Inter-individual variability and phenotypic plasticity: the effect of the environment on the biogeography, population structure, ecophysiology and reproduction of the sandhoppers *Talorchestia capensis* and *Africorchestia quadrispinosa*

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Abstract

Climatic envelope models focus on the climatic variables affecting species or species assemblages, and are important tools to investigate the effect of climate change on their geographical ranges. These models have largely been proposed in order to make successful predictions on species' persistence, determining which variables are likely to induce range expansion, contraction, or shifting. More recent models, including the ability and the cost for individuals to respond promptly to an environmental stimulus, have revealed that species may express phenotypic plasticity able to induce adaptation to the new environment. Consequently, understanding how species evolve to a changing climate is fundamental. From this perspective, investigating intraspecific responses to an environmental variable may contribute to better understanding and prediction of the effect of climate change on the geographical range and evolution of species, particularly in the case of widespread species. In this context, the present study aimed at establishing how environmental variables (focussing mainly on temperature) may have contributed to shape the spatial distribution, physiology, reproductive biology and connectivity of two species of Southern African sandhoppers (*Talorchestia capensis* and *Africorchestia quadrispinosa,* Amphipoda, Talitridae). Most of the work was carried out on *T. capensis,* due to its widespread spatial distribution.

A first investigation of the biogeography of *T. capensis* and *A. quadrispinosa,* revealed that, for both species, spatial patterns of abundance, size and sex ratio were not explained by the Abundant Centre Hypothesis (greater abundance at the core of a spatial range), but rather guided by bio-physical forces. Precisely, the abundance of sandhoppers was driven by the morphodynamic state of the beach, salinity and temperatures, with strong differentiation among sites that reflected local environmental conditions. In support o of these findings, strong population structure in the genetics of *T. capensis* was found (three main groups) when investigating its phylogeography and genetic connectivity. Although such defined structure

may suggests cryptic speciation, the concomitant within-population variation in the COX1 region of mtDNA, also highlighted the importance of individual genetic variability. High individual variability was also found in the response of *T. capensis* to temperature, both in its physiology (thermal plasticity) and its reproductive biology (maternal effects).

Since temperature is one of the main variables affecting the coastal marine systems of southern Africa and the metabolism of animals in general, its effect on the physiology and reproduction of *T. capensis* was therefore investigated. Thermal responses to increasing/decreasing temperatures were assessed for separated populations of *T. capensis*. Individual variability was reported in the oxygen consumption of *T. capensis* in response to temperature (high variation around the means, especially for increasing temperatures). Among population differences in thermal sensitivity were significantly correlated with air temperature variability experienced over the past 23 years, highlighting the importance of historical temperature fluctuations to the current thermal physiology of these sandhoppers. Temperature also had an important effect on the reproductive plasticity of *T. capensis*. Different temperatures induced mothers to adjust the size of their offspring (i.e. egg size), with larger eggs produced at lower temperatures. Interestingly, females showed strongly significant among individual variation in the size of the eggs.

Given the importance of understanding rapid responses of organisms to climate change and considering the fundamental role played by phenotypic plasticity in evolution, the overall study revealed the significance of individual plasticity and variability in response to the environment and highlighted its importance. Particularly, studying the thermal physiology of separated populations and understanding within population reproductive plasticity in response to temperature, helped to clarify how differences among individual responses have important consequences at the population level, possibly explaining the widespread distribution of *T. capensis*.

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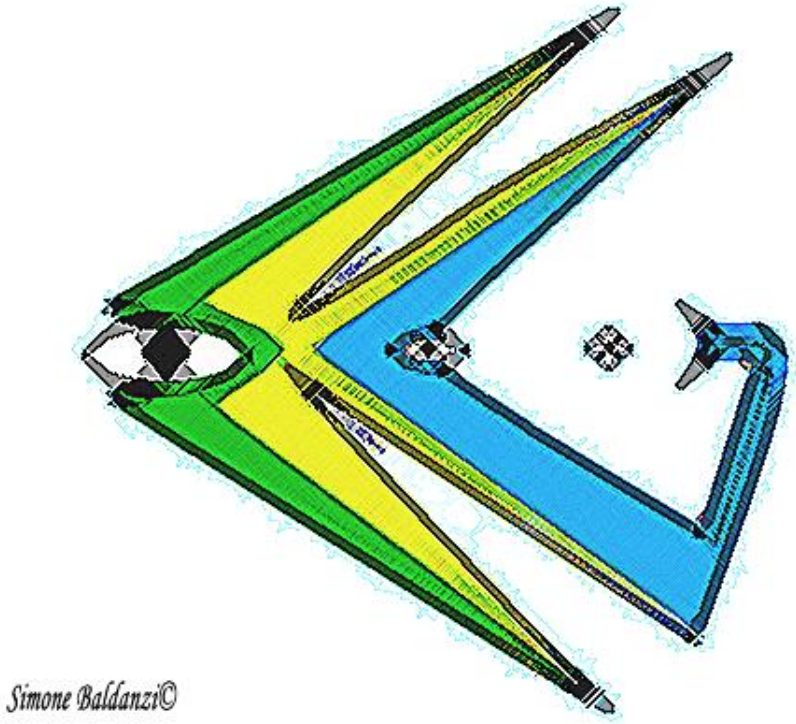
"Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure"

Nelson Rolihlahla Mandela

(1918-2013)

-Chapter 1-

General Introduction



*"Some people would use the term, **evolution**, only for the internally transmitted genetic material […] I think we are more than just our genes […] we entered in an area of **self-designed evolution**".*

Prof. Stephen Hawking

(Life in the Universe, lecture)

Current climate change is leaving a significant footprint on the biodiversity, distribution and ecography of species (Parmesan, 2006; Dawson et al., 2011). Nowadays scientists agree that the fundamental causes of climate change are anthropogenic (Chown et al., 2010), pointing out that human activities are leaving inevitable, harmful and calamitous consequences for large parts of the world (Rockström et al., 2009).

The rate at which anthropogenic climate change is speeding up natural fluctuations in climatic variables will likely lead to local population declines, range contractions and even extinctions (Parmesan et al., 2005). Over the past 40 years, some species have been extending their ranges toward the poles and populations have been migrating, developing, or reproducing earlier in the spring than previously (Parmesan and Yohe, 2003). It is however complicated to estimate whether climate change is really affecting a species range, as often little is known about the historical range of distribution of a species and some predictions on the effect climate change/global warming are sometimes overestimated (Loehle and LeBlanc, 1996).

Understanding how and at what rates species respond to rapid changes in climate is however urgent, given the demonstrated rapid loss of biodiversity (Dawson et al., 2011). To preserve biodiversity and reduce the effect of climate change on animals and plants, much recent effort has been directed to understanding the evolutionary mechanisms behind species' responses to climate change (Franks and Hoffmann, 2012). How can climate change influence the evolution of species? How fast should species evolve to respond to "new environmental challenges"? If fast mechanisms are involved in response to climate changes, how will temperature fluctuations or variations in salinity, pH and CO_2, induce such rapid responses? Is phenotypic plasticity the key to understanding how the environment affects the evolution of species? Such questions are still open, but a broad range of researchers, including ecologists, molecular and evolutionary biologists and philosophers of science all agree on the overall importance of such topics (Pigliucci and Muller, 2010).

Several authors have demonstrated that climate changes can lead to heritable genetic adaptation as a response to recent seasonal changes due to a rapid shift in climate (Bradshaw and Holzapfel, 2006). Empirical examples show us how undeniably important it is to study the effect of climate change on the evolutionary adaptation of species (Franks and Hoffmann, 2012). Unfortunately, to provide full evidence for genetic changes in response to climate change, researchers need to carry out observations and experiments on one or a few species over several decades, requiring much dedication (often an entire career) and high costs (Bradshaw and Holzapfel, 2006). Nevertheless, the ability of organisms to adapt genetically to rapid changes is considered crucial (Bradshaw and Holzapfel, 2006), often determining evolutionary "winners or losers" (Stilman, 2003; Somero, 2010; Hoffmann and Sgrò, 2011).

Despite its importance, however, the genetic basis of evolutionary adaptation (i.e. genetic evolution) still remains poorly understood (Reusch and Wood, 2007). Few case studies have really established a genetic basis of adaptive evolution in a quantitative trait in response to a change in climate (Franks and Hoffmann, 2012). As we see repeatedly in the literature, plants are useful and successful model organisms in evolutionary biology (see Mendel's works and his classic model organism) and recent studies showed, as examples, the genetic basis behind the shifting in flowering time for several plants in response to climate change (Hendry and Day, 2005; Inouye, 2008; McMahon et al., 2011).

Recently, for most reported cases of phenotypic change in the wild in both plants and animals, phenotypic plasticity (the ability of a genotype to express different phenotypes in different environments, Pigliucci 2001) has been largely proposed as an alternative and complementary mechanism to genetic evolution in response to environmental changes (Pigliucci, 2001; Chevin et al., 2013). In fact, the idea that genetic adaptation by natural selection is not the only solution for responding to environmental changes, has often been advanced in the past, but only recently re-proposed (Pigliucci, 2001; Pigliucci et al., 2006; Lande, 2009; Pigliucci

and Müller, 2010). One reason is that natural selection occurs at the population level and with generations as the time unit, while environmental changes show to occur at a fast per-generation rate (Chevin et al., 2010; 2013). A 50 year old debate among scientists and (with even more emphasis) between philosophers of science and evolutionary biologists, is the real occurrence of, and the interaction between, processes such as phenotypic plasticity, genetic assimilation and phenotypic accommodation (Pigliucci and Murren, 2003; West-Eberhard, 2003; Pigliucci et al., 2006; Crispo, 2007). Genetic assimilation, the process by which an environmentally induced phenotypic plasticity can become genetically fixed, is a term derived from Schmalhausen's idea (Schmalhausen, 1949), first used by Waddington (Waddington, 1953, see review of Crispo, 2007 for a comprehensive definition of the term). Such concept has recently been re-proposed and re-elaborated (but see criticisms in de Jong, 2005 and responses in Pigliucci et al., 2006) thanks to new advances in fields like molecular biology, ecological epigenetics and developmental biology (Jablonka and Lamb, 2005; Richards, 2006; Richards et al., 2010; Bossdorf et al., 2008; Pigliucci and Muller, 2010). Phenotypic evolution can be described as follows (*sensu* Pigliucci and Murren, 2003; West-Eberhard, 2003): phenotypic plasticity allows the expression of relatively well-adapted phenotypes under novel conditions (e.g. a new environment experienced after dispersal, range expansion or rapid climate change); hence allowing a population to persist (concept of "Phenotypic accommodation" and "initial spread" of the new trait, *sensu* West-Eberhard, 2003). Natural selection will then act on the novel phenotype resulting in the assimilation of the new trait in the environment ("genetic assimilation" *sensu* West-Eberhard, 2003 and Pigliucci and Murren, 2003). Natural selection (if this operates constantly) on a given trait in the new environment, may result in the loss of plasticity for that phenotype, for example due to the high costs in maintaining such plasticity once the environmental stimulus ceases (Pigliucci et al., 2006). Phenotypic (through phenotypic plasticity, accommodation and genetic

assimilation) and genetic evolution (through natural selection) will consequently hold/maintain a hierarchical relationship: phenotypic plasticity (a *developmental process* which generates new traits through environmental stimuli) will be shaped by natural selection (an *evolutionary mechanism*) to give birth to a new *evolutionary outcome* through genetic fixation of that phenotype in the new environment (Pigliucci and Murren, 2003; Pigliucci et al., 2006; Lande, 2009; Pigliucci and Muller, 2010).

Phenotypic evolution is consequently an important adaptive mechanism that could explain some focal biological responses like the *lag phase* and subsequent population explosion of many invasive species (Pigliucci et al., 2006), physiological responses to acute stress (Shulte et al., 2011) and transgenerational maternal effects (Ghalambor et al., 2007). Recent advances in the fields of ecological epigenetics and developmental biology have brought even more light onto the molecular basis underpinning rapid individual responses to environmental changes (Jablonka and Lamb, 2005). The concept of epigenetics was firstly used by Waddington (linking the two words of *Genetics* and *Epigenesis*, see Van Speybroeck, 2002 for an historical review) who originally defined it as *''the branch of biology which studies the causal interactions between genes and their products which bring phenotypes into being''* (Waddington, 1956 reported in Jablonka and Lamb 2002, *p. 83*). Nowadays, the term epigenetics has assumed a slightly different significance and its mechanisms includes DNA modifications (e.g. DNA methylation, histone modification, non-coding RNA activity) able to induce genetic activity without altering the underlying DNA sequence (Jablonka and Lamb, 2002, 2005). Epigenetic mechanisms may contribute to phenotypic evolution because they are consequences of environmental stimulus (Jablonka and Lamb, 2005; Richards, 2006; Richards et al., 2010), thus playing a fundamental role facilitating rapid response to new environments (Ho and Burggren, 2010).

Among all the environmental variables inducing individual plastic responses, temperature certainly plays a major role (Kingsolver and Huey, 2008), being a central topic of many studies dealing with the effect of climate change on animal and plant eco-physiology (Spicer and Gaston, 1999), reproduction (Marshall and Uller, 2007; Fernàndez et al., 2009), biogeography (Sagarin et al., 2006) and evolution (Angilletta, 2009). It is widely recognised that temperature has a profound effect on biological functions at several hierarchical levels, ranging from molecules to ecosystems (Clarke, 2003; Somero, 2010), strongly affecting the maintenance of homeostasis (Hazel, 1995).

Due to the current climate change, scientists have started to re-evaluate the impact of elevated temperatures on the ecology of species (Krenek et al., 2012). Here, ectothermic organisms are of special interest as their physiological performance is highly dependent on environmental temperature, often resulting in a direct scaling between basal metabolic rate and temperature (Universal Temperature Dependence models, Gilooly et al., 2001). More complex mechanisms involving an evolutionary optimisation between costs, benefits and ecological lifestyle, however, seems to be more appropriate when temperature affect ectotherms metabolism (Clarke, 2004; Clarke and Fraser, 2004).

Studies on genetic and phenotypic diversity over the geographic range of a species are important, as they allow us to make predictions on the responses of a species to variations in temperature (Krenek et al., 2012). In fact, investigating pattern of genetic diversity and phenotypic adaptation to environmental variables within a species geographical range may contribute to better understand which populations may be more sensitive to climate changes (Guo et al., 2012). Importantly, investigations of this kind can underpin patterns of evolutionary temperature adaptation to the current thermal heterogeneity by determining which ectotherms have a high acclimatisation capacity and which can only occur at specific temperatures (Sanford and Kelly, 2011). From this perspective, phenotypic plasticity may

allow higher tolerance to changing thermal conditions, while local temperature adaptation might be detrimental (Ghalambor et al., 2007; Sanford and Kelly, 2011). Much of our current understanding of the ecological effect of climate change however, is based on the erroneous assumption that body temperatures of ectotherms are equal to air temperatures (Buckley et al., 2013). More realistically, air temperatures together with sea surface temperatures, solar radiation, humidity, and wind speed interact with the phenotypes of organisms to produce complex patterns of body temperatures in space and time (Denny and Gaylord, 2010).

