl containing 500 µg proteins) was applied to 11 cm ReadyStrip IPG strips (Bio-Rad), pH 3-10 (linear), overnight for active rehydration at 50 volts (V) and then subjected to IEF using a Protean IEF Cell (Bio-Rad). Focussing conditions were as follows: 250 V for 20 min, followed by a linear gradient from 250 V to 8000 V over 2.5 hours, and at 8000 V for a total of 60000 V h. The maximum current did not exceed 50 µA per gel. After IEF, the IPG strips were equilibrated for 20 minutes in equilibration buffer 1 (6 M urea, 2% SDS, 0.05 M Tris-HCL (pH 8.8), 50% glycerol, and 2% w/v 1,4-DTT) followed by another 20 min in buffer 2 (identical to buffer 1, but containing 2.5% iodoacetamide instead of DTT).

For second-dimension separation (2-DE gel electrophoresis), the equilibrated IPG strips were inserted on top of the prepared SDS-polyacrylamide gels (18 cm x 18 cm) and sealed with 0.5% w/v agarose. The running buffer was standard Laemmli buffer for SDS-PAGE (modified using 0.2% w/v SDS). The gels were run at room temperature (20°C) at 200 V until the bromophenol blue (marker) front reached the bottom of the gel. After electrophoresis, 2-DE gels were fixed overnight in 50% methanol and 10% acetic acid to remove SDS. The gels were washed 3 times for 30 minutes with Milli-Q water, and then stained with Coomassie Brilliant Blue G-250 (CBB G-250) for 24 hrs in closed glass containers placed on a shaker. The gels were washed 3 times for 15 minutes each with Milli-

Q water, and then destained with 1% acetic acid and again washed 3 times with Milli-Q water before image acquisition (see below).

5.2.4.3. Image and statistical analysis

The gels were scanned at an optical resolution of 400 dpi using the GS-800 densitometer (Bio-Rad, Hercules, CA, USA), and analysed using the PDQuest software (ver. 8.0; Bio-Rad), which models protein spots mathematically as a three-dimensional Gaussian distribution and determines the maximum absorption after correction of the raw image and background subtraction. Since 5 gels for each treatment, size and species were of different quality (not shown), 3 high quality gels were chosen for further analysis. Automatic spot detection in each gel was verified by visual inspection in order to ensure spots were all properly detected. Spot intensities were normalized using total density values, and then spot analysis was performed using both qualitative and quantitative modes. Spots that displayed significant statistical differences ($p < 0.05$; student‟s *t*-test on PDQuest software) and with 2-fold or greater change in mean volume with respect to the control were considered differentially (up or down regulated) expressed at the total protein level. The spot analysis in this study assumed normal distribution of spot volumes in replicate gels within each group (non-stressed or stressed treatments).

A dendrogram was constructed using square Euclidian distances (using group average, Resemblance: S17 Bray Curtis similarity on PDQuest software) summed over spots and Ward‟s method for all protein spots in 24 samples to estimate similarities in the global expression pattern between the control and the heat treatment gels.

5.3. Results

5.3.1. Two-dimensional gel images of *Afrolittorina* species

Two-dimensional gel images of both size classes of *Afrolittorina* spp. showed that species, size and treatment all had effects on the protein profiles, thus the proteome of the species investigated (see Fig. 5.1.1-4). Hence, there were differences in the proteomes between and within *Afrolittorina* spp. as a function of species, size and treatment (see below). In addition, there was differential (up and down regulation; indicated by arrows and circles) expression of certain protein spots in non-stressed and heat stressed 2-DE gels in both size classes of *Afrolittorina* spp. (see Fig. 5.1.1-4).

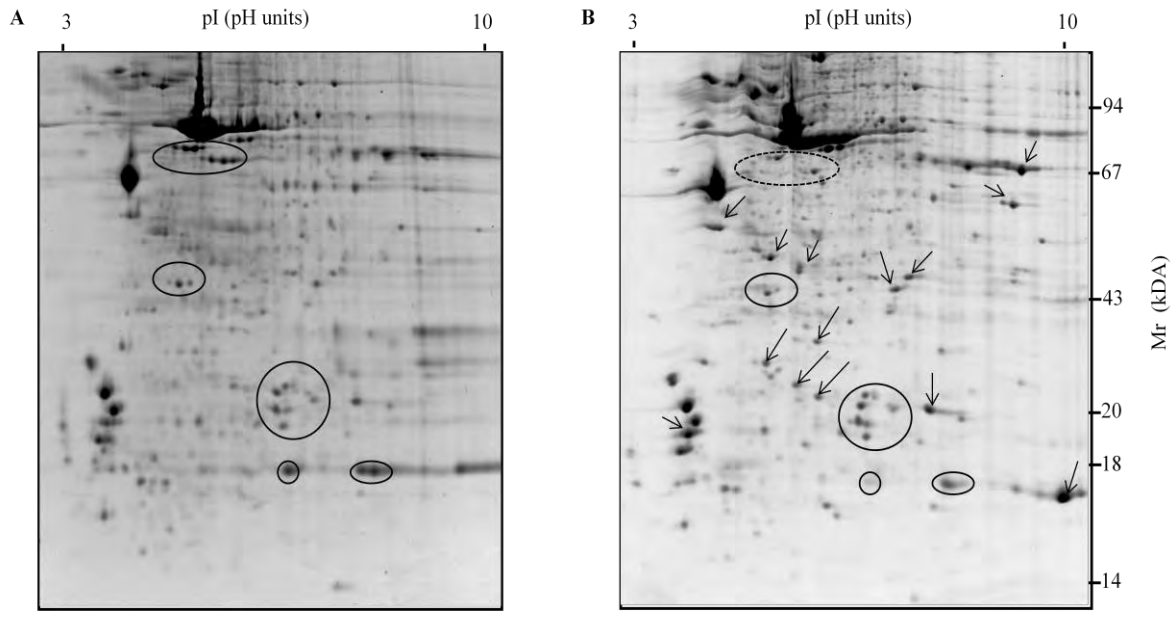

Figure 5.1.1. Representative two-dimensional gel images of (A) non-stressed and (B) heat stressed small individuals of *A. africana*. Arrows and circles indicate protein spots that were differentially expressed between control and treatment groups; dotted circles indicate spots tentatively identified as „Hsps" on the basis of Mr (70 kDA) and pI (pH = 5).

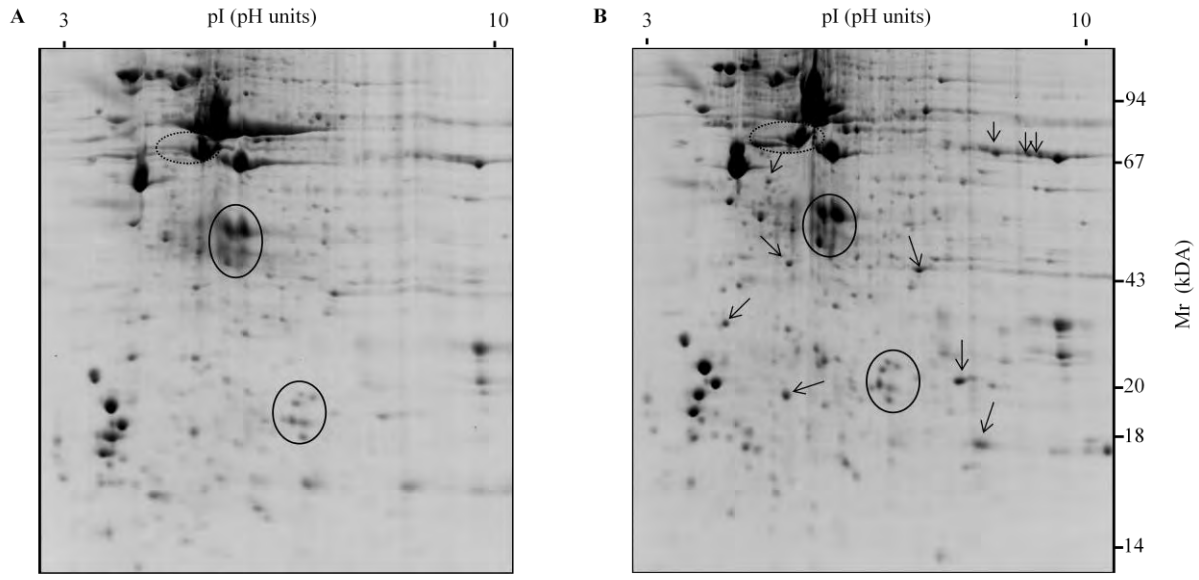

Figure 5.1.2. Representative two-dimensional gel images of (A) non-stressed and (B) heat stressed large individuals of *A. africana*. Arrows and circles indicate protein spots that were differentially expressed between control and treatment groups; dotted circles indicate spots tentatively identified as „Hsps" on the basis of Mr (70 kDA) and pI (pH = 5).