Complex mosaics of interaction between temperature and organisms may influence reproductive strategies and patterns of species richness in the oceans (Fernàndez et al., 2009). As an example, Fernàndez and coauthors (2009) reported contrasting effects of temperature on species richness between direct and indirect developers of several taxa along the Chilean coast, suggesting that this variation depends largely on larval developmental modes. It is therefore important to examine/address how local variation in physiological and reproductive traits may influence patterns at broader scales, providing useful insights on how organisms respond to environmental changes at several hierarchical levels, ranging from individuals to species assemblages (Gaston et al., 2009).

Within a species, individual responses may substantially differ as result of individual specialisations, reflecting a diverse array of physiological, behavioural, and ecological mechanisms that can generate intra- and inter-population variability (Bolnick et al., 2008). Variation among populations within a single species may be important when that species shows a wide range of distribution, because different phenotypes are produced, resulting in high degree of phenotypic plasticity at the population level (Sanford and Kelly, 2011). In this view, individuals gain a fundamental role as sources of phenotypic variability, which could be the key for species range expansion, persistence and evolution in an unpredictable and heterogeneous environment (Einum and Fleming, 2004).

Widely distributed species subjected to a broad range of conditions represent useful frameworks for evaluating environmental effects on phenotypic variability (Gomez et al., 2013). This is the case for widespread species that have colonised severe and unpredictable environments, like intertidal and supratidal sandy shores, where environmental and physical forces drive species richness and abundance (Defeo and McLachlan, 2011; Baldanzi et al., 2013). Invertebrates living at the interface between marine and terrestrial life, have evolved important mechanisms to persist in such harsh habitats, becoming ideal model organisms to understand the effects of environmental variability (i.e. temperature effect) on individuals and populations/species dynamics.

Aim of the study and structure of the thesis

The present study aimed at establishing how environmental variables (focussing on temperature) may have contributed to shaping the spatial distribution, physiology, reproductive biology and connectivity of two species of Southern African sandhoppers (*Talorchestia capensis* and *Africorchestia quadrispinosa,* Amphipoda, Talitridae).

Most of the work has been carried out on *T. capensis*, for its widespread distribution, encompassing at least three different thermal bioregions, making it a good candidate for the study of physiological responses to temperature. Furthermore, *T. capensis* was potentially a good model to carry out mesocosm reproductive experiments, mainly for its fast reproductive cycle (Van Senus, 1988). *A. quadrispinosa* was studied for its biogeography only, as reported in Chapter 2, proving to be a good model for testing predictions from spatial-ecological hypotheses (see below and Chapter 2 for details).

The entire study area covered the South African and Namibian sandy shores (Namibian coasts were surveyed only for the biogeography of *A. quadrispinosa*, as shown in Chapter 2), encompassing four main Bioregions (as defined by Lombard, 2004) and two important coastal current systems (the Agulhas and the Benguela currents). Sandy shores are highly variable,

unpredictable and physically dominated environments (Defeo and McLachlan, 2011) where species abundance, richness and biomass are mainly driven by the morphodynamic state of the beach and environmental gradients (Defeo and McLachlan, 2011; Baldanzi et al., 2013). Such environments are ideal for the study of how species respond to changes in environmental conditions, in terms of spatial distribution, phylogeny and connectivity, physiology and life history.

Furthermore, talitrids show active parental care, keeping eggs and juveniles in the female's brood for enough time to ensure sufficient conditions for survival (Morritt and Spicer, 1996a, b, c; Morritt and Richardson, 1988; Thiel, 1999). Such a strategy enhances maternal care, resulting in adaptive maternal effects if the future environment of the offspring is comparable to the maternal environmental conditions (thus "prediction" could be made) and/or when pre-existing "environmental cues" can be transferred across generations (Ghalambor et al., 2007). Sandhoppers therefore represent an ideal model organism for testing phenotypic plasticity in response to the environment.

The thesis first examines the biogeography of the two species of sandhoppers (Chapter 2), providing the first study of its kind for southern African sandy beach invertebrates. In this chapter, a possible centre to margin pattern explaining the spatial distribution of the two species was investigated, testing predictions from the Abundant Centre Hypothesis (Brown, 1984; Sagarin and Gaines, 2002). The study also provided a large scale investigation of the multivariate effects of several environmental variables on the geographical limits of distribution.

The third chapter deals with the phylogeny of *T. capensis* and its connectivity among separated populations, providing an important component for the entire study, as no previous studies have investigated the phylogeography of this species. Since *T. capensis* showed a range of distribution covering different bioregions with contrasting temperature regimes, the

last two chapters of this thesis contribute to a better understanding of the effects of temperature on *T. capensis* physiology (thermal plasticity and sensitivity) and reproductive biology (maternal effects).

The fourth Chapter tackles the metabolic response of separate populations of *T. capensis* to increasing and decreasing temperatures. Using oxygen consumption as a proxy for metabolic performance under laboratory conditions, the study investigates how climatic variability experienced by sandhoppers over a relatively long time period (historical data of air temperature) has shaped their thermal plasticity.

The last working chapter (Chapter 5) deals with temperature induced maternal effects on the early ontogeny of individuals of *T. capensis* from a single population. A mesocosm experiment was conducted to demonstrate the temperature-induced effect of the mothers' phenotypes on the size of their eggs, highlighting the importance of a type of phenotypic plasticity in response to temperature known as "Maternal Effects".

The last chapter of this thesis presents final conclusions and important remarks in the form of a Synthesis.

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-Chapter 2-

Environmental domains and range-limiting mechanisms: testing the Abundant Centre Hypothesis using Southern African sandhoppers

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Introduction

The complex dynamic and interlocking effects of climate change on organisms and their environments can lead to dramatic changes in the distribution of species and ultimately, loss of biodiversity (Parmesan et al., 2005; Ackerly et al., 2010). Accordingly, predicting shifts in species ranges and the underlining mechanisms behind such changes, has become a central challenge in conservation biogeography (Whittaker et al., 2005).

Range expansion/contraction and distributional shifts occur naturally and continuously, but can be accelerated by changes in climate and by human activities (Franco et al., 2006; Tolley et al., 2008) such as pollution, environmental degradation, changes in land use and the introduction of invasive species (Ward et al., 2005; Tolley et al., 2008). Modelling approaches understanding species distributions have focused most intensively on the description of a bioclimatic envelope that characterises the natural distribution of a species (Pearson and Dawson, 2003). Such simplification is a necessary response to the complexity of the real world, but a more realistic understanding of species distributions must also include a wide range of abiotic and biotic variables (Whittaker et al., 2001). Such an approach assigns a central role to the spatial domains of natural variables, with climatic variables having a dominant effect from regional to global scales, while other variables, such as biotic interactions, have more localised effects (Willis and Whittaker, 2002; Pearson and Dawson, 2003).

At regional scales, geographic patterns of abundance are fundamental to ecological issues, providing information on species range limits, gene flow among populations, population dynamics and species' responses to environmental change (Sagarin and Gaines, 2002a; Sagarin et al., 2006). It is widely accepted that the abundances of species are greatest at the centres of their distributional ranges and decline towards the margins (Brown, 1984; Sagarin and Gaines, 2002a; Samis and Eckert, 2007; Rivadeneira et al., 2010; Virgós et al., 2011;

Fenberg et al., 2011). This concept is the "Abundant Centre Hypothesis" (ACH hereafter). This idea has been explored by several authors (Brown, 1984; Enquist et al., 1995; Sagarin and Gaines, 2002a) and extensively used to understand ecological and evolutionary processes (Sagarin and Gaines, 2002a; Sagarin et al., 2006). Nevertheless, the concept remains largely theoretical and empirical evidence for the patterns predicted by the ACH is still weak (Tuya et al., 2008) and equivocal (Sagarin et al., 2006; Samis and Eckert, 2007; Rivadeneira et al., 2010; Fenberg et al., 2011). Sagarin and Gaines (Sagarin and Gaines, 2002a) reviewed a large number of published works that tested the ACH, and found that only 39% of these supported the ACH, probably because abrupt changes in biotic and/or environmental conditions may result in sharp, rather than gradual gradients in abundance (Brown, 1984; Tuya et al., 2008).

The need to evaluate variation in abundance at large geographical scales has been stressed by several authors with an emphasis on the need for large numbers of sampling sites, in order to detect the realistic edges of species distributions (Sagarin and Gaines, 2002a, b; Rivadeneira et al., 2010; Fenberg et al., 2011). Additional features such as genetic structure, physiological proxies, life-history traits or biophysical variables have been used to test the ACH as such factors can reflect both distributions and range boundaries (Gilman, 2006a, b; Lester et al., 2007; Rivadeneira et al., 2010). White et al. (2007) identified several types of relationships between size and abundance, assuming that the size-abundance relationship is a fundamental link between the individual and the population level. Rivadeneira et al. (2010) linked the distribution of abundance with variation in life history traits, such as sex ratio and the proportion of reproductively active females, concluding that sex ratio provided the strongest support for the ACH, with females being more abundant at the centre and males at the edges. Virgós et al. (2011), while testing the ACH on the European badger (*Meles meles*), concluded that body size is strongly related to food availability and resources, which are supposed to be

higher and of better quality at the centre of distribution and indeed they found individuals were larger at the core than the periphery.

Most tests of the ACH have focused on terrestrial species (Virgós et al., 2011), although there have been some studies of marine systems (Sagarin et al., 2002b; Defeo and Cardoso, 2004; Gilman, 2005; Gilman, 2006b; Rivadeneira et al., 2010; Fenberg et al., 2011). Intertidal and supratidal organisms are considered ideal models to test "range-wide hypothesis" (including the ACH) due to the linear geometry of their geographical ranges, reducing it at a one-dimensional pattern of distribution, where edges and centre are relatively easy to define (Sagarin et al., 2006). Here, I investigated the biogeography of two species of southern African sandhoppers (Crustacea, Amphipoda, Talitridae) to test the predictions of the ACH, and to understand the influence of environmental variables on their abundances.

How species respond to environmental variability is crucial in sandy beach ecology, as fluctuations in abundance at large spatio-temporal scales are fundamental to how these systems function (Defeo and Cardoso, 2004; Lima et al., 2000; Schoeman and Richardson., 2002; Cardoso et al., 2003; Defeo and McLachlan, 2011; Gomez and Defeo, 2012). Additional advantages of using this system to test the ACH are that it is particularly strongly forced by environmental factors and experiences little human impact. The effects of the environment on the relation between species range and population declines, are critical to effective tests of the ACH, as these two phenomena are generally correlated (Mace et al., 2010; Lawrence et al., 2012).

The two study species, *Talorchestia capensis* (Dana) and *Africorchestia quadrispinosa* (Barnard) show different distributions along the sandy shores of Namibia and South Africa, providing multiple tests of ACH predictions along a one-dimensional environmental gradient. *A. quadrispinosa* has a wide North-South distribution, encompassing two biogeographic regions (Harris et al., 2011), forming an ideal model to test the classic ACH (Rivadeneira et

al., 2010; Fenberg et al., 2011). On the other hand, *T. capensis* has a wide, but patchy distribution, from the west to the east coast of South Africa, encompassing three different biogeographic regions (the cool-temperate west, warm-temperate south and sub-tropical east coasts, following Harris et al., 2011), offering a highly diversified model to test the ACH.

I hypothesised that: 1) the geographic variation in abundance, size and sex ratio of these two species of southern Africa sandhoppers, should be explained by the predictions of the ACH. Particularly, I expected a good positive test for *A. quadrispinosa*, since its linear North-South distribution fits well with the classical inferences of the ACH (Rivadeneira et al., 2010; Fenberg et al., 2011); 2) among the environmental conditions experienced by these animals, the morphodynamic state of the beaches and temperature seem to be the most relevant parameters for a distributional range that extends across different latitudes (Defeo and McLachlan, 2011; Sunday et al., 2010) and are likely to have strong influences on abundances.

Materials and Methods

Study sites

The study area includes a long coastline encompassing three biogeographic regions: the cool-temperate Namaqua province on the west coast, the warm-temperate Agulhas province on the south coast and the subtropical East Coast province (Turpie et al., 2000; von der Heyden, 2009). The sampling area ran from Richards Bay (KwaZulu Natal, East coast, South Africa) to Wlotzkasbaken (West coast, Namibia) (Fig.1a).

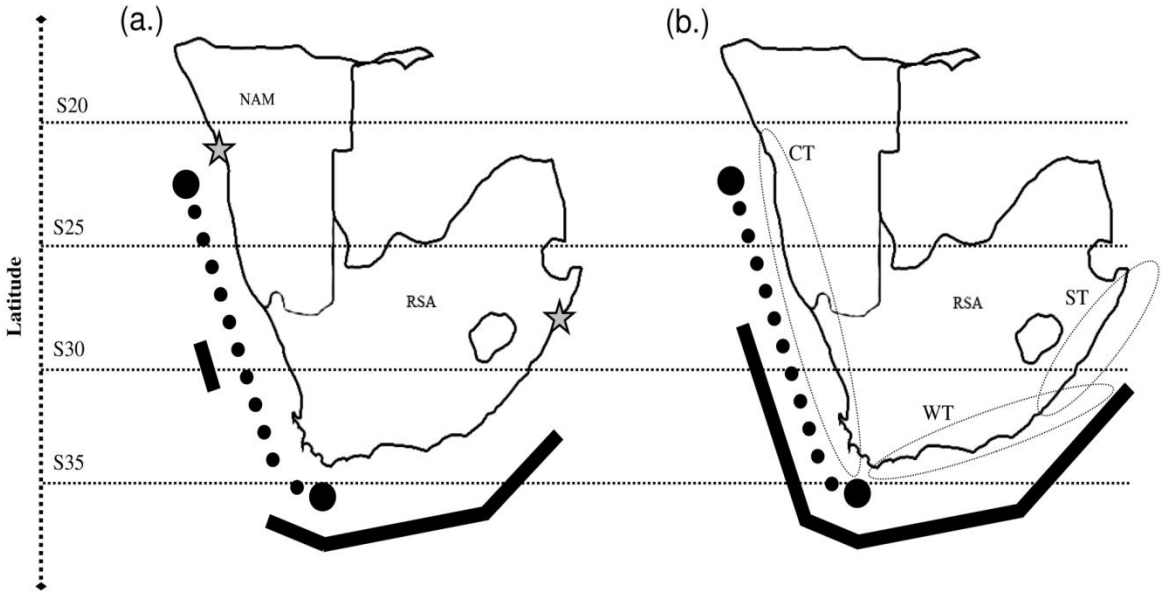

Fig. 1 Distributional range of the two species from the field surveys (a) and historical range data of distribution, based on the published data (b).
The dotted line represents the range of *Africorchestia*. *quadrispinosa* and the solid line the range of *Talorchestia. capensis*. The star symbols represent the range of the entire sampling area (a.) The ellipse indicates three biogeographical regions (b): Cool Temperate (CT), Warm Temperate (WT), Sub-Tropical (ST), [33]. The ST region has a transition zone which includes the limit of distribution of *Talorchestia capensis.* Latitudes are reported on the left side of the map.