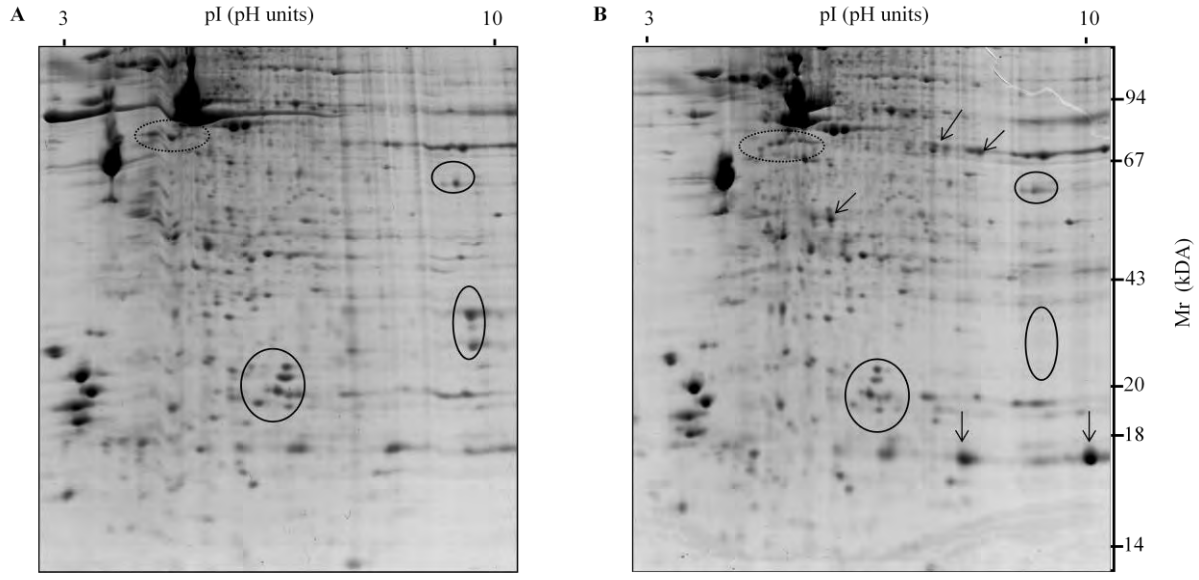

Figure 5.1.3. Representative two-dimensional gel images of (A) non-stressed and (B) heat stressed small individuals of *A. knysnaensis*. Arrows and circles indicate protein spots that were differentially expressed between control and treatment groups; dotted circles indicate spots tentatively identified as „Hsps" on the basis of Mr (70 kDA) and pI (pH = 5).

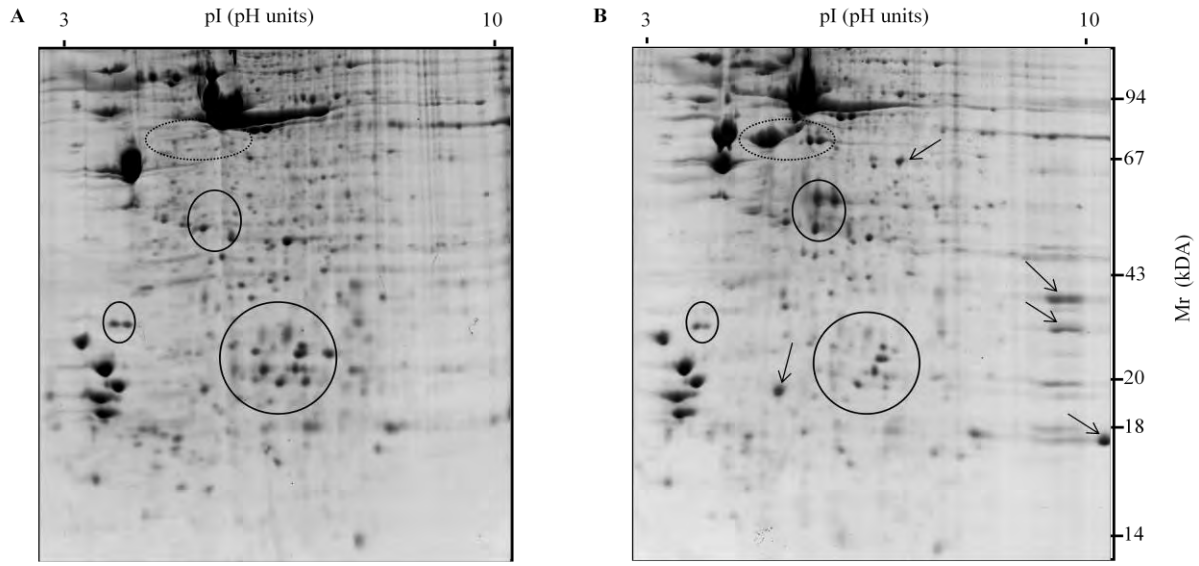

Figure 5.1.4. Representative two-dimensional gel images of (A) non-stressed and (B) heat stressed large individuals of *A. knysnaensis*. Arrows and circles indicate protein spots that were differentially expressed between control and treatment groups; dotted circles indicate spots tentatively identified as „Hsps" on the basis of Mr (70 kDA) and pI (pH = 5).

5.3.2. Protein representation in Afrolittorina spp.

Apart from four non-stressed outliers (two small *A. africana* and one large and one small *A. knysnaensis*; see Fig. 5.2), samples fell into two clear distinct groups (see Fig. 5.2). Each group comprised a single species (*A. africana* or *A. knysnaensis*) with the two size classes largely intermingled, especially in the case of *A. knysnaensis* (see Fig. 5.2A). The single exception was one *A. knysnaensis* (circled in red) individual that was grouped with *A. africana* (see Fig. 5.2). Thus, except for a few samples, there was some grouping according to size and treatment for *A. africana*, while there was no such grouping for *A. knysnaensis* with samples largely interspersed. Furthermore, there was a reasonable degree of inter-individual variation for both species, with grouping occurring around the 50-60% level of similarity (see Fig. 5.2A); also supported by the MDS plot results (see Fig. 5.2B).

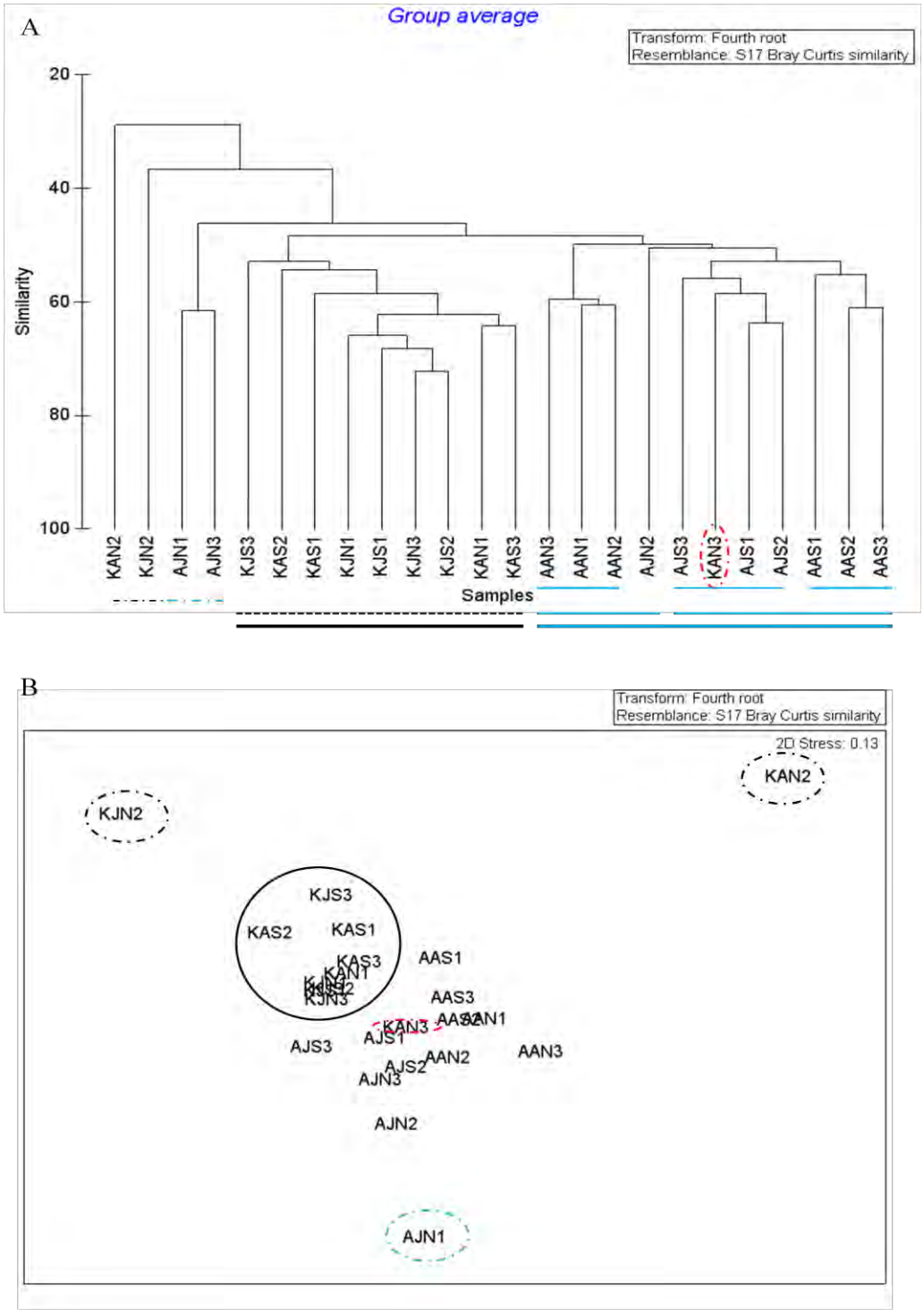

Figure 5.2. A Dendrogram (A) and Non-metric MDS plot (B) showing similarities in the global expression pattern of protein spot volume data of 24 samples. Solid lines indicate grouping according to species (blue for *A. africana* and black for *A. knysnaensis*), treatment or size; blue and red dotted lines and circles indicate where there is no such grouping and/or outliers.

Three-way ANOVA of gel data revealed that the only significant effect was species, with no effect of treatment or size, and no significant interactions (see Table 5.1). Thus, there were clear differences in numbers of protein spots between the two *Afrolittorina* spp., with *A. knysnaensis* showing more protein spots than *A. africana* irrespective of size or treatment (see Table 5.2 and Fig. 5.3). Although the difference was non-significant, small individuals of both groups generally showed higher numbers of spots than large individuals (see Table 5.2 and Fig. 5.3), though non-stressed large individuals of *A. knysnaensis* showed the highest values of all (see Table 5.2 and Fig. 5.3). Except for large individuals of *A. knysnaensis*, for which the non-stressed group showed more protein spots than the stressed group, large and small individuals of stressed groups showed higher spot numbers than non-stressed groups (see Table 5.2 and Fig. 5.3); though the difference was not significant (see Fig. 5.3). However, numbers of differentially expressed proteins were higher for *A. africana* than for *A. knysnaensis* (see Table 5.3 and Fig. 5.4), and this was irrespective of size or treatment.

Table 5.1. Three way-ANOVA results of protein spots for non-stressed and stressed *Afrolittorina* spp. from warm temperate region of South Africa.

Variables	**Degree of Freedom**	**Mean Square**	**F- ratios**	**P values**
Species	1	158113	11.6217	***0.003591***
Size	1	8894	0.6537	0.430654
Treatment	1	20184	1.4836	0.240874
Interactions				
Species*Size	1	25091	1.8442	0.193300
Species*Treatment	1	7704	0.5663	0.462671
Size*Treatment	1	2091	0.1537	0.700226
Species*Size*Treatment	1	5104	0.3752	0.548808

Bold and Italics numbers indicates significant difference.* = interaction/s

Table 5.2. Mean (± SD) number of protein spots for littorinid snails of the genus *Afrolittorina* from the warm temperate region of South Africa.

	A. africana		***A. knysnaensis***	
Treatment	**Large**	**Small**	**Large**	**Small**
Non-stressed	315.00±75.55	428.66±163.47	607.00±125.37	533.00±153.86
Stressed	419.33±12.66	512.00±100.58	581.33±125.37	603.00±78.619

N = 3 for each group

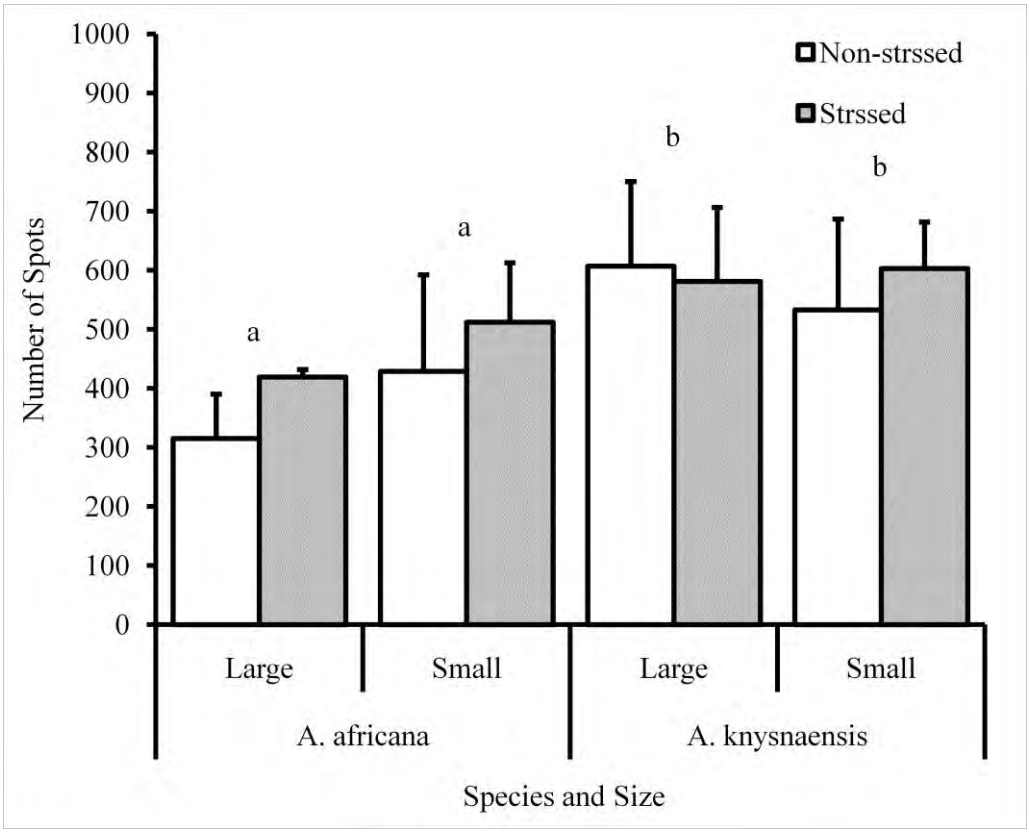

Figure 5.3. Mean number of protein spots for non-stressed and stressed *Afrolittorina* species. Histograms are means plus SD of three replicate gels. Letters indicate homogenous groups as determined using 3-way ANOVA.

In *A. africana*, stressed individuals showed higher protein spot numbers than non-stressed individuals (see Table 5.2 and Fig. 5.3); the differences in mean values being 104 and 84 for large and small individuals, respectively. In addition, small individuals of both groups showed higher numbers of protein spots than large individuals (see Table 5.2). There was slight difference in differential expressed proteins between large and small individuals (see Table 5.3 and Fig. 5.4).

On the other hand, for *A. knysnaensis*, unexpectedly non-stressed large individuals had higher numbers of protein spots (approximately 26) than stressed individuals; while for small individuals the results was as expected, with more protein spots (approximately 70) in stressed than non-stressed individuals (see Table 5.2). Once again in the non-stressed group, large individuals showed higher protein spot numbers than small individuals; while for the stressed group, small individuals showed more protein spots than large individuals (see Table 5.2 and Fig. 5.3). As for *A. africana*, there was a clear difference in differential expressed proteins between large and small individuals (see Table 5.3 and Fig. 5.4).

Table 5.3. Number of differentially (up and down regulated) expressed proteins between non-stressed and stressed *Afrolittorina* spp. from the warm temperate region of South Africa.

	A. africana		***A. knysnaensis***	
Analysis	**Large**	**Small**	**Large**	**Small**
Stati	31	31	20	12
Quali	10	14	9	0

Stati = mean differential expressed protein spots according to Students‟ t-test ($p < 0.05$); Quali = differentially expressed protein spots according to 2 fold or more change only (does not account for replicate variability)

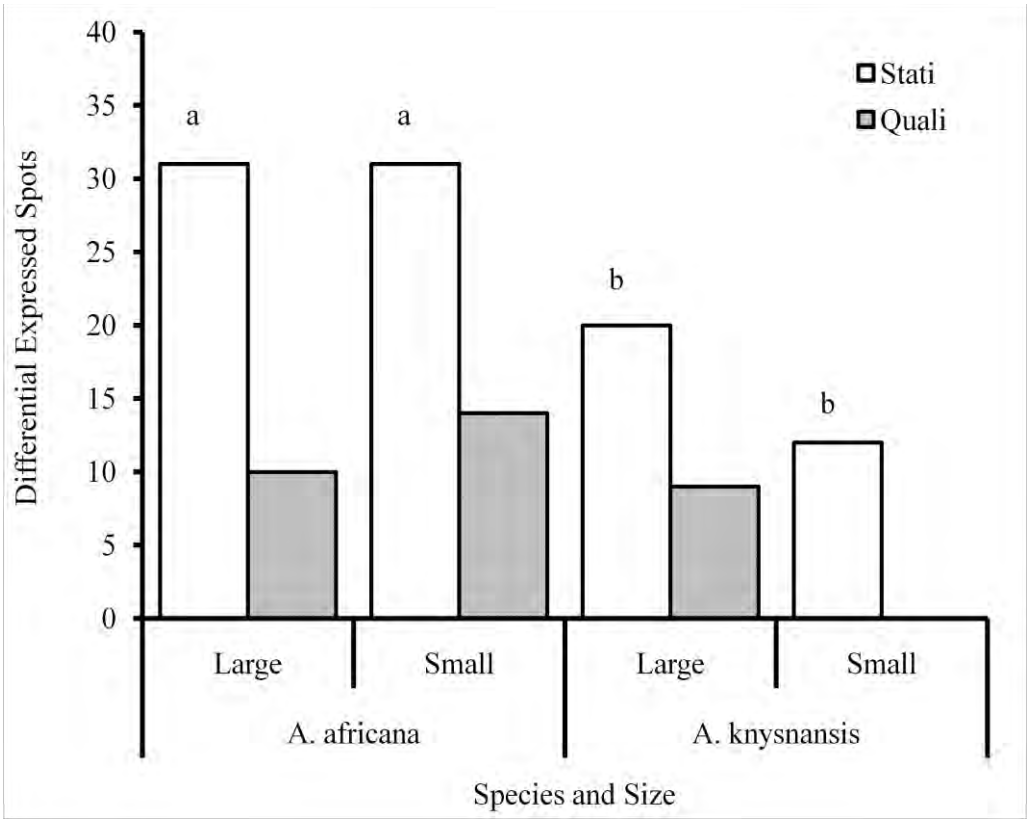

Figure 5.4. Number of differentially (up and down regulated) expressed proteins between non-stressed and stressed *Afrolittorina* species. Histograms are means of differential expressed proteins. Stati = mean differential expressed protein spots according to Students‟ t-test; Quali = differentially expressed protein spots according to 2 fold or more change only (does not account for replicate variability). Letters indicate significant differences between species, based on the statistics data (Students‟ t-test, $p < 0.05$).

5.4. Discussion and conclusion

Environmental temperatures (i.e. changes and extremes) are known to have effects on the physiology and distribution of intertidal animals including littorinid snails (see below). This is because animals‟ physiological processes are influenced by body temperature which is in turn under the control of environmental (i.e. habitat) temperature (see Tomanek and Somero, 1999; Dahlhoff *et al*., 2001; 2002; Helmuth *et al*., 2002; 2010; Pörtner, 2010; Tomanek, 2008; 2010; Somero, 2010; etc). In addition, temperature can penetrate physical barriers and damage the structure of virtually all macromolecules including proteins (see Place and Hofmann, 2001; Hickey and Singer, 2004; Serafini *et al*., 2011; Fields *et al*., 2012a), resulting in cell or whole organism malfunction and/or death. Thus, in addition to its effects on the rate of physiological or biochemical processes, temperature also affects the structure of macromolecules, and in turn plays an important role in limiting the distribution (geographical and vertical) patterns of animals, especially marine intertidal ectotherms.