The geographical coordinates for each site were taken using a global position system receiver (Etrex, Garmin) and are reported in the Appendix. In order to collect animals at the highest site-resolution possible, sample sites were no more than 100km apart, based on Google Earth® imagery. Once at a location, I established the best area according to accessibility and

beach width as a minimum width was necessary to allow the setting of traps, (see below). Based on this, sandy shores with or without detritus were both investigated. Animals were collected during winter, 2010 (South Africa, from June to August) and 2011 (Namibia, June). Two separate surveys were necessary due to the long distances covered and logistic constraints.

Study species

Sandhoppers are semi-terrestrial crustaceans in the Order Amphipoda. The Talitridae is the only family including truly terrestrial amphipods and, although many are found close to the sea on the upper parts of the shore, some occur inland (Lincoln, 1979). The species investigated in this study were: *Talorchestia capensis* (Dana, 1853) and *Africorchestia quadrispinosa* (K.H. Barnard, 1916).

Sandhoppers burrow into moist sand during the day, avoiding the stresses of heat and desiccation (Williams, 1995; Morritt, 1998) and emerge at night, when the air temperature is cooler, and the risk of predation is reduced (Mardsen, 1991a, b; Poulin and Latham, 2002a; Cardoso, 2002). Numerous studies report a strong link between the diet of sandhoppers and detrital macrophytes (e.g. Adin and Rier, 2003; Crawley et al., 2009), although other studies suggest a more complex opportunistic feeding strategy that allows sandhoppers to utilise alternative sources of food, such as diatoms, Johnston et al., 2005) and even conspecifics (Duarte et al., 2010). Porri and coauthors (2011), using stable isotope analysis, found no trophic link between the sandhopper *Talorchestia capensis* and the detritus underneath which animals were found.

Collection and laboratory analysis

Sandhoppers were collected at each site using pit-fall traps that were set up above the high water mark at dusk and emptied the following morning at sunrise, during neap tides. This allowed us to capture sandhoppers that migrated between the intertidal and the supratidal,

giving samples that integrated sandhopper abundances across the shore. The sampling unit was made up by four traps (made of half two-litre plastic bottles, filled up with soapy water) set on the four corners of two plastic baffles that were buried crossed into the sand in an 'X' arrangement (Fig. 2).

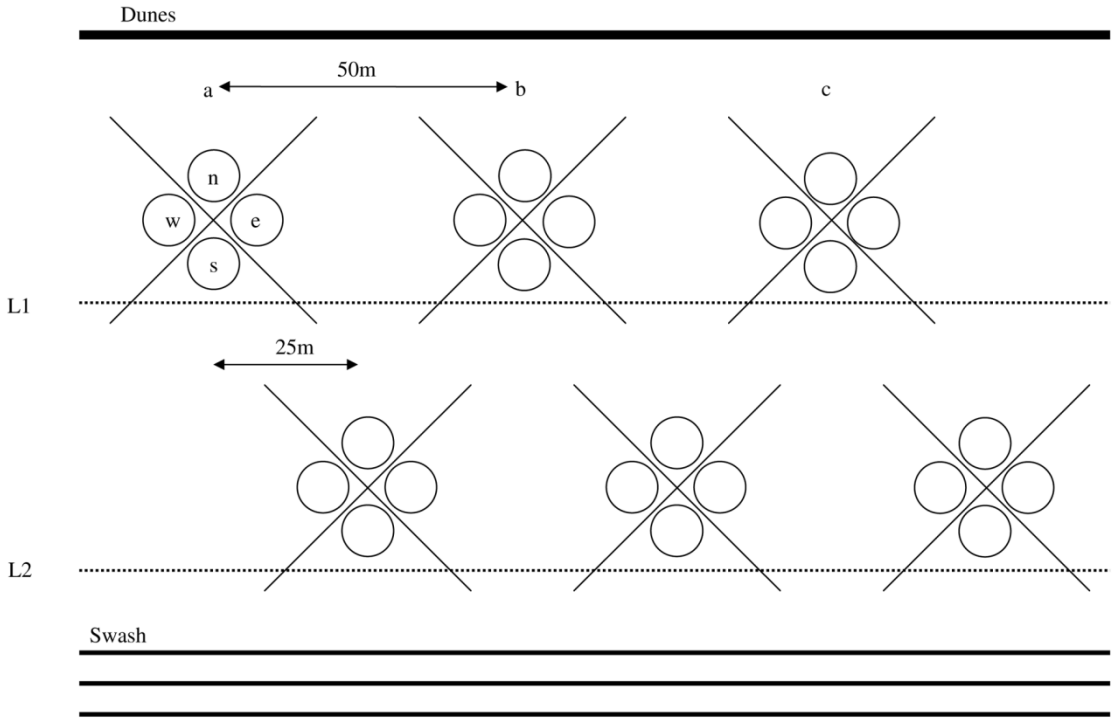

Fig. 2 Scheme showing the sampling design used at each sampling site.
The four traps has been set at the corner of the two baffles in order to maximize the collection and retrieve information on the orientation of the migratory activities (unpublished data). The traps has been named has follows: n=north; s=south; e=east; w=west. The position and the name of the traps do not coincide with the cardinal points, but the arrangement is purely related to the position relative to the shore. For instance, the trap named "n" is the one facing the dunes, while the "s" is toward the swash line. Consequently, the traps "e" and "w" are set, respectively at the right and left of the X arrangement. The two separated levels were assessed in order to investigate pattern of abundance at microscale and the effect of new fresh detritus (normally occurring at the L2) on the zonation of juveniles and ovigerous females (unpublished data).

Two levels were assessed: level 1, (L1) at the Spring Tide High Water Mark (STHWM) and level 2, (L2), at the Neap Tide High Water Mark (NTHWM). Each level included three replicates. Collections from the four corners were pooled to form a single sampling replicate. The three replicates within a level were 50m apart and the two levels were displaced by 25 m relative to one another: for example, the first replicate of Level 1 (L1a) was displaced by 25 m

alongshore relative to L2a. This "chessboard" arrangement allowed us to cover a total area 125 in length and an average of 5m wide (depending on the tidal range) (Fig. 2).

Animals were collected during seaward migration (occurring just after dusk) and landward migration, (at sunrise). After a 12h collection period, all traps were emptied into 500µm metal sieves, to collect adults, early juveniles and, when present, eggs. Specimens were stored in 75% ethanol and transported to the laboratory for further analysis. This design was chosen after a preliminary study carried out at four different sites on the south coast of South Africa during which I compared two different methods involving overnight pitfall-traps (which covered an area similar to that described above) and a core-transect method. The latter method included the use of three transects perpendicular to the shore, from the top of the dune to the swash: a core of sand (20 cm depth and 10 cm wide) was taken at each transect, every three meters landward and seaward, starting from the drift line. The sand was sieved immediately using a 1mm mesh. Since no animals were collected using the core-transect method, I opted for the pitfall traps. Pitfall traps are used to collect sandhoppers worldwide during their nocturnal migration (Chelazzi et al., 2005; Pavesi and Matthaeis, 2009) and the use of two levels should cover the entire range of migration of sandhoppers. Therefore I am confident on using such technique to test prediction from the ACH.

Animals were identified following Griffiths (1976) counted, measured and sexed using a stereomicroscope (32x and 64x magnification). The total body length (size), measured at 8x magnification, was taken from the base of the first antenna to the base of the telson (Pavesi and Matthaeis, 2009). On the basis of the body length, individuals were grouped into 0.5 mm size classes (Pavesi and Matthaeis, 2009). Males were distinguished by the presence of an enlarged 2nd gnathopod and genital papillae. Females do not show an enlarged 2nd gnathopod and could be distinguished by the presence of oostegites. Individuals lacking secondary sex characters were classified as juveniles (Pavesi and Matthaeis, 2009). Since the identification

of juveniles to species was not possible (especially when more than one species was collected at a site), only adults were considered for the analysis of abundance and size (see below).

Environmental parameters

Several environmental parameters (temperatures of water and sand, water salinity and percentage cover of detritus on the shore) were recorded during the deployment of traps. Sea temperature at the swash line and sand temperature measured at 10 cm depth for each sampling unit were taken using a mercury thermometer. Sand temperature was recorded twice, at dusk, during the deployment of the traps and at sunrise, during the collection. The double measurements minimised variability due to time of day. Salinity was measured using a handheld refractometer (Atago, S-10E). Percentage of detritus cover was estimated using a grid quadrat (50cm x 50cm): ten haphazard measurements were taken along the detritus line. Any organic matter found in the sampling area, under which animals occurred was considered detritus and the percentage was zero if no detritus was found.

Several measurements were used to define the physical state of the beach: beach slope, beach width and grain size. I did not measure breaker height at the time of collection, which can be used to define beach morphodynamic type, but which is unreliable and difficult to measure accurately (McLachlan pers. comm., also see McLachlan and Dorvlo, 2005 for details of the Dean parameter). Instead the description of beach morphodynamic state was based on beach slope, beach width and grain size as measured in the field (McLachlan pers. comm.). Beach slope was measured by two operators using a manual level to detect changes in slope every 3m from the swash area to the high tide mark. This is a modification of Emery's method (Emery, 1961). The beach width was considered as the portion of beach between the swash at low tide and the high tide mark. Sand samples were collected using a core sampler of 3.5cm in diameter to a depth of about 20cm. Sand samples were transported to the laboratory for granulometric analysis following a modified Falk and Ward procedure (Falk and Ward,

1957). After analysis, the following indices were calculated: Area, a measure of intertidal area obtained by dividing tide range by the beach face slope (McLachlan and Dorvlo, 2005), Beach Index, similar to Area but including a measure of sand particle size (BI, McLachlan and Dorvlo, 2005), Beach Deposit Index (BDI, Soares, 2003), an index that does not consider the tidal range. Indices were calculated using the following formulae:

$$\text{Area} = \log \times (\text{Tide} \div \text{Slope})$$

$$\text{BDI} = (1 \div \tan B) \times (a \div Mz)$$

$$\text{BI} = (\text{Sand} \times \text{Tide}) \div \text{Slope}$$

where: Tide is the maximum spring tidal range (meters); Slope or tanB is the beach slope; B is the average intertidal Beach slope, a = 1.03125 is the median grain size of the sand particle size classification; Mz is the average intertidal sand size (mm) and Sand is the mean sand particle size (phi units + 1) (McLachlan and Dorvlo, 2005; McLachlan et al., 1993; Short, 1996). The dimensions of the indices are: log meters (Area), log phi·m (BI). BDI is dimensionless.

The morphodynamic state of a beach is well known to have a strong effect on the biota. To ensure that the analyses were not distorted by mixing shores of different states, I categorised each shore based on BI index, (following McLachlan and Dorvlo, 2005). All shores were classified by this system as "Intermediate" and consequently were included in the analyses.

Data analyses

For each site, several sandhopper' variables were calculated: Absolute Abundance (AbA), Relative Abundance (RA), Relative Size (RS) and Sex Ratio. AbA is the number of individuals reported from the collections obtained by pooling all replicates and levels. RA was obtained by dividing the number of individuals for each site (AbA) by the maximum abundance found at any site within the range (Sagarin and Gaines, 2002a). This was done to

allow reasonable comparisons among sites and species (Enquist et al., 1995; Sagarin and Gaines, 2002b; Rivadeneira et al., 2010).

RS was calculated by dividing the size of individuals (mm) by the maximum size of any conspecific from any site. A Student t-test was used to assess differences in size between the sexes using data pooled for all sites. Sex ratio was calculated as the proportion of males to females (males/females). Chi-squared tests (χ^2) were used to determine whether sex ratio values differed from the expected 1:1 ratio.

To test the predictions of the ACH on abundance, size and sex-ratio, a non-parametric constraint space analysis was used, following procedures used by Enquist et al. (1995) and Sagarin and Gaines (2002a, b). These models are commonly used to describe patterns of abundance of species throughout their ranges (Sagarin and Gaines, 2002a; Sagarin et al., 2006; Rivadeneira et al., 2010). To evaluate whether abundance, size or sex ratio varied with position within the distributional range, a Range Index (RI) was calculated using the expression proposed by Brown and Sagarin and Gaines (Brown, 1995; Sagarin and Gaines, 2002a)

$$RI=2\times(LS)\div R$$

where L is the position (i.e. the distance in km) of a location relative to the northern or western range limit, S is the midpoint (in km) of the geographical range, and R is the extent of the geographical range (km). The RI index ranges between -1 and 1, so that sites with values close to 0 are considered to be near the centre of distribution and values close to -1 and 1 are near the western/northern and eastern/southern edges respectively. The degree of fit of each model (see Fig. 3 for a schematic representation, modified from Sagarin and Gaines [2002a] and Fenberg et al., [2011]) to the observed data was evaluated by calculating the residual sum of squared deviations (RSS) for sites exceeding the constraint boundaries generated by each

model. The significance of the observed RSS values was evaluated by generating 1,000,000 randomized values of RI, RA, RS and Sex Ratio. The fit of the model was considered significant when the observed RSS value was lower than the 5th percentile of the randomized distribution. The degree of support for each model was evaluated by calculating the Akaike's Information Criterion (AIC), selecting all models with Akaike weights > 0.25 (Sagarin and Gaines, 2002a; Sagarin et al., 2006; Rivadeneira et al., 2010). Analyses were carried out using a routine in R (R Development Core Team, 2007). All the works referred to above that describe the ACH consider a North-South range of distribution as the position of sites expressed in Latitude (Sagarin and Gaines, 2002a). I used a scale of kilometres instead, to adapt the expressions to distributions that follow the South African coast, as was done by Tuya et al. (2008) for endemic reef fishes of South to Western Australia. Kilometres were accurately calculated using the Ruler tools in Google Earth® imagery, measuring the distances between sites, from a height of 5 km. The coordinates of the sites recorded in the field (Appendix) were uploaded to assess the exact location of the sampling areas.