The effect of temperature on physiology (including protein homeostasis) is important for intertidal animals, particularly littorinids since they live in harsh and fluctuating environments (see McMahon, 1990; 2001b; Jones and Boulding, 1999; Emson *et al*., 2002; Muñoz *et al*., 2005; 2008; Judge *et al*., 2011). In addition, intertidal animals are amongst those expected to experience the strongest impacts of global warming (see Tebaldi *et al*., 2006; Thuiller, 2007; Tomanek, 2008; Stillman and Tagmount, 2009; Provan and Maggs, 2012). Thus, in the light of rapid global warming, the question arises as to how intertidal animals that are already living close to their physiological limits will respond to or deal with temperature changes and extremes in their habitats. Shifts in distribution ranges, and possible extinction are common symptoms of climate change in intertidal ectotherms (see Backeljau *et al*., 2001; Warwick and Turk, 2002; Kassahn *et al*., 2007; Tomanek, 2008; 2012b; Piñeiro *et al*., 2010; Nguyen *et al*., 2011). Thus, some species will show a contraction or expansion in distribution (geographic and within shores) ranges, while others will disappear (go extinct) in response to global warming (see above).

Therefore, knowledge of protein expression patterns or cellular (molecular) stress response is necessary to understand the direct link between climate change and an animal‟s physiological response (see Kültz, 2003; Somero, 2002; 2010; Tomanek, 2008; 2010; 2012a, b; Piñeiro *et al*., 2010; Tepler *et al*., 2011; etc). Although, it is critical to understand how organisms will respond to environmental change and fluctuations due to global warming, differential expression of proteins (proteome plasticity), especially in littorinids in response to temperature change has not yet been explored (see below). This is despite the fact that littorinids and other intertidal ectotherms respond to elevated temperatures (changes or extremes) by launching a heat shock (Hsps) response (see Feder and Hofmann, 1999; Lee and Boulding, 2010; Tomanek, 2008; 2010; Judge *et al*., 2011; Marshall *et al*., 2011, etc).

Recently, two-dimensional electrophoresis (2-DE) based proteomics has emerged as a highly useful tool to study global protein expression patterns following heat and other stress in a variety of animals. This is because 2-DE based proteomics can display differentially expressed proteins between a treatment and a control group or under specific conditions (see above). In addition, the proteome is increasingly being studied to identify key molecules involved in normal physiological pathways (see Kimmel and Bradley, 2001; Gardeström *et al*., 2007; Teranishi and Stillman, 2007; Sørensen, 2010; Tomanek and Zuzow, 2010). A similar approach was used in this study to understand the impact of heat stress on global protein expression pattern in littorinids of the genus *Afrolittorina* from a temperate region.

The two *Afrolittorina* spp. studied here have distinct geographical distribution patterns that can be hypothesized to reflect differences in their proteome and molecular responses to thermal stress. As expected, their proteomes differed, with significantly higher numbers of protein spots in both size classes of *A. knysnaensis* than *A. africana*. This reflects their geographical distributions; *A. africana* extends just into the subtropical parts of the coast, while *A. knysnaensis* is found in the cool temperate region (see McQuaid and Scherman, 1988; Grant and Lang, 1991; McQuaid, 1992; d‟Errico *et al*., 2008). Thus, the differences in geography and the conditions experienced can explain the differences in proteomes found in these species. Although *Afrolittorina* spp. are of temperate origins (see Hartnoll, 1976; Reid, 1989; 2002; Williams *et al*., 2003; Reid and Williams, 2004), the occurrence of *A. africana* in

the subtropical region suggests that it is more tolerant of heat stress than *A. knysnaensis*, which is predominantly found in the cool temperate region.

However, it must be noted that *A. africana* is restricted to lower levels on the shore in the subtropics where it also adopts different habitat use, preferring shallow pools and their edges, suggesting that conditions in this biogeographic region are stressful for it. Therefore, it would be important to investigate the effect of geographical distribution (regions) in the proteomes of these two species since they are found in more than one region, and so adapted to different conditions. Thus, studying the proteome differences between and within species will help explain the proteome differences found in this study. This is supported by studies on ecotypes of the snail *Littorina saxatilis* (Martínez-Fernández *et al.*, 2008; 2010a; Diz *et al*., 2012b), mussels of the genus *Mytilus* (Lopez *et al*., 2001; 2002a; Diz and Skibinski, 2007; Tomanek and Zuzow, 2010) and teleost fishes of the genus *Fundulus* (Rees *et al*., 2010), which showed that species adapted to different habitats (i.e. conditions) have different proteomes. See also below.

The two species overlap extensively in the warm temperate region where they co-exist and use the same microhabitats (see McQuaid and Scherman, 1988, McQuaid, 1992; d'Errico *et al*., 2008). Thus, one could expect a slight or no difference in the proteomes of the two species in the warm temperate region. Results of thermal tolerance (see Chapter 3; McQuaid and Scherman, 1988; Marshall unpub. data) and heart function measurements (see Chapter 4) indicate a small (1-2°C) difference in heat tolerance and endpoint temperatures (EPTs) between these two species in the warm temperate and other regions. Therefore, the greater number of protein spots in the proteomes of in *A. knysnaensis* might be explained by its lower resistance to heat stress than *A. africana* as shown by results for thermal tolerance and heart function.

Situations where species overlap in distribution and show different responses can be explained by different microhabitat use (however, no study has investigated the microhabitat use by these two *Afrolittorina* spp.), but in this case shell colour may also be important as is the case in other animals (see Wilkens and Fingerman, 1965; Markel, 1971; Phifer-Rixey *et*

al., 2008; Miller and Denny, 2011). The brown-black *A. knysnaensis* is expected to absorb more radiation and heat up to a greater degree than the light-coloured *A. africana* (see McQuaid and Scherman, 1988; McQuaid, 1992; 1996a). Even though black or dark bodies are known to absorb a larger fraction of solar radiation, the heat gained remains near the surface and is easily removed by either re-radiation, convection or air cooling (see Britton and Morton, 2003; Phifer-Rixey *et al*., 2008). This might also apply to *A. knysnaensis* since the body temperatures of the two species did not differ despite their colour differences (unpub. data). In addition, one cannot expect differences in heat loads (stress) between the two species due to colour differences since waterbaths were used as the heat source in this study.

Few studies have looked at protein (proteome) changes or expression patterns between closely related species (congeners) which co-exist and prefer the same microhabitats so that they experience similar environmental conditions (see below). For example, Serafini *et al*. (2011) found differences in the proteome of sea squirts of the genus *Ciona* collected from the same level (subtidal) when exposed to acute heat stress, with *C. intenstinalis* showing higher levels of constitutive molecular chaperones than its congener *C. savignyi*. Silvestre *et al*. (2010) found differences in the proteome between the green (34 of 551 detected protein spots showed variation in abundance) and white (9 of 580 detected protein spots showed variation in abundance) larvae of the sturgeon *Acipenser medirostris* reared under the same conditions and exposed to the same heat stress. In Dilly *et al*. (2012), animals of the hydrothermal vent polychaete genus *Paralvinella* showed differences in protein expression patterns, with the extremely thermotolerant species *P. sulfincola* showing upregulation of glutathione and Hsps and downregulation of nicotinamide adenine dinucleotide (NADH) and succinate dehydrogenase, while the cold-adapted congener *P. palmiformis* showed an increase in Hsps only.

Most studies on congeners were done on animals from or adapted to different habitats (geographical or vertical), which therefore experience different conditions, or focused on a single species (see below). In general, animals from warm environments (e.g. tropical and intertidal species) show different proteomes compared to those from cold environments (e.g. temperate and subtidal species). Thus, animals from cold environments show greater changes

in protein expressions profiles than those from warm environments when exposed to the same temperatures (see below; Dilly *et al*., 2012; Serafini *et al*., 2010). For example, Fields *et al*. (2012b) found that protein expression patterns varied among specimens of *Geukensia demissa* from different locations, with 31 out of 448 proteins changing in abundance in the northernmost (Maine) group compared to 5-11 proteins in the four southern groups. Sanders *et al*. (1991) showed that the high intertidal zone limpet *Collisella scabra* showed higher levels of Hsp70 and lower molecular weight Hsps than the congeneric species *C. pelta*, which lives lower on the shore in the upper midtidal. Tomanek and Zuzow (2010) found species-specific changes (which were more pronounced in the cold adapted *Mytilus trossulus* than in the warm adapted *M. galloprovincialis*) in protein expression patterns (e.g. proteins involved in molecular chaperoning, protein degradation, cytoskeleton, energy metabolism, life span, etc.) of two species exposed to acute heat stress. Tomanek (2005) found a decrease in the synthesis of most highly expressed Hsps in the heat-sensitive, low- to subtidal snails *Tegula brunnea* and *T. montereyi* compared to the heat-tolerant mid- to low-intertidal congener *T. funebralis*.

This was also supported by results for Hsps, with animals from hot environments expressing more Hsps than those from cold environments (see Roberts *et al*., 1997; Carpenter and Hofmann, 2000; Fangue *et al*., 2006; Madeira *et al*., 2012c; etc). Thus, congeners or species from different habitats show different protein expression or cellular responses to heat stress. Also see results of genomics and/or transcriptomics studies (see Kassahn *et al*., 2007; Teranishi and Stillman, 2007; Richier *et al*., 2008; Buckley and Somero, 2009; Stillman and Tagmount, 2009; Lockwood *et al*., 2010; Place *et al*., 2012; Schoville *et al*., 2012; etc).

With the exception of non-stressed large individuals of *A. knysnaensis*, small individuals had a greater number of proteins spots in their proteome than did large individuals. This was expected, and is explained by their position on the shore as seen for thermal tolerance (see Chapter 3). Small individuals (assumed to be juveniles) of *Afrolittorina* spp. are found lower on the shore where they are frequently wetted by incoming tides while large individuals (assumed to be adults) occupy higher shore levels and are only wetted by wave splash during high tides. Thus, the greater number of protein spots in the proteome of juveniles/small individuals in this study might be explained by the synthesis of inducible Hsps. On the other

hand, adults, which are found at the highest levels on the shore might benefit from constitutive Hsps with no need to synthesise new Hsps during heating. This predicts differences between size classes in protein expression in response to heat stress, which was the case in this study. Small animals showed a greater difference between control and treatment individuals than large animals (see Table 3.2 and Fig. 5.3).

However, juveniles are not always found lower on the shore, nor are adults always higher (see Vermeij, 1972; Boulding and Van Alstyne, 1993; Saier, 2000; Emson *et al.*, 2002). For example, juveniles of *A. knysnaensis* have been described as generally occurring higher on the shore than adults (McQuaid, 1981a, b; d‟Errico *et al*., 2008), though this was in the cool temperate region where heat stress may be less critical and was assumed to relate to wave action. Since it was not investigated if small individuals are sexually mature or not, my results need to be treated with caution. If adults are indeed found higher on the shore than juveniles, the results suggest that the basis for resisting heat stress may differ between large and small individuals. Such size-specific differences may thus either account for or reflect different distribution patterns on the shore.

Alternatively, the perception or experience of heat stress may differ with size. For example, one size class (adults in this case) may experience stress and respond, while the other (juveniles) does not experience the stress and so does not show a proteomic response. Since no study has looked at the effect of size, it will be premature at this stage to make conclusions about size-specific differences in proteome response to heat stress. However, it must be noted that animals show changes in proteome during development or ontogeny (see above; Sveinsdóttir *et al*., 2008; Diz *et al*., 2012b). Thus, an animal‟s proteome may change as early as the first stage of development with continued change through acclimatization of the adult and adaptation of the following generations (see Tomanek, 2010; 2012a).

The greater number of protein spots in the proteome of non-stressed large individuals of *A. knysnaensis* (true results) than for both classes and groups was unexpected, and warrants further investigation. This might be explained by the fact that high intertidal animals such as littorinids (in this case large individuals) show or maintain high constitutive Hsps (see

Sanders *et al*., 1991; Robert *et al*., 1997; Nakano and Iwama, 2002; Sorte and Hofmann, 2005; Berger and Emlet, 2007; etc) and this can be true for other proteins. Though energetically expensive, expression of constitutive Hsps (also expressed during normal conditions) may protect existing protein pools during periods of acute and chronic stress, and thus reduce the subsequent higher costs of *de novo* protein synthesis (see Somero, 2002; Halpin *et al*. 2004; Podrabsky and Somero, 2007; Dong *et al*., 2008a; Sørensen, 2010). Therefore, adults of *A. knysnaensis* might employ a strategy of maintaining a greater number of constitutive Hsps and other stress proteins than juveniles and both sizes of *A. africana*.

It appeared that the protein expression pattern was affected by heat stress (see Fig. 5.1.1-4 and 5.4). Thus, subsequent statistical analysis revealed dramatic heat stress-dependent changes in several protein spots, the magnitude of which was greater in both classes of *A. africana* than for *A. knysnaensis*. Unfortunately, none of these differentially expressed proteins were excised from the gels and identified. Among these differentially expressed proteins, some were downregulated and others were upregulated (see Fig. 5.1.1-4). The downregulated and upregulated heat responsive proteins can be treated as part of protein expression signatures (PES), and could help us to understand the molecular or cellular responses to heat stress in these species and other littorinids or ectotherms.

Intertidal ectotherms are subjected to thermal and desiccation stress, and at temperatures near the upper limit of thermotolerance, stress proteins, in particular Hsps, are the major proteins synthesised. Thus, during heat stress, Hsps (mostly inducible) are rapidly synthesised and are responsible for the protection of protein homeostasis (see Feder and Hofmann, 1999; Sørensen *et al*., 2003; Tomanek, 2002; 2008; 2010; Sørensen, 2010; Zerebecki and Sorte, 2011). This is because heat shock proteins are molecular chaperons that prevent aggregation of heat-damaged proteins and facilitate their renaturation or removal following a heat shock (see Feder, 1999; Feder and Hofmann, 1999; Sørensen *et al*., 2003; Sørensen, 2010). Heat shock proteins are also involved in thermal tolerance and acclimation (see Buchner, 1996; Krebs and Bettencourt, 1999; González-Riopedre *et al*., 2007; Dong and Dong, 2008) through stabilization, protection and repair of macromolecular structure and function (see Place and Hofmann, 2001; Pörtner, 2002b; Kültz, 2003; Meistertzheim *et al*., 2007; Podrabsky and Somero, 2007), and as such need to be upregulated during exposure to

sublethal temperatures. In that case, one would expect *Afrolittorina* species to upregulate Hsps and other stress proteins following acclimation as seen in other studies (see Kültz and Somero, 1996; Tomanek; 2005; McLean *et al*., 2007; Ibarz *et al*., 2010; Tomanek and Zuzow, 2010; Fields *et al*., 2012a). Therefore, it would be important to investigate the effect of acclimation, especially season, on the proteome and/or heat shock response of *Afrolittorina* spp. and other littorinids. This will help explain why littorinids in this study showed little and/or failed to acclimate both thermal tolerances and metabolic rates (see Chapter 3 and 4).

Studies using proteomics have also shown that various marine animals including intertidal ectotherms upregulate Hsps (e.g. Hsp70 or 90) in response to heat stress (see below). For example, Sanders *et al*. (1991) found that the intertidal limpets *Collisella scabra* and *C. pelta* showed upregulation of Hsp70 and lower molecular weight Hsps in response to heat (acute or chronic) stress. In Tomanek and Zuzow (2010), the cold tolerant *Mytilus trossulus* upregulated three Hsp70 isoforms at 32°C, while the warm adapted *M. galloprovincialis* did not. In addition, *M. trossulus* showed increasing levels of several Hsp70 and small Hsps isoforms at lower temperatures than *M. galloprovincialis*. This is also supported by results on genomics or transcriptomics (see Lund *et al*., 2002; Buckley *et al*., 2004; 2006; Buckley and Somero, 2009; Stillman and Tagmount, 2009; Truebano *et al*., 2010; Lockwood *et al*., 2012; etc) studies. For example, Clarke *et al*. (2008) found that the bivalve *Laternula elliptica* and the gastropod *Nucella concinna* significantly upregulated the Hsp70 gene in response to increased temperatures. Logan and Somero (2011) also found that the eurythermal fish *Gillichthys mirabilis* acclimated at different temperatures upregulated the Hsp70 gene in response to acute heat stress.

Although proteins (e.g. Hsps) were only tentatively identified in this study by looking at the molecular weight of the spots (70 kDA) and their isoelectric point (pH of 5) see Fig. 5.1.1-4, spots provisionally identified as Hsps and other stress related proteins were significantly upregulated in response to heat stress. Such upregulation is likely to offer protection to heat exposed animals, although intertidal animals generally have high levels of constitutive Hsps, and so do not need to synthesize new Hsps (see above). Thus, littorinids could benefit from constitutive Hsps as a long-term response to heat stress. Only future investigations can confirm the nature of this hypothetical correlation between high production of Hsps or stress

proteins and tolerance or response to heat stress. This could be done by investigation of the heat shock response using the widely used Hsps (e.g. Hsp70/90) methods (see above).

Along with these heat stress related proteins, expression of other proteins such as chaperones, metabolic and cytoskeleton proteins might also change substantially in response to heat stress, as is the case in other studies (see below; Gardeström *et al*., 2007; Tomanek, 2010; Tomanek and Zuzow, 2010). Serafini *et al*. (2010) found changes in a number of protein functional groups, with cytoskeleton proteins showing higher levels of expression in *Ciona intenstinalis* than *C. savignyi* after exposure to heat stress. Fields *et al*. (2012b) found that warm and cold acclimation caused changes in cytoskeleton, energy metabolism, oxidative stress, and chaperone proteins in the mussels *Mytilus trossulus* and *M. galloprovincialis*. In addition, cold-adapted *M. trossulus* showed increased abundances of chaperone proteins at 19°C, while warm-adapted *M. galloprovincialis* did not. Silvestre *et al*. (2010) found that the proteins involved in protein folding and degradation, energy supply and structural proteins showed increased abundance in the larval stages of the sturgeon *Acipenser medirostris*, while those involved in the synthesis of proteins other than Hsps showed a decrease in abundance. Overall, my results suggest that both stress and other proteins (e.g. metabolism and cytoskeleton related proteins) are strongly induced by heat stress.

On the other hand, proteins that are not involved in maintenance or protection of animals from heat are likely to be suppressed under heat stress. In fact, severe heat shock involves a variety of other effects including suppression of protein synthesis other than Hsps production (see Roelofs *et al*., 2008; Tomanek, 2012a, b). This is because production of such proteins can be energetically expensive (see Somero, 2002; Clarke, 2003; Stillman, 2002; Sokolova *et al*., 2012; Fitzgerald-Dehoog *et al*. 2012) so that animals need to suppress or cease their synthesis to save energy. Unsurprisingly, certain protein spots were down regulated or completely disappeared after heat exposure (see B in comparison with A in Fig. 5.1.1-4). Such down regulation may be associated with metabolic depression in response to heat stress.

There are many strategies that marine organisms employ to tolerate heat stress, and one of these involves metabolic depression. For instance, littorinids tend to depress their metabolic

rates and energy metabolism in response to heat stress (see Chapter 4; Sokolova and Pörtner, 2001b; 2003; Marshall and McQuaid, 2010; Marshall *et al*, 2010; 2011). In other marine species, genes or proteins involved in metabolic, energy production, cell growth and proliferation, protein biosynthesis and cell cycle arrest or apoptosis pathways were down regulated in response to heat stress (Buckley *et al*., 2006; Gracey *et al*., 2008; Boutet *et al*., 2009; Tomanek and Zuzow, 2010; Dilly *et al*., 2012; Fields *et al*., 2012a; Silvestre *et al*., 2012; etc). Therefore, one would expect the down regulation of a large number of proteins in response to heat stress in order to conserve energy as one of several compensatory responses. My results provide preliminary evidence to support this hypothesis.