A Distance–based Linear Model (DistLM, Legendre and Anderson, 1999) procedure was performed to analyse the relationship between the abundance of sandhoppers and environmental traits, physical variables and indices, i.e. sand temperature, water temperature, salinity, percentage of debris coverage, slope, grain size (Mz and Sand), Tide (maximum spring tidal range), Area, BDI and BI. Slope is reported as log(1/Slope) since it is considered for a good predictor for regional patterns of the abundance of sandy beach fauna (McLachlan and Dorvlo, 2005). Multi-collinearity for the environmental variables was detected between Area and Tide, Area and Slope, Mz and Sand, after examining the Draftsman's plots (Clarke and Gorley, 2006) and Area and Sand were therefore removed.

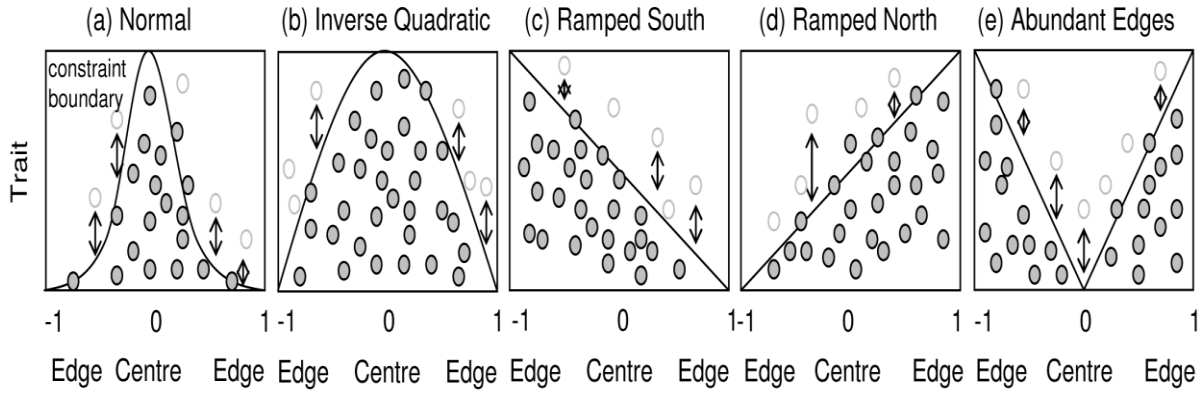

Fig 3 Five Hypothetical models proposed for explain the patterns of distribution of abundance, size and sex ratio along the geographical range of the two species of sandhoppers
Normal model (a), Inverse Quadratic (b), Ramped South (c), Ramped North (d), Abundant edges (e). I calculated the residual sum of square deviations (RSS, deviations indicated by arrows) for the observed data that exceeded the constraint boundary (open dots). The grey dots represent the analysed trait values.

For the model used for the DistLM, I selected the AIC (Akaike Information Criterion), basing the analysis on the Bray-Curtis resemblance measure after square root transformation of the abundance data (Anderson et al., 2008). The data contained a high proportion of zero's and therefore a dummy variable with a value of 0.0001 was added to the Bray-Curtis similarity matrix to moderate spurious similarities where no species were recorded in two compared samples (Clarke and Gorley, 2006). All analyses were carried out using PRIMER (ver. 6.1.12) and PERMANOVA + (ver. 1.0.2) (Clarke and Warwick, 2001; Anderson et al., 2008).

Results

Geographical range and pattern of abundance, size and sex ratio

A comparison between historical distribution data and the results from the present manuscript is summarised in Fig 1a,b. The most abundant species was *A. quadrispinosa*, with a total of 12496 adults collected. Its highest concentration occurred within the centre of its distribution (from Port Nolloth to Cape Columbine, Appendix). *T. capensis* showed high abundances of individuals (total n = 8 398 adults), though 90 % were collected from a single site (Hondeklipbaai, Appendix). For size and sex ratio, *T. capensis* had the largest animals, with a significant difference between the sexes (males: 10.8 ± 1.4; females: 9.9 ± 1.4; t-test, $p<0.0001$), but no significance differences in the proportions of females to males. *A. quadrispinosa* individuals were smaller than *T. capensis*, with no difference between males and females (males: 8.8 ± 2.8mm; females: 8.7 ± 2.7mm). Significant differences in the proportions of females to males ($\chi^2=44.36$; $p<0.005$) were observed. The geographic pattern of relative abundance, size and sex ratio differed between the two species with no predominant pattern (Fig. 4). Sex ratio did not fit any of the models for either species. *A. quadrispinosa* showed the best degree of fit with a ramped pattern explaining abundance (Ramped North; Table 1a, left panel; Fig 4, upper panel) and male and female size (Ramped South; Table 1b, 1c; Fig. 4, middle panel). For *T. capensis*, only the distribution of female size showed a significant fit, with the Inverse Quadratic model (Table 1c, right panel; Fig. 4, middle panel), while abundance, male size and sex ratio did not show any patterns related to any of the tested ACH models.

Environmental domains driving abundance

The results of the DistLM on the environmental factors showed that BDI, Slope, salinity, % of detritus and sand temperature were strong predictor variables in the distribution of the two species (Fig. 5). The DistLM showed the best results for *A. quadrispinosa* abundance

distribution, with 68.7% of cumulative variation explained (dbRDA1 and dbRDA2). Higher priority should be given to the dbRDA1 axis than the dbRDA2 axis, with salinity having the stronger effect. Sand temperature and percentage of detritus cover had a similar, but less strongly correlated relationship with abundance. The analyses also reported an effect of the log(1/slope) on the abundance data of *A. quadrispinosa*. The DistLM showed that the best fit for *T. capensis* abundance and distribution was obtained using three predictor variables, though even combined, these explained very little of the variation in the data cloud (cumulative variation explained 24.5%) : BDI, log(1/slope) and salinity. Abundance was mostly explained by the dbRDA1 (21.1% of total variation) and among the three variables, log(1/slope) was the strongest predictor for *T. capensis.*

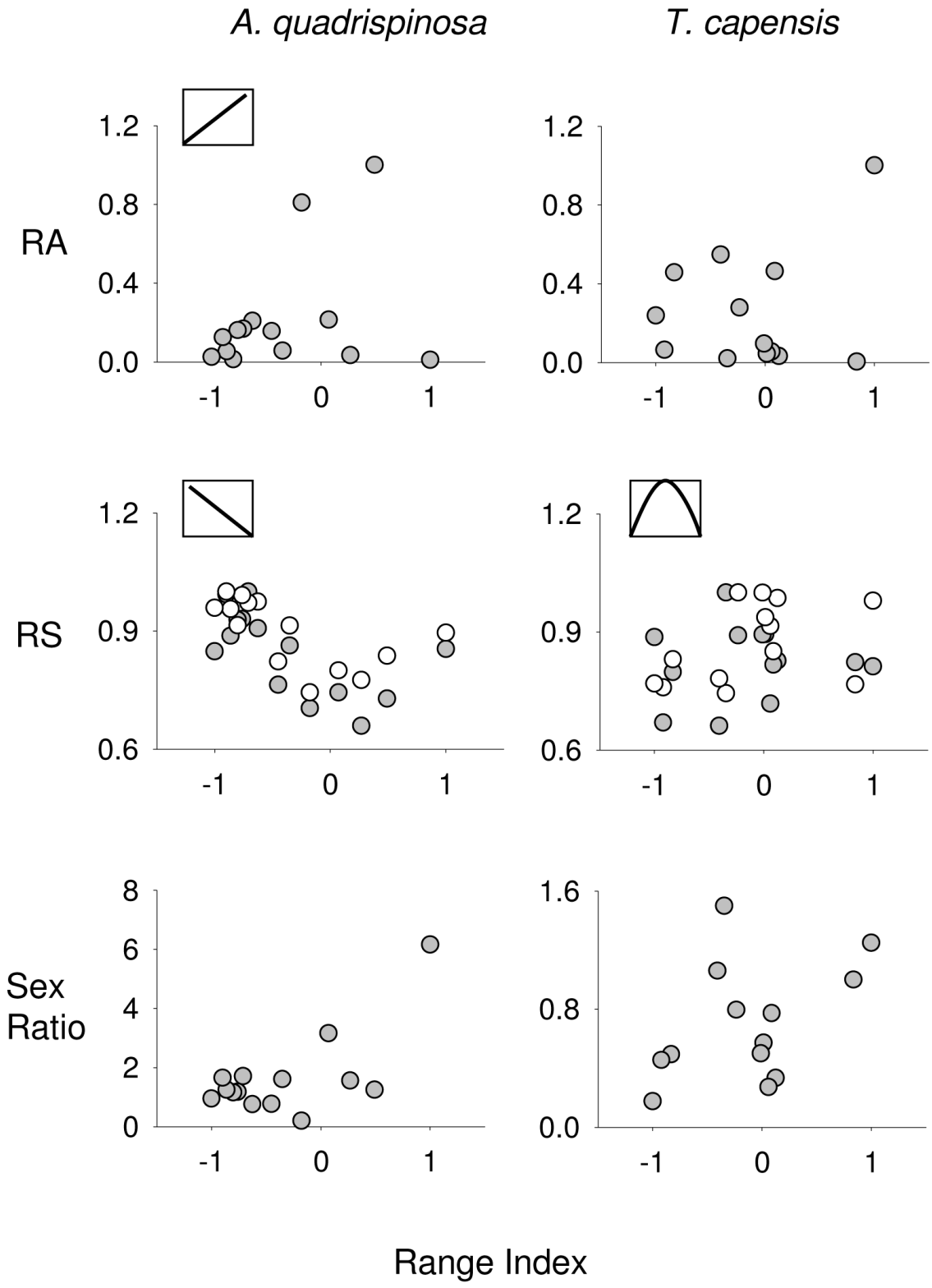

Fig. 4 Pattern of geographic distribution of abundance (upper panel), size (middle panel) and sex ratio (lower panel) for the two species of sandhoppers (top of the figure)
The range is reported as Range Index (see Material and Methods), where -1 = southern/eastern range; 0 = centre; +1 = northern/western range. The model which best fitted the observed is reported as a small icon for each pattern of distribution. The IQ model (see Material and Methods) is referred to the female size distribution. Ra = relative abundance; RS=relative size; filled dots: males; open dots: females.

Table 1. Degree of fit of each models for abundance, male size, female size and sex ratio.
*Significant values for RSS. **higher degree of support for each model (AICwt>0.25) Bold: fitted model No=Normal; I.Q.=Inverse Quadratic; A.E.=Abundant Edges; RN=Ramped North; RS=Ramped South. RSS=residual sum of square; AIC=Akaike Information Criterion; AICwt=AIC weight

	Africorchestia quadrispinosa				*Talorchestia capensis*			
Model	RSS	5^{th} percentile	AIC	AICwt	RSS	5^{th} percentile	AIC	AICwt
(a) abundance								
No	1.0092	0.2927	-76.24	0.00	0.2412	0.0104	-112	0.00
I.Q.	1.0007	0.0427	-76.46	0.00	0.0715	0.0041	-142.4	0.00
A.E.	0.4974	0.0125	-89.93	0.00	0.1282	0.0495	-123.8	0.00
R.N.	0.0701*	0.1385	-142.9	1.00**	0.2402	0.0091	-112.1	0.00
R.S.	1.1308	0.0149	-73.4	0.00	0.0032	0.0010	-220.1	1.00
(b) male size								
No	6.0077	5.2794	-31.65	0.00	2.6125	2.4092	-52.46	0.00
I.Q.	2.6588	2.155	-52.02	0.00	1.9407	1.6826	-59.9	0.19
A.E.	1.6798	1.558	-59.5	0.00	3.4973	2.9577	-41.17	0.00
R.N.	4.8759	3.8633	-36.86	0.00	2.7922	2.5875	-50.8	0.00
R.S.	1.2536*	1.5218	-70.82	0.99**	1.7306	1.4005	-62.76	0.80
(c) female size								
No	6.8369	6.1522	-28.41	0.00	2.4942	2.6886	-53.62	0.01
I.Q.	3.2122	2.6582	-47.3	0.00	1.7786*	1.868	-62.08	0.88**
A.E.	1.9965	1.9814	-55.19	0.02	4.2328	3.3354	-36.4	0.00
R.N.	5.4417	4.6036	-34.12	0.00	3.0313	2.972	-48.75	0.00
R.S.	1.7177*	1.9163	-62.95	0.98**	2.105	1.6629	-57.86	0.11
(d) sex ratio								
No	21.944	17.924	0.7408	0.00	0.3592	0.2792	-102.1	0.00
I.Q.	14.25	12.194	-10.05	0.44	0.0396	0.0541	-157.2	1.00
A.E.	13.101	11.231	-8.155	0.17	1.0152	0.2877	-72.1	0.00
R.N.	17.362	16	-5.114	0.04	0.712	0.3373	-84.96	0.00
R.S.	14.507	10.824	-9.606	0.35	0.1393	0.0412	-125.8	0.00