In summary, there were differences in global proteins between and within species, with *A. knysnaensis* showing higher protein expression than *A. africana* of both size classes. This suggests differences (distinct) in molecular strategies, and thus the cost of living, used by the two species to survive heat stress in the littoral zone. I predict that *A. knysnaensis* might employ a strategy of maintaining higher constitutive Hsps and other stress related proteins, while *A. africana* synthesize or induce more Hsps when exposed to heat stress. Of interest was the differential expression of certain proteins (the magnitude of which was greater in both sizes of *A. africana* though the proteins were not identified), after exposure to heat stress. Importantly, expression of other proteins appears to be suppressed; however, further *in vitro* tests should be performed to confirm this finding. Nevertheless, demonstrated differential expression patterns of proteins appear to be a part of biochemical compensatory mechanisms (a short-term adaptive response) in littorinids to heat stress. Thus, to cope with heat stress, intertidal animals could adjust the expression patterns of proteins as a short-term adaptation (i.e. acclimation). This is a common strategy adopted by organisms to tolerate abiotic stressors, known as a "plastic proteome response" (see López *et al.* 2001; McLean *et al*., 2007; Silvestre *et al*., 2012; Tomanek, 2012a, b).

In addition, the differentially expressed proteins might allow these species and other littorinids or intertidal ectotherms to survive environmental (temperature) change during climate change. However, for firm conclusions and to test this hypothesis, future studies should identify and confirm the functional role of heat stress responsive proteins (e.g. Hsps and other stress proteins or enzymes) in stress tolerance. Rapid advances in proteomic studies

in non-model marine organisms have just begun to uncover the proteins that are plastic and are responsible for stress tolerance. Thus, the application of proteome maps generated using conventional 2-DE technique and recent advances in mass spectrometry for protein identification will provide insights into the effects of environmental temperature change on animals at molecular levels. This will be complimented by developments and advances in sequencing animals‟ genomes (see Galindo *et al*., 2010; Canbäck *et al*., 2012). This study therefore underlines the importance of proteomics as a tool in environmental change. In fact the concept has already been employed in a number of studies in marine gastropods such as snails and bivalves (see Gardeström *et al*., 2007; Joyner-Matos *et al*., 2009; Tomanek and Zuzow, 2010; Fields *et al*., 2012a, b; etc), crustaceans (Kimmel and Bradley, 2001; Wang *et al*., 2007a; Serafini *et al*., 2011; Dilly *et al*., 2012; etc), fishes (Kültz and Somero, 1996; McLean *et al*., 2007; Ibarz *et al*., 2010; Silvestre *et al*., 2012; etc).

CHAPTER 6: Synthesis

Temperature is the main driving force behind many ecological processes, and many studies have established the effects of temperature (changes and extremes) on the distribution and abundance as well as the phenology and physiology of animals, including marine ectotherms (Miller, 2006; Calosi *et al*., 2008; Harley *et al*., 2009; Somero, 2010; 2011; 2012; Walther, 2010; Chapperon and Seuront, 2012; etc). This is particularly important given the problem of "global warming" with climate change related rises in temperature (air and sea surface) and extreme heat events already threatening biodiversity (Fields *et al*., 1993; Clark *et al*., 2000; Thuiller, 2007; Omann *et al*., 2009) and this has been linked to the contraction or expansion of distribution ranges and local extinctions (Barry *et al*., 1992; Backeljau *et al*., 2001; Walther *et al.,* 2002; Clark, 2006; Sorte *et al*., 2010; etc).

As such, there is a need to understand the physiological and behavioural responses (i.e. adaptations) of animals to perturbations in environmental temperature, as well as the biochemical mechanisms that allow these responses. Thus, it is important to understand the mechanisms that determine an organism"s thermal niche, especially in the case of ectotherms whose body temperature (and thus performance) is determined by that of the environment (Helmuth *et al*., 2002; 2006a, b; 2010; Sørensen and Loeschcke, 2007; Pincebourde *et al*., 2008; Lima *et al*., 2011; etc). This is particularly true for littorinids as they live in harsh and fluctuating environments (i.e. the extreme interface between land and sea), often living close to their physiological limits, and temperature gradients generally correlate with species distributions and abundances (McMahon, 1990; 2001b; Jones and Boulding, 1999; Emson *et al*., 2002; Muñoz *et al.*, 2005; 2008; Judge *et al*., 2009; 2011; etc).

Temperature pervasive effects on physiological and biochemical systems are reflected in the suite of temperature adaptive differences observed among species and/or populations from different thermal niches, such as species with different distributions along the subtidal to intertidal or tropical to polar gradients (McMahon, 1990; 2001b; Stillman and Somero, 1996; 1999; Sokolova and Pörtner, 2001b; 2003; Somero, 2002; 2010; Helmuth *et al*., 2002; etc).

Whole organism thermal tolerance, metabolic rates and protein synthesis are amongst the physiological and biochemical traits that exhibit adaptive variation related to distributions; and some, but not all, of these thermally sensitive traits show acclimation (adjustment), which leads to adaptive shifts in thermal optima and limits or thresholds (Markel, 1974; Fields *et al*., 1993; Stenseng *et al*., 2005; Calosi *et al*., 2008; Feidantsis *et al*., 2009; Tomanek, 2008; etc).

This study used different approaches to investigate physiological and biochemical responses to heat stress in littorinid snails from different latitudes, regions and shore levels. An understanding of animals‟ temperature tolerances, and the plasticity or flexibility of those tolerances enables us to make inferences about what will happen to their distributions and abundances during climate change. In addition, the genetic diversity of *Afrolittorina* spp. was also investigated as it is accepted that marine animals and plants with low genetic diversity will be more threatened by the effects of global warming than those with high genetic diversity (Fields *et al*., 1993; Visser, 2008; Hoffman and Willi, 2008; Brierley and Kingsford, 2009; Provan and Maggs, 2012). Thus, genetic (high) diversity might buffer the effects of global warming by providing resilience (Ehlers *et al*., 2008; Bergmann *et al*., 2010; Barshis *et al*., 2010), but this needs to be proven or investigated. If fact, fitness in heterogeneous and/or uncertain environments is positively correlated with higher heterozygosity, thus genetic diversity (Nevo, 1978; Noy *et al*., 1987; Hawkins, 1995; Laudien *et al*., 2003; Schmidt *et al*., 2007). Thus, species with adequate genetic diversity or variation to generate phenotypes (Pigliucci *et al*., 2006) with different tolerances and optima, may be „winners‟ during global warming (Somero, 2010).

The results indicate differences in the physiological and biochemical responses of the study species to heat stress that seem to relate to differences in biogeography, phylogeny and species ecology. Thus, thermal tolerance, heart function and proteomics data indicate that there are inter- and intraspecific differences in the responses to heat stress of littorinid snails as a result of temperature adaptive differences amongst species and/or populations from different habitats. The tropical and subtropical species, which occupy the eulittoral fringe, showed higher tolerances (thresholds and limits) than the subtropical/temperate species which are found in the eulittoral zones. This was supported by heart performance, with tropical species showing good metabolic regulation followed by the subtropical and temperate

species, respectively. This agrees with the hypothesis that temperature tolerances in marine animals show a decrease from the tropics to polar regions in both eulittoral fringe and lower shore species (McMahon, 1990; 2001b; Calosi *et al*., 2008; Somero, 2010; Sorte *et al*., 2011; Sunday *et al*., 2012).

However, there were few or no differences in the thresholds and limits or the heart performance of *Afrolittorina* spp. from different regions. On the other hand, the proteome results for these *Afrolittorina* spp. showed differences in global proteins between and within species, with both size classes of *A. knysnaensis* showing higher protein expression than those for *A. africana*. However, differential expression of certain proteins after exposure to heat stress was greater in both size classes of *A. africana* than in *A. knysnaensis*. This suggests differences in molecular strategies, and thus the cost of living, used by these two species to survive heat stress in the littoral zone. The two congeners seem therefore to utilise different approaches for resisting heat stress. *A. knysnaensis*, with a distributional range into generally cooler conditions seems to employ a strategy of maintaining higher constitutive Hsps and other stress related proteins, while *A. africana*, which extends into warmer conditions, synthesizes or induces more Hsps when exposed to heat stress. This reflects the fact that the temperate/subtropical species is better equipped to cope with high temperatures than the temperate species.

I have shown that diversity in the metabolic rates, thresholds and/or limits as well as proteomes in this study is related to evolutionary adaptive responses to probable maximal habitat temperatures. Thus, latitudinal and vertical temperature adaptations lead to shifts in limit and threshold temperatures. Differences or shifts in limits and thresholds have been found to reflect evolutionary adaptations in other animals, including littorinids (Dahlhoff *et al*., 1991; Stillman and Somero, 1996; 1999; Stenseng *et al*.; 2005; Braby and Somero, 2006). The same is true for protein profiles or proteomes (López *et al*., 2002a; Diz and Skibinski, 2007; Martínez-Fernández *et al.*, 2008; 2010b; Diz *et al*., 2012b). The threshold and limit temperatures of the study species were several degrees above current maximum air temperatures, and well above predicted climatically derived estimates of global warming. This suggests that the study species live far from their upper thermal tolerance limits and that the current global warming trend is still unlikely to be dangerous to them.

Predictions of climate change are traditionally derived from modelled changes in air and sea surface temperatures which in the case of high shore littorinids and other ectotherms may be misleading as much of their heat can be derived from either contact with the substratum or from solar radiation (Britton and Morton, 2003; Broitman *et al*., 2009; Miller and Denny, 2011; Zippy and Helmuth, 2012). Thus, air and sea surface temperatures are largely irrelevant to high shore animals‟ body temperature in the field, but higher solar or UV radiation and substratum temperatures are important (Wethey, 2002; Clark, 2006; Gilman *et al*., 2006; Helmuth *et al*., 2006a, b; 2011; Chapperon and Seuront, 2011a, b).

By demonstrating the existence of fixed physiological and biochemical differences between species from different geographic regions, this study provides evidence that environmental temperature adaptation at the organismal, physiological and molecular levels is important for the maintenance of dissimilar biogeographies, and likewise for distribution among shore levels. The results also confirm that littorinids can tolerate high temperature stress, and are therefore well suited to life in the intertidal zones where temperature and other stresses are extreme and can change abruptly. In the short term, littorinids are tolerant of the high temperatures that they are likely to experience on the shore, and can also survive temporary exposure to supernormal temperatures. In fact, littorinids can regulate their metabolism within the sub-lethal temperature range experienced under natural conditions (this study; Sokolova and Pörtner, 2003; Marshall and McQuaid, 2010; Marshall *et al*., 2010; 2011) and can adjust the expression of proteins (e.g. Hsps) as part of a biochemical response to heat stress (this study; Lee and Boulding, 2010; Judge *et al*., 2011; Marshall *et al.*, 2011).

The present study reveals that both the stresses found and mechanisms or strategies utilized in physiological and biochemical adaptations to high temperature exposure by subtropical and temperate littorinids are similar to those utilized by the littorinids from the tropics. Therefore, high thermal tolerance, metabolic depression and/or regulation as well as the induction of heat shock proteins as temperature increases are physiological and biochemical adaptations of this group of marine animals for life high in the intertidal zone, allowing them to live higher than almost all other marine organisms.

Although my results suggest that littorinids have high tolerances to temperature, it is clear that these animals are already living close to their thermal limits as shown by their limited capacity to adjust those tolerances, and the fact that distribution within-shore alters with region. Thus, in the event of global warming and climate change related rise in solar radiation hence substratum temperature, the distribution of littorinids and other intertidal ectotherms may be more affected than those of subtidal ones. Recent studies suggest that some intertidal animals may have very low plasticity in their ability to acclimate to higher temperatures (Stillman and Somero, 1999; Tomanek and Helmuth, 2000; Stillman, 2002; 2003; 2004; Somero, 2005; 2009; 2010; 2011). Because littorinid snails regularly experience high temperatures for extended periods, they may be unable to further adjust their physiological and biochemical response as seen in this study, and as such may be more affected by global warming. However, littorinids and other intertidal ectotherms might benefit from behavioural and morphological adjustments (see below).

If the upper shore limit of the study species is a consequence of increased aerial body temperature, then we should observe downwards shifts in their upper limits of zonation when or where aerial temperatures increase, while in terms of geographical distribution, we should expect poleward shifts. In fact, the zonation patterns of marine animals are primarily influenced by environmental gradients and it has been suggested that the upper vertical limits of intertidal organisms are inversely correlated with temperature (McMahon, 1990; Charles *et al.*, 1992; Warwick and Turk, 2002; Harley and Helmuth, 2003; Miller and Denny, 2011). *A. africana* is already restricted to the low shore in the subtropics, where it even prefers temporary and shallow pools (pers. obs.). In the tropics, *E. vidua* is mostly found in the lowermost eulittoral fringe, and together with *E. malaccana*, which inhabits the eulittoral fringes, these species migrate to lower levels (e.g. eulittoral zones) during hot summer months where they even prefer biogenic habitats and form aggregations during low tide (GA Williams and DJ Marshall pers. comm.; Williams *et al*., 2011; Cartwright and Williams, 2012; Stafford *et al*., 2012). However, no study has investigated the vertical distribution and abundance as well as behavioural responses of *Afrolittorina* spp., *E. natalensis* and *L. glabrata* or other littorinids found in South Africa.

Increased temperatures could not only reduce the vertical distribution of *Afrolittorina* spp., but could also reduce their eastern and western biogeographic limits. In addition, these species showed low genetic diversity within and among populations, suggesting their vulnerability to the effects of rising temperatures as suggested (see above). On the other hand, the southern limits of *Littoraria glabrata* (with low genetic diversity; Silva *et al*., 2013) and *Echinolittorina natalensis* might extend polewards to the warm temperate region. At the moment, *E. natalensis* and *L. glabrata* are found as far south as the vicinity of East London in the warm temperate region. Likewise, *Echinolittorina malaccana* and *E. vidua* and other tropical littorinids might extend further into the subtropics.

There are already indications that climate change will favour the poleward spread of species characteristic of warmer temperature regimes (Schiel *et al*., 2004; Lima *et al*., 2007; Sorte *et al*., 2010; Xavier *et al*., 2010), but such generalization should be made with caution since animals respond differently (Helmuth *et al*., 2002; Rivadeneira and Fernández, 2005; Poloczanska *et al*., 2008; Provan and Maggs, 2012). Thus, animals or populations may respond differently (with „winners" and „losers"; see Somero, 2010; Lucas and Griffiths, 2012) to climate change owing to additional local environmental effects, interspecific ecological interactions and dispersal capacity (Fields *et al*., 1993; Genner *et al*., 2003; Angilletta Jr. *et al*., 2006; Byrne *et al*., 2010; Kordas *et al*., 2011), amongst others.

For example, upwelling (which might intensify in the future) and/or near-shore cold waters in the south coast of South Africa (Bakun, 1990; Clark *et al*., 2000; Riegl, 2003; Harrison and Whitfield, 2006; Lucas and Griffiths, 2012) might limit *E. natalensis* and *L. glabrata* from extending further into the warm temperate region. In addition, it is likely that populations or species that are already well established in other regions, but near their tolerance limits, will be as negatively affected by increasing temperatures and related environmental changes as local ones (see Branch 1984 in Clark, 2006; Lima *et al*., 2007; Lucas and Griffiths, 2012). In fact, animals or species are assumed to be more stressed and/or have decreased performance at the edges of their distribution ranges (see Somero and Hofmann, 2004; Osovitz and Hofmann, 2007; Roelofs *et al*., 2008; Wernberg *et al*., 2011; Zippay and Helmuth, 2012) and they are more vulnerable to the effects of climate change. In addition, these animals are

critically important in determining species‟ responses to climate change (see Hampe and Petit, 2005; Ehlers *et al*., 2008; Provan and Maggs, 2012).

Fossil evidence and distributional surveys show that biogeographic range shifts are associated with climate change in marine environments as in terrestrial environments (Clarke *et al*., 1992; Sagarin *et al*., 1999; Schiel *et al*., 2004; Mieszkowska *et al*., 2006; Sunday *et al*., 2012; etc). There have been dramatic changes in the distribution of South African commercially fished stocks such as pilchards, anchovy and sardines which have shifted from the west coast to the south coast, while tropical and subtropical fishes are moving southwards (Clark *et al*., 2000; Clark, 2006; Lucas and Griffiths, 2012). Similar changes have been noted in other animals such as mussels, crabs, rock lobsters, seals, seabirds and various zooplankton species as well as plants such as kelps, algae, seaweeds, etc. (Shannon *et al*., 1988; Clark *et al*., 2000; Clark, 2006; Crawford *et al*., 2008; Lucas and Griffiths, 2012). Thus, South Africa‟s marine organisms have been/are moving as climate change warms the Agulhas current in the east coast, and cools the southern Benguela upwelling system on the west coast and the near-shore south coast marine environment (Clark *et al*., 2000; Lucas and Griffiths, 2012).

In addition, changes in temperature can alter species‟ co-existence equilibria and modify species distributions as a result of changes in the outcomes of their interactions (Southward *et al*., 1995, Schneider and Helmuth, 2007; Doney *et al*., 2012; Zippay and Helmuth, 2012). *A. africana* and *A. knysnaensis* co-exist in the warm temperate region, where they even occupy the same microhabitats (pers. obs.; McQuaid, 1992; d‟Errico *et al*., 2008). Therefore, under global warming, there might be changes in their co-existence equilibria and distributions, with the possibility of *A. knysnaensis* being restricted to lower (cooler) shore levels than *A. africana* which might remain in the upper (hot) levels. *A. knysnaensis* is already restricted to low shores towards its eastern biogeographic limits (East London to the vicinity of Durban), while *A. africana* occupies higher levels in the same shores (pers. obs.; McQuaid and Scherman, 1988; McQuaid, 1992; d‟Errico *et al*., 2008). Thus, differences in thermal physiology between these two species suggest that *A. africana* may have competitive advantages over the less heat tolerant *A. knysnaensis* during global warming. This might be true for *E. natalensis* and *L. glabrata* as well as other littorinids that co-exist in the

subtropics. In fact, warm-adapted genotypes (i.e. animals) happen to outperform cold-adapted genotypes (see Asbury and Angilletta Jr., 2010; Angilletta Jr. *et al*., 2010).

The outcomes of this study yielded novel insights which could advance our knowledge of the responses of littorinids and other ectotherms to predicted environmental temperatures, changes and extremes. The findings underline the importance of integrating information from different levels and disciplines in order to understand the responses of animals to climate change (Clarke and Crame, 1992; Guderley and St-Pierre, 2002; Pörtner *et al*., 2006; Moore *et al*., 2007; Chapman *et al*., 2011; etc). Integration of multidisciplinary and integrative approaches will provide considerable potential advances in the understanding of animals‟ responses to climate change (Osovitz and Hofmann, 2007; Sørensen and Loeschcke, 2007; Somero, 2010; 2011; 2012; Walther, 2010). For example, studies are focusing on behavioural and morphological responses as thermoregulatory mechanisms additional to physiological and biochemical mechanisms that animals might use to survive and/or buffer the effect of rising temperatures (Kearney *et al*., 2009; Chapperon and Seuront, 2011a, b; Miller and Denney, 2011; Tuomainen and Candolin, 2011; Zippay and Helmuth, 2012).