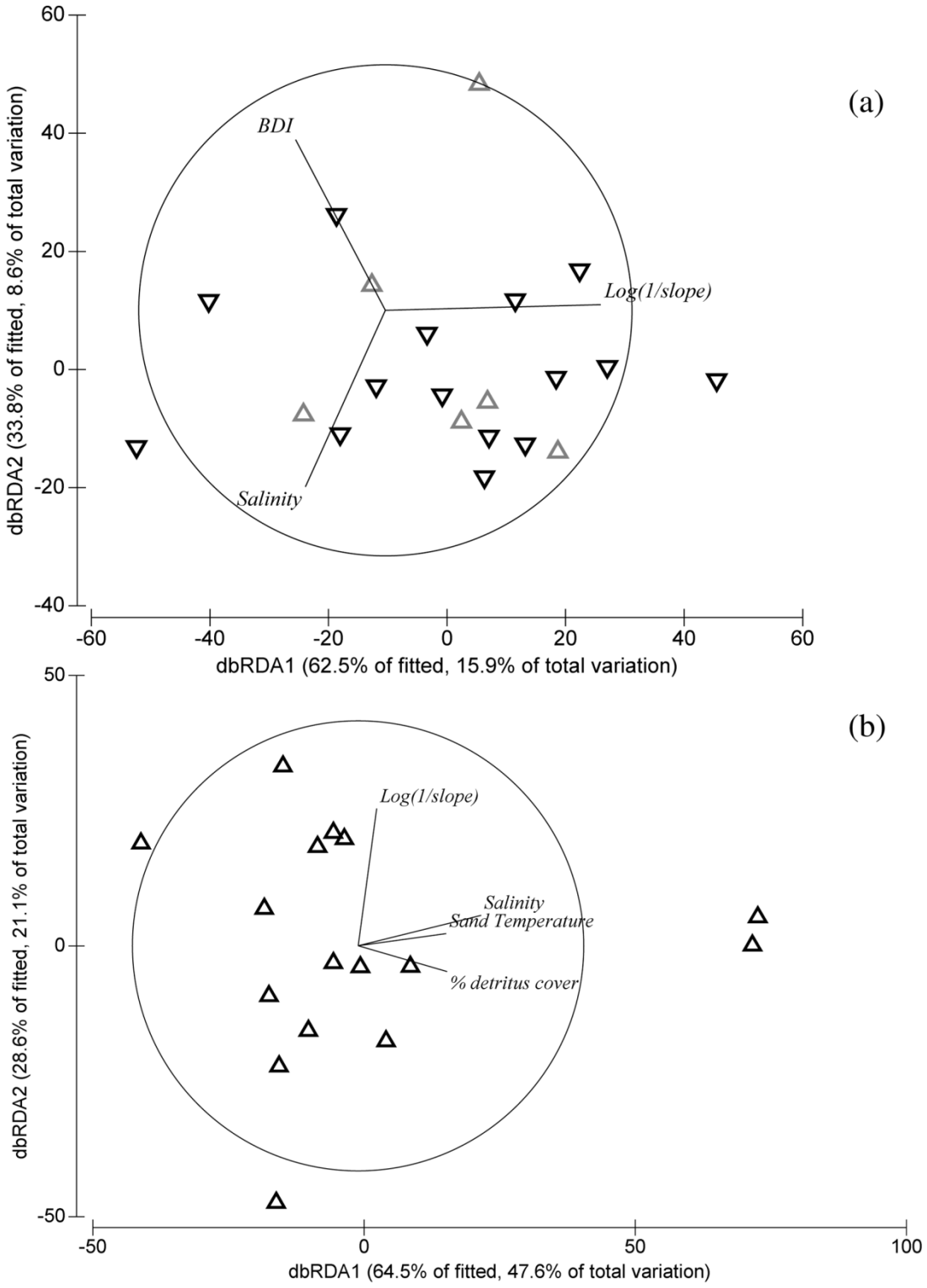

Fig. 5. Distance Based Linear Model (DistLM) of abundance distribution along the southern African coasts of *Talorchestia capensis* (a) and *Africorchestia quadrispinosa* (b).
The plots represent the absolute abundance data for each site of collection. The grey triangles show the sites in the Cool Temperate bioregion; the black triangles, the sites in the Warm Temperate bioregion. The dbRDA values are reported for the first (dbRDA1) and second axes (dbRDA2). The base variables which best explain the distribution of abundance are reported in the graph as vectors. (resemblance: Bray Curtis similarity; transformation: square root; correlation type: Pearson, correlation>0.2)

Discussion

The Abundant Centre Hypothesis postulates the presence of an optimal centre of distribution, where species are more abundant, primarily because environmental requirements are assumed to be optimal in the centre, and degrade towards the margins (Brown, 1984; Enquist et al., 1995). The ACH is based on the fact that both abundance and distribution are driven by biotic and abiotic environmental factors (Enquist et al., 1995) and on the assumption that these environmental requirements are spatially auto correlated, so that sites close to one another are supposed to meet species requirements to a similar degree (Sagarin and Gaines, 2002a). Consequently, sites located far from the "optimal centre" are less likely to meet these requirements (Brown, 1984; Sagarin and Gaines, 2002a).

Sandy beaches are physically dominated systems and extremely variable in space and time (McLachlan and Dorvlo, 2005).The importance of the morphodynamic state of a beach on species richness, abundance, growth and reproduction is a debated argument (Defeo and McLachlan, 2011). The Swash Exclusion Hypothesis states that dissipative beaches have higher species richness, abundance and biomass than reflective ones, consequently a single site (i.e: a single beach) does not necessarily have the same characteristic as an adjacent one (McArdle and McLachlan, 1991). In contrast, the Habitat Safety Hypothesis, which separates supralittoral from intertidal forms, states that supralittoral species (such as sandhoppers) have higher abundances, individual growth, survival and reproduction rates on reflective than on dissipative beaches (Defeo and McLachlan, 2011). Furthermore Gomez and Defeo (2012) found that supralittoral crustaceans increased in abundance from dissipative to reflective beaches in South America, a tendency opposite to that of intertidal animals, which increased from reflective to dissipative.

Considering these fundamental principles, I tested the ACH for the first time on sandhoppers. Our support for the ACH, tested using the distributions of abundance, size and sex ratio for sandhoppers, was equivocal and differed between the two species examined, with the strongest support coming from the most abundant and most widespread species. The unclear pattern of abundance could be associated with the technique used to collect sandhoppers, which probably is not an ideal for testing predictions from the ACH. Even if pitfall traps are the most accurate technique used to capture these creatures in their environment, I recognise this as a possible limitation of the quality of the data. For further investigations concerning the use of large-scale geographical hypotheses explaining the abundance of sandhoppers, I recommend the use of a more reliable technique or even a more accurate design including the replication of the sampling unit within the same beach. The analyses on the environmental variables confirmed the importance of the morphodynamic state of the beach as a fundamental driving-factor for the abundance of sandhoppers. Important here was the fact that, although all our shores were categorised as intermediate in state, within that category, beach slope still had a critical effect of the fauna.

Africorchestia quadrispinosa

Not surprisingly, *A. quadrispinosa* was the species that best fitted the model predictions. The large scale continuous distribution and the north-south orientation of its geographical range, provide a very suitable model to test the ACH.

The distribution of abundance and size of *A. quadrispinosa* followed a ramp-shaped pattern, with animals being more abundant towards the northern limits. Ramped patterns are generally attributed to unexpected changes in habitat or environmental conditions (Brown, 1984; Samis and Eckert, 2007; Tam and Scrosati, 2011). This could explain the rapid changes in abundance among several relatively closely positioned sites on the west

coast of South Africa (from Cape Columbine, to Port Nolloth) and Namibia (from the border to Swakopmund, see Appendix for GPS coordinates).

In contrast, the size distribution of *A. quadrispinosa* was south-ramped, with larger animals, both females and males, towards the southern edge of distribution. The relationship between size and range edges is highly debated, particularly in the case of endotherms, like mammals and birds (Virgós et al., 2011). Some studies suggest that larger individuals occur at the core of the range (in agreement with the ACH) where the habitat is most suitable (Brown, 1984; Sagarin et al., 2006; Meiri et al., 2009). Alternatively, individuals tend to be larger towards higher latitudes. Populations distributed along a North-South axis therefore tend to show larger individuals near one of the edges rather than the core (Goltsman et al., 2005; Meiri et al., 2009). Our results, confirmed this last tendency of larger size at higher latitudes, with *A. quadrispinosa* showing a ramped south distribution of size.

Talorchestia capensis

The size distribution of female *T. capensis*, was best fitted by an Inverse Quadratic model, providing positive support for a centre pattern hypothesis. Females of *T. capensis* were larger in size on temperate sandy beaches than in the sub-tropical and cool-temperate biogeographic regions, a trend also found for sandy shore isopods by Cardoso and Defeo (2004). In general, size is positively related to food availability and quality, which should support the ACH (Virgós et al., 2011). The interaction between beach morphodynamics and sandhopper size and density is usually positively correlated, with dissipative and temperate beaches offering a more suitable habitat, even though a reverse trend has been shown (an increase of size and a decrease of density towards reflective beaches type) for supralittoral crustaceans, in several sandy beaches in South America (Defeo and Cardoso, 2002; Defeo and McLachlan, 2011). The results of the DistLM for *T. capensis*, suggest an

influence of the Slope, BDI and salinity on abundance. Beach morphodynamics interact very tightly with the amount of detritus, with beach morphodynamic state being the fundamental driver operating through its effect on food availability. Two substantial gaps appeared in the distribution of *T. capensis*, making it discontinuous. A gap on the south-coast is explained by the absence of suitable habitat as this stretch of coast forms continuous rocky shores. The 600km gap on the west coast is more difficult to explain as it includes stretches of sandy shore. Sampling over such a large geographic scale necessarily provides only a snapshot of abundances (although the same pattern of distribution as the present one was confirmed by collections for genetic analysis as reported in Chapter 2) and temporal variation could explain unexpected absences from sites. It is not uncommon for sandhoppers to show seasonal changes in their within-shore distribution, as well as geographic differences. Tsubokura et al. (1997) found that sandhoppers burrow more deeply and farther inland during winter, migrating down shore and burrowing less deeply during spring (Tsubokura et al., 1997). In general talitrids are concentrated along the high tide mark, burrowed underneath the largely macrophytes detritus on the shore (Tsubokura et al., 1997), although on the south coast of South Africa *T. capensis* can show higher abundances towards the dune base or even into the dune slacks rather than in the intertidal (Van Senus, 1988). Information on population genetics helped to clarify whether the observed gaps were artifactual by explaining the degree of isolation between population centres (see Chapter 3 for details).

In general, the predictions of the ACH gained little support from the observed data and, consequently, the hypothesis of a general model of an optimal centre of distribution of abundance, size, and sex ratio must be broadly rejected for these southern African sandhoppers. Indeed conformation with the predictions of the ACH has been described as "more the exception than the rule", (Sagaring and Gaines, 2002a, *p.993*) and is often

considered to over-simplify species distributions (Tam and Scrosati, 2011) or to work only for a north-south range of distribution Rivadeneira et al., 2010; Fenberg et al., 2011). Nevertheless, the most suitable test organism, *A. quadrispinosa*, provided the strongest support for the predictions of the ACH. This suggests that the ACH may be applicable, but only in certain cases where organisms are abundant and show clear patterns of distribution. An accepted tool for conservation planning is the development of distribution models able to evaluate species ranges in relation to environmental changes (Elith and Leathwick, 2009).

The present study reported a mesoscale investigation of the biogeography of supralittoral amphipods, which contribute to the biomass of wrack associated macrofauna of sandy beaches and therefore play an important role in the bottom up trophic ecology of sandy beaches (Defeo et al., 2009). Understanding what regulates the boundaries of species range is crucial, especially given predictions of accelerated environmental change (Sagarin et al., 2006) and is particularly relevant for these systems as they are highly dynamic and respond strongly to environmental forcing (Defeo et al., 2009). Environmental effects operate hierarchically and changes perceived by individuals need not be reflected in the dynamics of a species' biogeography. Individual plasticity might therefore be a key factor when investigating the links between the environment and the distribution of organisms (Brown, 1984; Sagarin et al., 2006; Lester et al., 2007; Samis and Eckert, 2007; Rivadeneira et al., 2010; Fenberg and Rivadeneira, 2011).

The DistLM analyses showed that, even within the category of intermediate morphodynamic state, beach slope was particularly important. This is central as it offers an explanation for the weak support gained by to the ACH: individual stretches of beach are highly differentiated from one another, mainly due to physical differences. This is in contrast with the main assumption of the ACH that sites close to one another should

provide similar environmental conditions (Brown, 1984). The DistLM also showed an important effect of salinity on the distribution of the abundance for both species. Salinity is a fundamental factor for sandhoppers as they have colonised terrestrial environments, which requires extreme physiological adaptations (Morritt and Richardson, 1988). A concurrent variation in salinity and sedimentological variables are fundamental in shaping the spatial distribution of abundance in sandy beach macrofauna (Lercari and Defeo, 2006).

Although *T. capensis* abundance occurs on both the west and east coasts, encompassing three biogeographic regions with widely different temperature regimes, the distribution of *A. quadrispinosa* suggests that temperature is an important factor in shaping distributions and range limits as its southern limit of distribution ends in an area which is often considered as a transition between the cool temperate and warm temperate regions (Harris et al., 2011; Turpie et al., 2000). In accord with this, sand temperature was linked to the distribution of *A. quadrispinosa* abundance in the DistLM. Nevertheless, temperature generally had little effect in these analyses and this could be attributed to our methodology which provided only an instantaneous measure of temperature which is a much less integrated variable than salinity. Further investigations of the effects of temperature should include an integrate estimate of temperature using temperature data loggers, if possible (Booth and Freeman, 2006). Subsequent analyses of the thermal tolerances of these species helped to clarify the complex dynamics that drive their biogeographic ranges and make possible predictions of how these may shift (see chapter 4 for further details).

Despite the weak support for the ACH provided by these sandhoppers, the importance of environmental parameters in driving their distributions was clear, especially in systems and spatial scales where the abundance of animals is more related to physical than biological factors. One limiting and simplistic aspect of this and other tests of the ACH is the focus

on adult organisms and it is possible that the integration of environmental effects on different life stages and reproduction (which might generate maternal effects, see Chapter 5 for details), would clarify the synergies and/or constraints that result in the distribution of organisms along latitudinal gradients.

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-Chapter 3-

Population structure and connectivity of separated populations of Talorchestia capensis (Amphipoda, Talitridae)

Introduction

In marine systems, connectivity is defined as the exchange of individuals among geographically separated subpopulations that survive and reach reproductive maturity (Moilanen and Nieminen 2002; Warner and Cowen 2002; see Pineda et al., 2007, for the definition of reproductive population connectivity). Connectivity includes dispersal, settlement and post-settlement processes and it is linked to the concept of metapopulation, where landscapes are viewed as a network of habitat patches in which species occur as discrete local populations connected by the migration of individuals (Di Bacco et al., 2006). Dispersal contributes to the degree of genetic connectivity and, depending on the scales of physical transport/rafting (McQuaid and Phillips, 2000; Thiel and Haye, 2006), it affects the geographical patterns of genetic structure (phylogeography) within a species providing invaluable insights into historical and modern connectivity (Pineda et al., 2007). Intraspecific geographical discontinuities are often mirrored by genetic discontinuities among populations (Benzie, 1999). Such breaks occur where past and/or present-day barriers to dispersal allow genetic differentiation and prevent homogenisation of gene flow within a species (Teske et al., 2011a). In the marine realm, oceanographic conditions are the major drivers of phylogeographic breaks (Collin 2001; Sanford et al., 2003; Tellier et al., 2009; Zakas et al., 2009), although differences in abundance, timing of reproduction, life history traits, population and recruitment dynamics, and physiological traits can also regulate such discontinuities (Broitman et al., 2001; Rivadeneira et al., 2002; Ragionieri et al., 2009; Kelly and Palumbi, 2010; Carson et al., 2010; McQuaid, 2010). In coastal areas, phylogeographic breaks in marine organisms have mostly been associated with strong environmental gradients, like currents and upwelling cells, which all interfere with along-shore dispersal (Pelc et al., 2009; Brante et al., 2012; Teske et al., 2011a).

In South Africa, coastal species are often divided into genetic lineages whose distributions are linked with the marine biogeographic provinces of southern Africa (Teske et al., 2007; 2009; 2011a). The major breaks have been identified at three main localities that, in most cases, coincide with the disjunctions between such provinces (Fig. 1; Teske et al., 2011a). The break separating the south-western from the south-eastern provinces seems to be located between the Cape Point and Cape Agulhas (Western Cape, South Africa), where most of the species showed distinct lineages (Evans et al., 2004; Teske et al., 2007; Von der Heyden et al., 2008). The south-east break differs considerably among species and is located somewhere between Algoa Bay and the Wild Coast, in the Eastern Cape (Zardi et al., 2008; Teske et al., 2009). A third break separating subtropical from tropical lineages has been identified at the St Lucia estuary, QuaZulu-Natal (Teske et al., 2007; 2009).