This is also true for biotic interactions which might modulate species‟ responses to climate change (Pearson and Dawson, 2003; Moore *et al*., 2007; Hawkins *et al*., 2008; Poloczanska *et al*., 2008; Chapperon and Seuront, 2011a, b) and/or influence the net benefits of behavioural and physiological responses (Angilletta Jr. *et al*., 2006; Helmuth *et al*., 2006b). Of most importance is the study of thermal ecology of intertidal animals (Helmuth *et al*., 2002; 2006a, b; 2010a, b; 2011; Gilman *et al*., 2006; Wethey and Woodin, 2008; Finke *et al*., 2009; Lima *et al*., 2011; etc), as this might help us to understand and predict the responses of animals to rising temperatures. In addition, studies are also looking at the responses of early life stages (e.g. eggs and larvae) to climate change as these are considered to be more vulnerable to environmental changes (Coelho *et al*., 2000; Przeslawski *et al*., 2005; 2008; Byrne *et al*., 2009; 2010; Parker *et al*., 2009; Walther *et al*., 2010), while most existing studies have been done on late (adult) stages. Particularly important will be the need to investigate the effects of multiple interacting factors that occur in nature (Harley *et al*., 2006; Pörtner, 2008; Häder *et al*., 2007; Somero; 2011; Zippay and Helmuth, 2012).

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Appendices

Appendix 2.1. Representatives of mitochondrial (mtCOI) and ribosomal (28S rRNA) nucleotides sequences for *A. africana* and *A. knysnaensis*.

2.1.1. mtCO1 sequences

>A.africana 615 bases
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2.1.2. 28S rRNA Sequences

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>MB_03 745 bases

Appendix 2.2. List of mitochondrial (mtCOI) and ribosomal (28S rRNA) sequences (excluding singlets; see Figure 2.2-2.3) of *A. africana* and *A. knysnaensis*.

mtCOI	**28S rRNA**
A.knysnaensis_BA_1_BA_4_BL_10_BL_8_BR_03_BR_05_BR_101_BR_2_BR_3_BR_4_BR_6_CA_01_CA_011_CA_012_CA_02_CA_1_CA_2_CA_8_CA_9_CB_04_CB_05_CB_10_CB_6_CR_010_CR_03_CR_05_CR_08_CR_09_CR_1_CR_101_CR_104_CR_3_CR_4_CR_41_CR_51_CR_6_CRB_1_CRB_2_CRB_3_CRB_4_CRB_5_CRB_7_DS_1_DS_10_DS_2_DS_4_FH_04_FH_7_FH_8_FHB_1_FR_07_FR_09_FR_10_FR_12_FR_2_FR_6_FR_7_FR_8_FR_9_GO_10_GO_101_GO_106_GO_2_GO_3_GO_4_GO_6_GO1_GR_1_GR_10_GR_101_GR_106_GR_6_GR_7_GR_8_HB_2_HB_6_HH_1_HH_103_HH_1031_HH_114_HH_2_HH_3_HH_4_HH_5_HH_6_HH_7_HH_8_HH_9_HM_2_HM_3_HM_4_HM_5_HM_6_HM_7_HM_8_HM_9_HN_1_HN_4_HN_7_HNB_1_JB_1_JB_10_JB_103_JB_109_JB_110_JB_2_JB_3_JB_4_LB_02_LB_03_LB_04_LB_05_LB_1_LB_2_LB_6_LB_8_MB_2_MB_5_MB_7_MB_8_MB_9_MZ_2_MZ_8_PA_2_PA_5_PA_6_PA_7_PA_8_PA_9_PB_3_PB_5_PB_6_PB_7_PB_9_PE_1_PE_4_PE_5_PJ_1_PJ_10_PJ_3_PJ_4_PJ_5_PJ_6_PJ_7_PL_10_PL_2_PL_6_PL_7_PL_9_PN_01_PN_02_PN_03_PN_07_PN_08_PN_09_PN_4_PN_6_PNB_1_POB_02_POB_03_POB_06_POB_1_POB_2_POB_3_POB_4_PR_1_PR_2_PR_4_RE_1_RE_2_RE_3_RE_4_RE_5_RE_6_RE_7_RE_8_RG_1_RG_10_RG_2_RG_3_RG_5_RG_6_RG_7_RG_8_RG_9_SB_1_SB_12_SB_14_SB_15_SB_2_SB_5_SB_7_SE_02_SE_2_SE_9_SF_01_SF_02_SF_03_SF_04_SF_05_SF_06_SF_3_SF_7_SF_8_SF_B1_SX_1_TT_07_TT_10_TT_201_TT_6_WN_1_WN_10_WN_11_WN_14_WN_15_WN_2_WN_5_WN_6_WN_7_WNB_1_WNB_2_YZ_11_YZ_4_YZ_5	BR_1_BR_3_CA_012_CR_104_CR_5_CR_8_CRB_4_CRB_5_GR_1_GR_101_GR_2_GR_6_GR_7_HH_103_HH_5_HH_6_HK_6_JB_1_LB_02_LB_2_PA_4_PA_5_PA_8_PE_1_PJ_3_PJ_5_PJ_8_PN_09_PN_3_PN_9_POB_3_POB_4_RE_6_RG_10_RG_6_RG_7_RG_8_SB_1_SB_6_SBB_1_SBB_3_SF_4
A.africana_BA_10_BA_7_BR_017_BR_12_BR_14_BR_15_BR_7_BS_1_BS_10_BS_102_BS_103_BS_104_BS_105_BS_106_BS_108_BS_3_BS_6_BT_10_BT_4_BT_5_BT_8_CR_111_CR_112_CR_113_CR_115_CR_12_CR_13_CR_14_CR_141_CR_15_CRB_10_CRB_6_CRB_8_DS_011_DS_012_DS_12_DS_13_DS_14_DS_15_DS_17_FH_014_FH_015_FH_14_FH_15_GO_11_GO_15_HH_10_HH_11_HH_111_HH_13_HH_132_HH_15_HH_213_HK_12_HM_012_HM_11_HM_12_HM_13_HM_15_JB_13_LR_02_LR_1_LR_10_LR_2_LR_3_LR_4_LR_9_ML_01_ML_03_ML_04_ML_06_ML_1_ML_10_ML_2_ML_3_ML_4_ML_5_ML_6_MLW_1_MR_005_MR_01_MR_02_MR_03_MR_05_MR_3_MRW_1_MRW_2_PA_11_PA_12_PA_13_PA_14_PA_15_PA_16_PA_17_PA_18_PAW_1_PD0_11_PDO_01_PDO_1_PDO_12_PDO_3_PDO_4_PDO_5_PDO_6_PDO_8_PDO_9_PDOW_1_PE_10_PE_11_PE_12_PE_13_PE_14_PE_15_PE_16_PE_19_PE_20_PE_3_PE_7_PJ_113_PL_11_PL_14_PR_14_PR_6_PRW_1_RG_13_RG_16_RG_17_SE_013_SE_12_SE_13_SE_14_SFB_11_SH_1_SH_11_SH_3_SH_4_SX_11_SX_12_SX_14_WN_24_ZK_003_ZK_02_ZK_03_ZK_04_ZK_1_ZK_2	BR_05_BR_101_CR_1_CR_14_CR_4_CR_6_CR_7_CRB_1_GO_4_HB_1_HB_2_HB_5_HH_10_HH_2_HH_4_HH_8_HH_9_JB_2_LB_6_MB_2_PA_7_PE_5_PJ_1_PJ_7_PL_6_SE_2_SF_9_YZ_9
CR_11_FH_18_HH_313_HK_15_LR_5_LR_6_ML_05_PE_17_PE_18_PJ_115_PJ_215_SB_17_SEW_1_SX_13	BS_101_BS_104_BS_105_BS_108_CR_115_CR_141_GO_12_GO_13_JB_10_ML_8_MR_5_PA_11_PA_13_PA_17_PA_19_PJ_215_PR_03_SE_09_SE_9_ZK_2_ZK_5
DS_18_FH_13_FH_16_GO_12_HH_12_PL_15_SX_15	BT_10_DS_012_DS_12_DS_15_DS_18_FH_13_FH_15_HK_12_ML_10_ML_9_PA_20_PE_8_RG_18_SB_11_SB_15_WN_21_ZK_1

PA_4_PJ_9_POB_04	CRB_7_HH_12_HH_15_HM_15_ML_3_MR_3_PA_16_PA_18_PE_10_PE_16_PE_18_PE_20_PJ_111_PJ_115_PJ_13_PJ_214_ZK_4
BR_13_BR_211	BS_1_CRB_6_CRB_8_CRB_9_DS_16_FH_18_ML_7_MR_1_PA_12_PA_15_PN_7_PR_4_SB_17_SB_18_SB_3_WN_23
BR_1_BR_8	BR_4_HB_3_HH_1_HH_114_HH_3_JB_3_PJ_6_PL_7_PL_8_POB_1_POB_5_PR_9_YZ_4
BS_109_BS_110	BR_12_BS_102_BS_110_CR_13_GO_14_MR_4_MR_6_PE_3_PE_7_PR_5
GO_13_GO_14	BT_4_CRB_10_HH_13_MR_2_PE_13_PR_011_PR_14_PR_6_RG_15
GR_2_POB_07	BA_2_BR_15_BS_1081_HH_132_HH_213_HK_15_RG_12
PJ_214_RG_15	BS_103_BS_4_HH_111_HM_012_MB_1_PA_14_ZK_6
MZ_07_SE_15	BA_6_FH_014_FH_015
WN_21_WN_22	BR_211_BS_109_FH_16
ZK_001_ZK_01	DS_013_DS_14_PR_06
	BS_107_BT_7_SE_013
	CR_12_CR_15
	CR_3_RG_2
	CRB_3_RG_9
	MB_03_SB_2
	BS_10_SEW_1