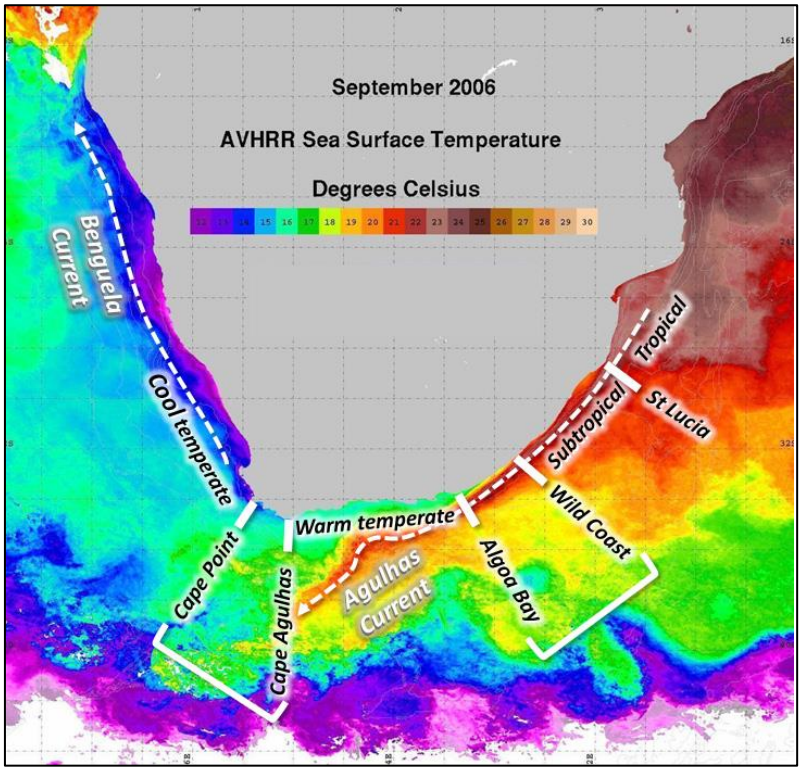

Fig. 1 Southern African oceanography and coastal phylogeographic breaks
Main currents flow along the coasts of Southern Africa (Benguela and Agulhas currents) influencing the coastal temperature regime. The warm Agulhas current flowing from east to west contributes to create three main biogeographic provinces (Tropical, Subtropical and Warm-temperate). The Benguela current flow northward along the West coast of South Africa and contribute to create the Cool temperate bioregion. Transition area occur in the cape region (from the Cape Point to the Agulhas). Phylogeographic breaks (white solid line) are often associated with the oceanography of South Africa and, in most of the cases, coincide with transition areas between provinces.

Despite this general pattern, differences in the positions of phylogeographic discontinuities are common among South African marine invertebrates (Teske et al., 2011a), especially between planktonic species and direct developers (Teske et al., 2007). Teske and coauthors (2007) comparing population structure, demographic history and gene flow of five South African invertebrates with contrasting dispersal potential (long-lived planktonic larvae, abbreviated larval development and direct developers) found lower levels of genetic structure among planktonic species than direct developers. Conversely, direct developers showed the lowest rates of gene flow, confirming the low dispersal ability of these animals (Teske et al., 2007). Generally, direct developers, lacking a larval dispersal stage, show a geographical distribution range that is wider than species with planktonic larvae (Thiel and Haye, 2006). Such a pattern is common between sympatric, congeneric species with contrasting developmental modes (direct vs indirect), such as two species from the genus *Littorina* in the North Atlantic, which show differences in the extent of their respective geographical range (Johannesson, 1988). The reasons why direct developers often show wider ranges than planktonic larvae, despite a lower dispersal potential (Kelly and Palumbi 2010), have been attributed to their rafting dispersal (Thiel and Gutow, 2005a, b) facilitated by current systems and oceanographic features (Thiel and Haye, 2006). In this way, animals, which have undertaken an evolutionary road away from truly marine environment, can still survive in the oceans and transported by currents (Persson, 2001).

Currents and oceanographic features however, still remain the major forces to drive dispersal and marine connectivity (Pineda et al., 2007). Passive dispersal of brooders (direct developers) is a transport of juveniles and/or adults over short or long distances through patches of rafting material, allowing animals to massively and simultaneously colonise new geographic areas, maintaining high population connectivity (Waters and Roy 2004, Donald et al., 2005, Fraser et al., 2009, 2011; Nikula et al., 2010; Haye et al., 2012).

Waters and Roy (2004) proposed that the widespread distribution of the sea star *Patiriella exigua*, showing a non-monophyletic African lineage and a monophyletic Australian group, was due to a Pleistocene eastward dispersal across the entire Indian Ocean, probably achieved by rafting on wood or macroalgae. Rafting facilitates low to moderate levels of gene flow between populations of marine benthic brooders (Thiel and Haye, 2006) and taxa for which rafting had been inferred as a potential dispersal mechanism often displayed high levels of connectivity, a defined population structure and no Isolation by Distance (IBD, Le Gac et al., 2004; Colgan et al., 2005; Hart et al., 2006; Haye et al., 2012).

The intensity and the periodicity of the rafting episodes are also relevant in influencing the dispersal and ultimately the genetic structure of marine populations (see Thiel and Haye, 2006). Based on the frequency of rafting episodes, three main rafting routes can be distinguished: frequent, intermittent and episodic. Frequent routes occur at local scales (bays, lagoons and estuaries), are typically facilitated by substrata of biotic origin (seagrass, saltmarsh vegetation, intermediate-sized algae and mangroves) and are relatively independent of currents. Intermittent routes are found along temperate continental shores, facilitated primarily by giant kelps which are transported mainly by means of currents. Episodic rafting routes, occurring over a large scale in the open ocean, are facilitated by volcanic pumice, floating trees and occasionally by giant kelps when these are pushed beyond intermittent routes by strong winds or currents (Thiel and Gutow, 2005a; Thiel and Haye, 2006). For several direct developers rafting on kelp has been proposed, demonstrating that the extent of their wide geographic distribution may be strongly dependent from kelp-mediated rafting routes and coastal currents (Knight-Jones and Knight-Jones 1984; Helmuth et al., 1994; Haye et al., 2012).

Sandhoppers, supratidal amphipods of the family Talitridae, are direct developers showing low active dispersal ability (Dahl, 1946; Wildish, 1970), with dispersal usually assigned to rafting (Persson, 2001). Sandhoppers are often associated with wrackbed or any organic material present on the shore, where optimal temperature and humidity ensure suitable habitats to survive and successfully reproduce (Wildish, 1970). Such ephemeral habitats are considered an important source of floating materials, facilitating dispersal by means of rafting (Thiel and Gutow, 2005a, b). The active parental care of these animals, which persists even after juveniles are released in the maternal brood (Morritt and Spicer, 1996a, b, c; Morritt and Richardson, 1988), could facilitate juveniles' survival to prolonged journeys on rafting materials (Thiel, 1999; Thiel and Haye, 2006). The sandhopper *Talorchestia capensis*, shows a widespread distribution along the South African coast (Baldanzi et al., 2013), which encompasses four South African Bioregions (as defined by Lombard, 2004).

In the present study, I investigated the population structure and gene flow of several populations of *T. capensis* using mitochondrial DNA as molecular marker. As for many sandhoppers, it is expected that *T. capensis* should show a defined population structure (Pavesi et al., 2011; 2012; 2013) and a phylogeny which should be linked with South African's biogeographic regions, as reported for most of the marine invertebrates (Teske et al., 2011a).

I hypothesise that separated populations of *T. capensis* should show connectivity and no IBD, a reflection of the dispersal mode proposed for these animals and the nearshore current systems of South Africa. Currents should maintain sufficient migration rates among geographically separated populations, especially in the South-East coasts due to a bidirectional currents system: the wind-driven eastwards currents flowing nearshore and

the net westward Agulhas flowing offshore. Such a pattern would be in agreement with several authors for South African coastal invertebrates (reviewed by Teske et al., 2011a).

Material and Methods

Study area and animal collection

Animals were collected from 17 locations along the South African coast, encompassing three bioregions (Fig 1): the sub-tropical, warm temperate, and cool-temperate. Localities within the Cape region (from Cape Point to Agulhas) are often considered to be in a transition area between the warm and cold temperate regions (Teske et al., 2011a). Locations were chosen on the basis of accessibility and the presence of animals (see below). Figure 2 shows the locations and the South African current systems, Agulhas and Benguela Currents, which dominate the south-east and the south-west coasts, respectively. GPS coordinates are reported in Appendix. Ten to thirty individuals per location (depending on available specimens) were collected by hand and stored in 100% ethanol (sex was not considered). The collection period ranged between 2012 and 2013.

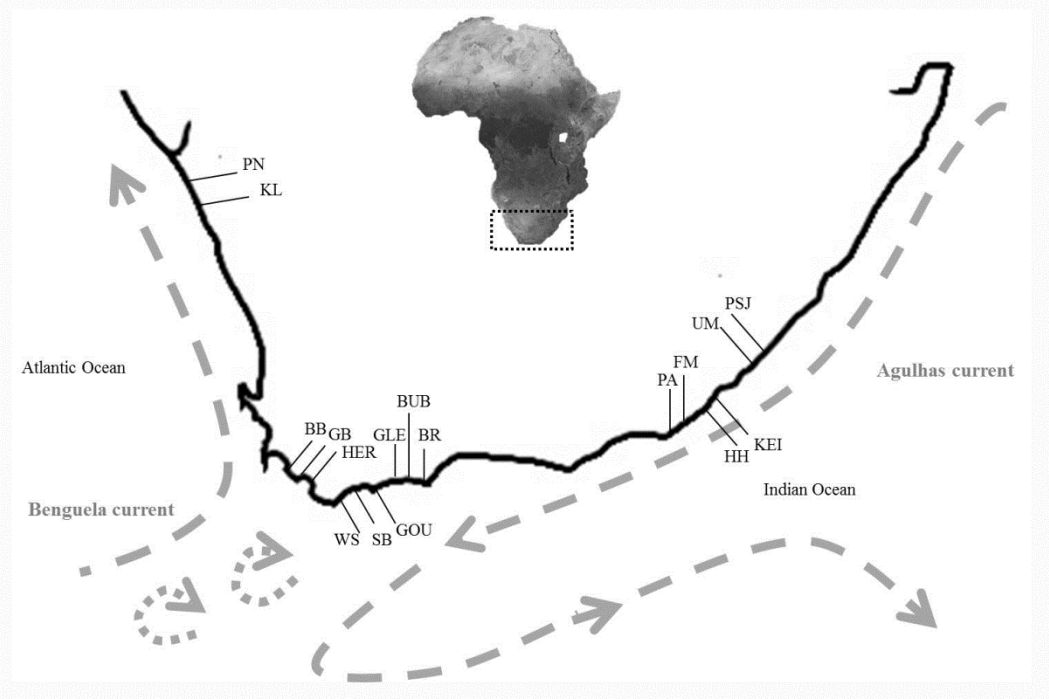

Fig. 2 Sampling locations along the South African coasts
PN:Port Nolloth; KL: Kleinsee; BB: Betty's Bay; GB: Gansbaai; HER: Hermanus; WS Witsand; SB: Still Bay; GOU: Gouritzmond; GLE: Glentana; BUB: Buffalo's Bay; BR: Brenton on Sea; PA: Port Alfred; FM: Fish River Mouth; HH: Haga Haga; KEI: Kei Mouth; UM: Mngazana; PSJ: Port St. Johns. The Agulhas and Benguela Current systems are represented by the grey dashed arrows.

DNA extraction, amplification and sequencing

DNA was extracted from the telson of the animals following a CTAB (cetyltrimethyl ammonium bromide) method. All the available animals were used as replicates for each population, in order to maximise the number of individuals from which to extract DNA. A portion of the mitochondrial cytochrome oxidase c subunit I gene (mtDNA, COXI) was amplified using the polymerase chain reaction (PCR). The forward primer CrustCOIF 5'-TCA ACA AAT CAY AAA GAY ATT GG-3' and reverse primer PeracCOIR 5'-TAT WCC TAC WGT RAA TAT ATG ATG-3' (Teske et al., 2007) were used to amplify all the specimens for all populations. The PCR profile comprised an initial denaturation step of 3 min at 94°C; 35 cycles of denaturation (30 s at 94°C), annealing (45 s at 48 to 50°C) and extension (75 s at 72°C); and a final extension step of 10 min at 72°C. PCR products were purified and cleaned with a DNA clean up kit (MSB® Spin PCRapace, Stratec®), cycle-sequenced both in the forward and reverse direction using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystem) and sequenced on an ABI 3100 genetic analyser. The sequences were edited using the software SEQUENCHER v4.8 (Gene Code Corporation, Ann Arbor, MI, USA) nucleotides in length were obtained and edited sequences were aligned using MEGA v5.05 (Tamura et al., 2011).

Data analysis

A phylogenetic tree was reconstructed to identify genealogical relationship among haplotypes using the Maximum Likelihood method (ML) and clade support was obtained from 1000 bootstrap replicates. Evolutionary distances were computed using the Tamura 3-

parameter method, after testing for the most appropriate using the software JModelTest2 (Guindon and Gascuel, 2003; Darriba et al., 2012). Models with the lowest BIC scores (Bayesian Information Criterion) were considered to describe the best substitution pattern. A Neighbor-Joining (NJ) and Maximum Parsimony (MP) analyses were also performed to obtain improved confidences in the genealogical relationships, using the Tamura3-parameter method as above. Clade support was obtained from 1000 bootstrap replicates, for both analyses. Genealogical relationships were performed using MEGA v5.05 (Tamura et al., 2011). An additional Median-Joining (MJ) haplotype network of mtDNA (COXI) haplotypes was drawn to identify grouping among the populations and examine haplotypes frequencies implementing a Maximum Parsimony calculation to eliminate non-parsimonious links among haplotypes (Polzin et al., 2003). The network was generated using the software NETWORK v4.6 (Fluxus Technology, Ltd; www.fluxus-engineering.com).

Differentiation among groups of populations was estimated by the analysis of molecular variance (AMOVA) as implemented in Arlequin v3.5 (Excoffier et al., 2005). The separations among groups were chosen *a priori* on the basis of previous phylogeographic studies carried out on southern African coastal invertebrates (see review by Teske et al., 2011a and references therein), which revealed two main phylogeographic breaks at Cape Agulhas and Algoa Bay (Fig. 1). Populations were grouped as follow: a western group including PN, KL, GB, HER and BB; a southern group, including SB, WS, GOU, BUB, GLE, BRE; a eastern group, comprising PA, FM, KEI, PJ and UM. The population of Haga Haga (HH) was excluded because comprising only one individual, thus permutations performed by AMOVA, were not possible. F*st* indices (Excoffier et al., 1992) values were calculated using haplotypes frequencies and Tajima and Nei distance method applied, as suggested for unequal nucleotide frequencies (Tajima and Nei, 1984). Significance of the

fixation indices, under the null hypothesis of no differentiation among groups, was tested using a non-parametric permutation approach (10,000 permutations of haplotypes among populations). In this case, the P value of the test is the proportion of permutations with F*st* values larger or equal to the observed one (Excoffier et al., 1992). Pairwise population comparisons using the Tajima and Nei method were also performed among all groups of populations. Analyses were carried out using the software Arlequin v3.5 (Excoffier et al., 2005).

The F_{ST} matrix resulting from the pairwise comparisons was used to perform a Mantel (1967) test to evaluate the possible relationship between geographic and genetic distances. Isolation by distance was checked for all populations and also within each geographically distinct group resulted by the phylogenetic analyses (see Fig 4 for the grouping/lineages). Geographical matrices were created based on log-transformed geographical distances among locations (Slatkin, 1993), retrieved from Google Earth imagery. Coastline distances among locations were calculated using the ruler tool from an altitude of approximately 5 km. The correlation between the two matrices, based on geographic and genetic data, was computed with 20,000 permutation iterations using the software MANTEL v1.19 (Cavalcanti, 2005).

To investigate gene flow among lineages, estimates of migration rates were calculated using a Bayesian approach and the COX1 sequences, implemented in the software MIGRATE-N v.3.216 (Beerli and Felsenstein, 1999; Beerli, 2009). All populations were grouped (see Results for groupings) and the migration rate from and towards each group was derived from its mutation-scaled migration rate value ($M=m*\mu$). This parameter represents the importance of variability brought into the population by immigration compared with the variability created by mutation (Beerli, 2009). Migration rates were first estimated running a full migration matrix model in which gene flow was unrestricted

among groups ("Panmictic" model, Fig. 3a) to determine whether gene flow could underpin a lack of isolation by distance among lineages. A more restricted model including gene flow among only adjacent populations was also considered ("Adjacent model", Fig. 3b): in such a model, migration among the S-E and the S-W groups were not considered, and only unrestricted migrations between S and S-E and between S and S-W were allowed. All computations were performed with two long Markov chains of 10 million generations and a static four-chain scheme with default heating values. Default uniform priors and slice samplers were used for the M (migration rates) and θ (population size) parameters, and starting run values were estimated from the F*st* measure as computed by the software. The effective numbers of migrants per generation were obtained by multiplying the θ parameter of each group by their migration rate (M) To estimate the best model. Logarithmic marginal likelihood scores (Bezier logML) for all migration models were obtained using/calculating? a thermodynamic integration with Bezier approximation (Gelman and Meng, 1998), as implemented in the software. Direct comparison of models was then assessed by transforming these likelihood scores into Log Bayes Factors (LBF) and Bezier Probabilities (BP), both performed using the method described in Beerli and Palczewski (2010)

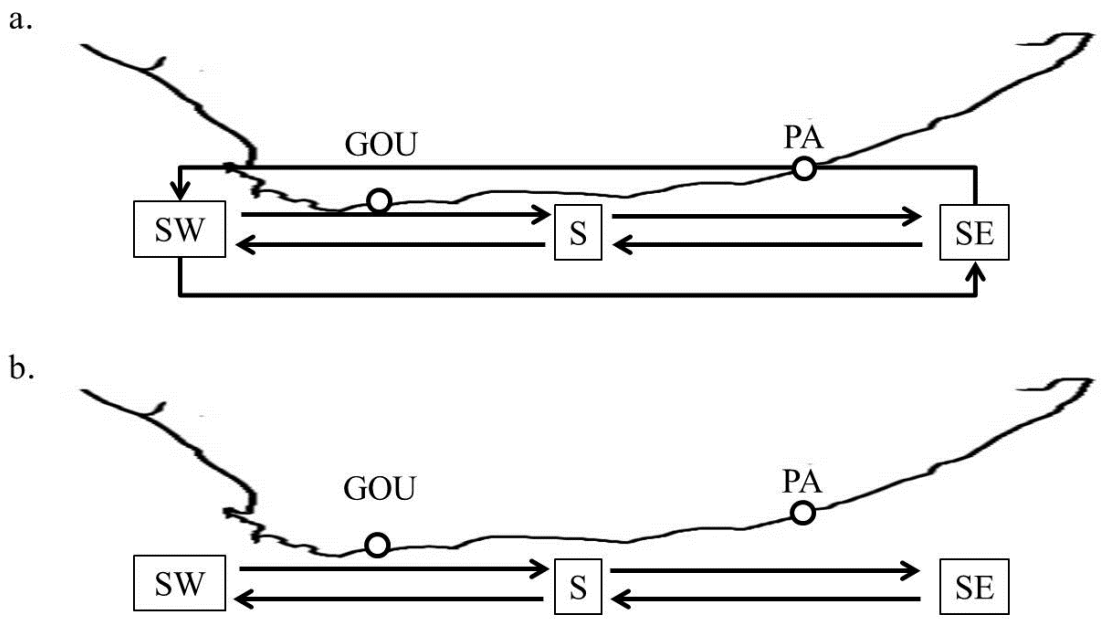

Fig. 3 Models proposed to estimate gene flow among lineages
The graphical scheme shows the two proposed migration models among the three lineages along the South African coastline. Models are: a. "Panmictic" model; b. "adjacent" model (see text for details). Arrows indicate the direction of migrants. SW=South-West lineage; S=South lineage; SE=South-East lineage. GOU and PA indicate the approximate separation among geographical distinct groups.

Results

The fragments had an A-T rich nucleotide composition (C =17.6%; A=25.9 %; T=39.3%; G=17.2%), as reported for other arthropod mitochondrial DNA (Simon et al., 1994). A total of 169 variable and 104 parsimony-informative sites were obtained from 80 individuals sequenced for a 585 pb fragment of COX1, resulting in 50 haplotypes. The overall average number of differences between pairwise haplotypes was 0.046. Not all the individuals collected were finally sequenced, as shown in Fig. 2.

Phylogenetic relationships among individuals from different populations were obtained from the phylogeny trees (Fig. 4). The ML tree showed three main lineages of *T. capensis*: a South-West (S-W) lineage, including individuals from the West coast (Port Nolloth and Kleinsee), the Cape region (Gansbaai, Hermanus and Betty's Bay) and the South coast (Witsand, Still Bay and Gouritsmond); a South (S) lineage, including individuals from the

South coast (Glentana, Buffel's Bay and Brenton-on-Sea) and individuals from Witsand and Still Bay (clustered within S-W) and a South-East (S-E) lineage (Port Alfred, Fish River Mouth, Kei Mouth, Mngazana and Port St. Johns) which also included individuals from the S-W (Still Bay and Gouritsmond) and S lineages (Glentana) (Fig. 4). The bootstrap replicates supported the separation of the main clades well, with the ML calculation giving the highest support (Fig. 4). Overlapping among the three lineages appeared to be approximately between Gouritsmond and Witsand on the South Coast, where individuals from S-W lineage shared haplotypes with both the S and S-E lineages (Fig. 4). The MJ haplotype network (Fig. 5) also showed three divergent lineages, confirming the genealogy reconstructed from the ML tree (Fig. 4). In the network, the S-W lineage showed the highest number of shared haplotypes (29 individuals sharing six different haplotypes), while the S-E lineage showed nine individuals sharing three haplotypes. In the S lineage only one haplotype was shared among three individuals.

Haplotypes were extensively shared among localities within each lineage (Fig. 4). An example comes from one haplotype shared by three individuals of the S-E lineage which are found in localities that should belong to the S and S-W lineages (Fig. 4). The AMOVA results based on the three groups defined *a priori* are reported in Table 1. The majority of variation explained was among the three groups (53.82%), while 28.36% was within populations. Among population variation within groups explained 17.82% of the variation. The AMOVA results were significant for all sources of variation ($p < 0.0001$). The fixation indices were as follow: F*sc*=0.38; F*st*=0.71; F*ct*=0.53. Pairwise F*st* values are reported in Table 2, together with their significance. The pairwise show a high differentiation among localities with populations from separate groups showing the highest F*st*. A Mantel test performed on all populations showed no statistical relationship between genetic and geographic distances (R=0.569; p=1.000). Within geographical distinct groups analyses

also showed no significant isolation by distance (S-W: R=0.549, p=0.99; S-E: R=0.08, p=0.36; S: R=0.598, p=0.99).

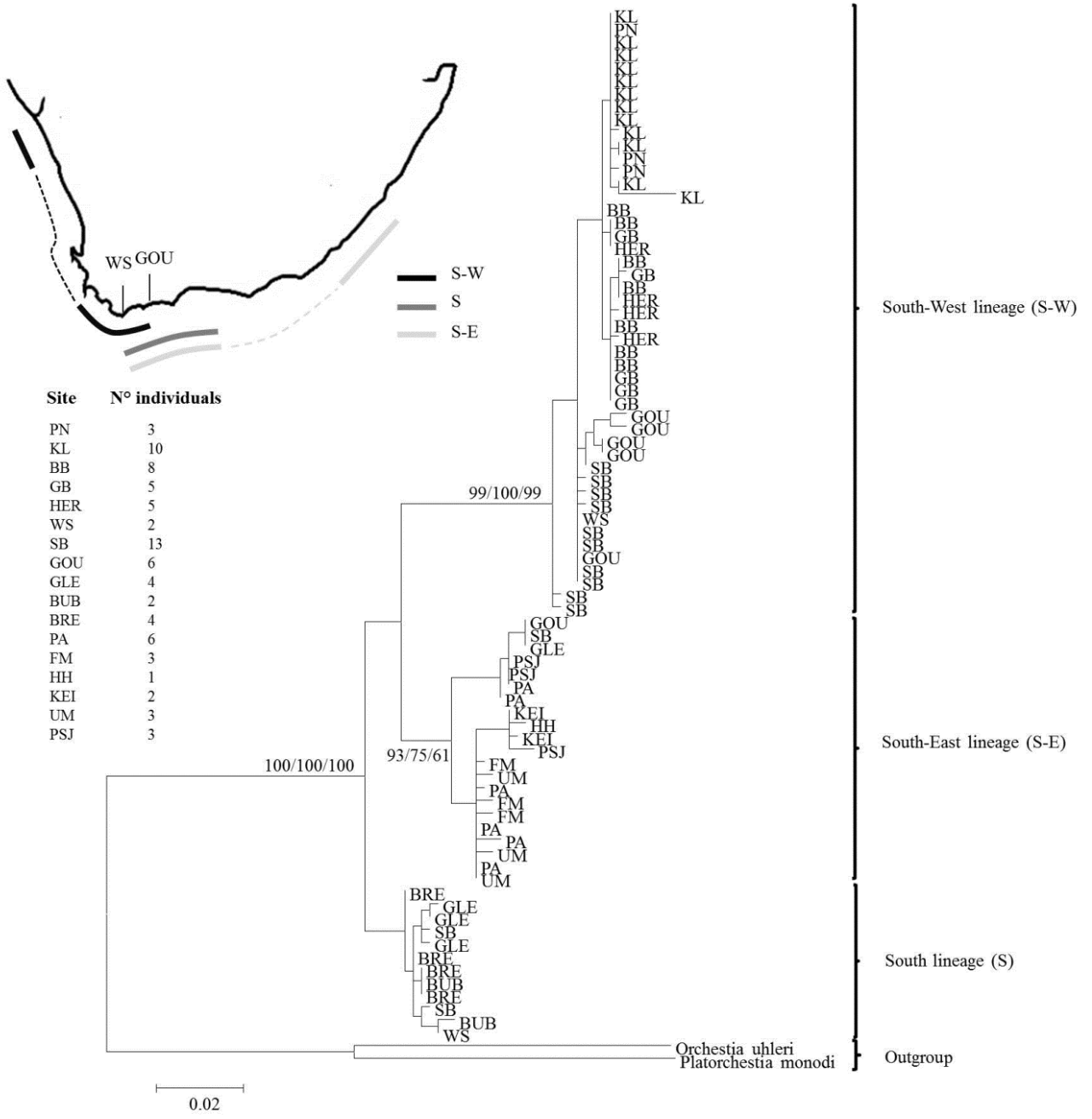

Fig. 4 Maximum Likelihood phylogenetic tree of mtDNA CO1 region of *T. capensis* Evolutionary model to infer phylogenetic relationships were computed using the Tamura 3-parameter method. Branch lengths are in the same units as the evolutionary distances. The percentages of bootstrap replicates in which the associated taxa clustered together are shown on the nodes (minimum accepted threshold: 75%). Maximum Likelihood, Neighbour Joining and Maximum Parsimony bootstrap percentages are shown as follows: ML/NJ/MP. The left upper quadrant shows the South African coastline with the distribution of the clades. Solid lines are the effective locations of the clade; dashed lines represent the expected locations but from which sequences were not retrieved. The terminals represent individuals, which are also reported in the table below the map. Refer to Figure 2 for location code.

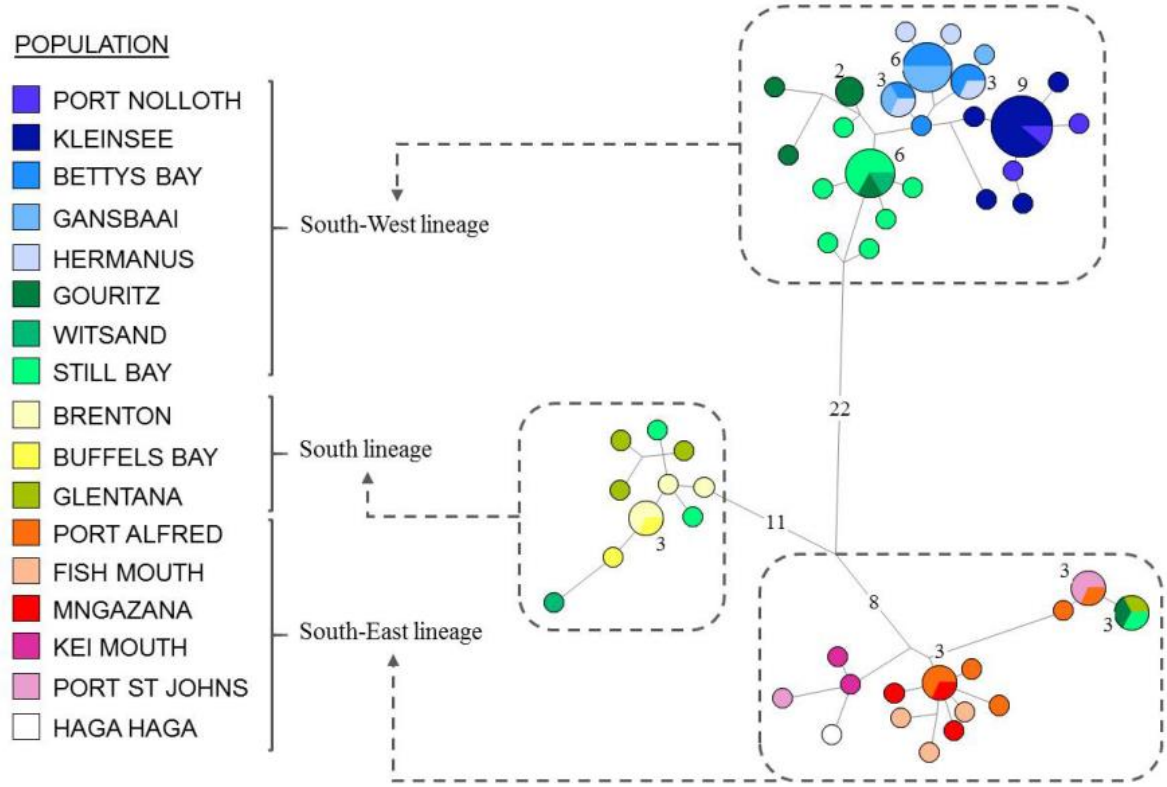

Fig. 5 MJ haplotype Network of mtDNA CO1 region of *T. capensis*
Haplotypes are shown as pie charts in which size is proportional to the number of individual sharing that haplotype. Numbers of individuals greater than 1 are reported next to each shared haplotype. Haplotype colours are shown in the legend on the left of the graph; each colour corresponding to different population and geographical origin. Number of mutational steps are reported for the three main branches forming the three lineages. All branches lengths are proportional to the number of mutational steps.

Table 1. Analysis of molecular variance (AMOVA) of population structure of *T. capensis*
d.f.=degree of freedom; SS=sum of squares. ***=p<0.0001

Source of variation	d.f.	SS	Variance components	Variation	p-values
Among groups	2	448.1	Va = 7.96	53.82%	***
Within groups	14	211	Vb = 2.63	17.82%	***
Within populations	63	264.4	Vc = 4.19	28.36%	***

Table 2: AMOVA pairwise F*st* values among populations of *T. capensis* and F*st* p-values

Pairwise F*st* values for all population comparisons are reported below the diagonal with the significant p values indicated in bold (p<0.05). Values on the diagonal (0.00) represent absence of genetic variance (F*st*=0.00) between individual of the same location. KL: Kleinsee; PN: Port Nolloth; HER: Hermanus; GB: Gansbaai; BB: Betty's Bay; GOU: Gouritzmond; WS Witsand; SB: Still Bay; BUB: Buffalo's Bay; GLE: Glentana; BR: Brenton on Sea; PJ: Port St. Johns; KEI: Kei mouth; UM: Mngazana; PA: Port Alfred; FM: Fish River Mouth.

Population	KL	PN	HER	GB	BB	GOU	WS	SB	BUB	GLE	BRE	PJ	KEI	UM	PA	FM
KL	0.00															
PN	0.06	0.00														
HER	**0.41**	0.40	0.00													
GB	**0.44**	**0.47**	0.18	0.00												
BB	**0.43**	**0.50**	0.15	-0.15	0.00											
GOU	**0.95**	0.95	0.93	**0.95**	**0.95**	0.00										
WS	**0.87**	**0.77**	**0.77**	**0.80**	**0.83**	-0.02	0.00									
SB	**0.95**	**0.96**	**0.94**	**0.95**	**0.96**	0.18	0.09	0.00								
BUB	**0.66**	0.29	0.34	**0.43**	**0.54**	0.00	0.07	0.35	0.00							
GLE	**0.30**	0.13	0.13	0.16	**0.19**	**0.55**	**0.50**	**0.59**	-0.08	0.00						
BRE	**0.34**	0.11	**0.13**	**0.18**	**0.23**	**0.63**	**0.56**	**0.70**	0.01	-0.03	0.00					
PJ	**0.90**	0.82	**0.82**	**0.85**	**0.88**	0.65	0.34	**0.75**	0.29	**0.53**	**0.57**	0.00				
KEI	**0.95**	0.96	0.93	0.95	**0.96**	0.94	0.54	0.95	0.36	**0.58**	**0.63**	0.31	0.00			
UM	**0.94**	0.94	**0.93**	**0.94**	**0.95**	0.91	0.60	**0.93**	0.50	**0.60**	**0.66**	0.39	0.70	0.00		
PA	**0.90**	**0.85**	**0.85**	**0.86**	**0.88**	**0.75**	**0.53**	**0.79**	**0.53**	**0.60**	**0.66**	0.05	0.37	0.04	0.00	
FM	**0.94**	0.93	**0.91**	**0.93**	**0.94**	0.88	0.59	**0.91**	0.48	**0.60**	**0.66**	0.38	0.63	0.09	0.10	0.00

Migration rates among the three geographical distinct groups were tested to see whether the lack of isolation by distance could be explained by high levels of gene flow among populations (Fig. 6). The unrestricted model showed gene flow among groups of populations with more than one individual per generation (except for the migration between S-W and S-E, m=0.7) indicating high historical connectivity among all populations (Mills and Allendorf, 1996). The model showed a West to East pattern of migration between S-W and S groups, an East to West between E and S-E groups and between distant areas (S-W and S-E) (Fig. 6a). Comparison among the two proposed models however, indicated that the "Adjacent" model (Bezier logML=-3401.59; LBF=0; BP=1) was more appropriate than the "Panmictic" model (Bezier logML=-3492.66; LBF=-182.14; BP=2.811 x 10^{-40}) in explaining the migrations among groups. Such model (Fig. 6b) showed an unidirectional Eastward migration between S-W and S groups, while a bidirectional flow between S-E and S groups.

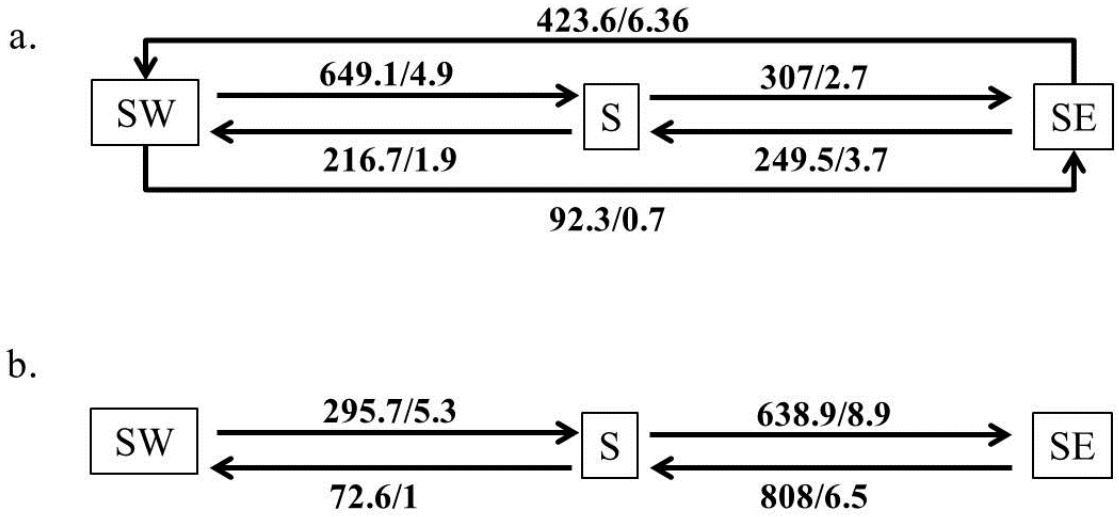

Fig. 6 Migration rates and number of effective migrants per generation for the two migration models
(a) "Panmictic model. (b) Adjacent model. Migration rates (M) and number of effective migrants per generations (m) are reported next to each migration direction (arrows) in the form of "M/m". The most appropriate model obtained is the "adjacent" model (b.)

Discussion

The present study investigated the genetic structure (based on mtDNA) and connectivity of the sandhoppers *Talorchestia capensis* along the coasts of South Africa. Sandhoppers, as direct developers (which lack an active planktonic stage) generally show low dispersal ability resulting in a highly defined population structure (Kelly and Palumbi 2010). *Talorchestia capensis* showed a clear genetic structure characterised by three potential separated lineages (with the highest variation explained among groups (see Table 1). Such pattern agrees with the "biogeography and phylogeography concordance hypothesis" (Avise et al., 1987) which suggests that the same factors (such as nearshore currents or estuary discharges) defining species distribution may also shape genetic boundaries within species (Avise et al., 1987). In Mediterrean populations, however, a defined population structure in sandhoppers seems to be species-specific. For example, *Macrorchestia remyi* showed very little genetic structure (De Matthaeis et al., 2000), while *Talitrus saltor* seemed to have a more defined phylogeny (De Matthaeis et al., 1998). More recently Pavesi et al (2013) proposed that the lack of genetic structure in semi-terrestrial populations of *M. remyi*, could be due to the fact that, even though the terrestrial environment would create differences among populations, occasional passive movements of migrants (i.e. marine rafting) could mask such differences. This may suggest that *T. capensis* have a more terrestrial than marine influence on its phylogeography. This is certainly plausible if one considers that these animals occupy a transition zone between terrestrial and marine environments and are often seen further inland towards the dune slacks (Van Senus, 1988).

The strong statistical support of among groups variation and the high number of mutations between the separated groups however, may strongly suggest cryptic speciation, as reported for COX1 profiles in a wide range of taxa (Hebert et al., 2003). Ragionieri et al.

(2009), investigating the biogeography of the mangrove crab *Neosarmatium meinerti*, found the presence of four cryptic species, providing genetic and morphological evidences. *Talorchestia capensis* showed three different groups with a higher number of mutations (between 10 and 20) in a comparable fragment length (4-5 mutations in a 600pb long fragment) to that one of Ragionieri and coauthors (2009), suggesting that speciation may have occurred. Given that, at least three cryptic species could be identified for *T. capensis*: one on the South coast which colonised those habitats first, one on the South-West-coast, and one on the South-East coast, both more recently generated. The three putative cryptic species overlap on an area that could be located between Still Bay and Gouritsmond (Western Cape, South Africa), suggesting that both the South-West and the South-East maybe expanding their range towards the South. Potential overlap on the edge between the South and the South-East species could occur, but this is difficult to address, given the low number of individuals available (especially in the gap between BRE and PA).

Although assigning a higher taxonomic level to the three main groups (confirming cryptic speciation) is challenging, I recognise the possibility of cryptic speciation for *T. capensis*. Cryptic speciation is, indeed, an important topic which has been discussed and proposed by different authors for southern African marine species (Gouws et al., 2004; von der Heyden et al., 2011; Teske et al., 2011a). A poor evaluation of cryptic speciation is often the cause of the underestimation of marine biodiversity and its detection has been considered fundamental when investigating biological invasion (Teske et al., 2011b). I recognise the low number of individuals analysed in this study as a limitation to the discussion of the results and I recommend that future studies investigating the phylogeography of *T .capensis*, should consider the existence of at least three cryptic species and increase the number of individuals and populations analysed. A more comprehensive study dealing with the taxonomy of South African sandhoppers is also recommended. Nevertheless,

speculation on the phylogeography and connectivity of separated groups of *T. capensis* (whether they represent either metapopulation or cryptic species) can be done, providing valuable insights for the main chapters of this thesis.

Given that, the geographical pattern found in the present study is consistent with most of the work carried out on marine invertebrates of southern Africa (reviewed by Teske et al., 2011a), which has identified that the phylogeography of coastal species is largely linked to the marine biogeographic provinces of southern Africa (Teske et al., 2006, 2009, 2011). The current study, however, found a break separating the South and the South-West from the South-East populations of *T. capensis* on a stretch of coast approximately between Witsand and Gouritsmond. This represents a slightly different result compared with the work mentioned above, which proposed the Agulhas region as a common geographical break for most of the invertebrates examined (Teske et al., 2011a). Teske et al. (2006), however, investigating the population structure and connectivity of several crustacean species, found an unclear boundary between the western and the eastern populations of the cumacean *Iphinoe truncatae* (a direct developer), which could be located somewhere along the south coast around the Goukou and Touws estuaries (south coast of South Africa). The dynamics of coastal phylogeographic breaks therefore appear to be complex, where interacting factors can influence population structuring and connectivity (Carson et al., 2010; Brante et al., 2012 and where "timing is everything" (McQuaid, 2010, pag. 938). For example, life history traits, timing of reproduction, changes in abundance and adaptation to local environmental conditions can be as important as physical barriers in determining phylogeographic breaks (Carson et al., 2010; Brante et al., 2012 Teske et al., 2013b).

The abundance of *T. capensis*, as for sandhoppers in general (Pavesi et al., 2013), seems to be highly dependent on the morphodynamic state of their habitats (Baldanzi et al., 2013). Thus, optimal species-specific environmental conditions could explain the unusual break

found in this study. During snapshot sampling in 2010, *T. capensis* abundance decreased among populations from the Cape region to the south-east coast (Baldanzi et al., 2013). Such a decrease in abundance could be attributed to the phylogeographic breaks (in this study that found between Witsand and Gouritsmond) as proposed by Broitman et al. (2001) and Rivadeneira et al. (2002) along the Chilean coast. The fact that the population structure of *T. capensis* is not affected by the Agulhas boundary (as individuals of the same lineage are found both west and east of Agulhas) suggests that *T. capensis* may be well adapted to the different environmental conditions (mainly temperature driven) typically found around the Agulhas break. In this view, physiological adaptation plays a fundamental role in determining the boundaries between populations (Teske et al., 2011a). Physiological adaptation to different environments has been proposed by Teske et al. (2007) to explain the break between the subtropical and temperate regions found for the mudprawn *Upogebia africana* along the South-East coast of South Africa. As reported in Chapter 4, differences in the physiology among separated population was clear, even though similar thermal sensitivity was detected between individuals of Gansbaai (S-W group) and Port Alfred/Mngazana (S-E), suggesting that even genetically separated population (or cryptic species) could show similar physiological plasticity.

An alternative explanation for the break found in this study could be the different dispersal abilities between sandhoppers living in ephemeral habitats and those who live in open and exposed sandy shores (Pavesi et al., 2012). For instance, since sandhoppers most likely disperse by means of rafting (Persson, 2001), habitats close to estuaries or with a higher amount of wrack and macroalgae (e.g. the Cape sites, characterised by high amount of kelp wrack and those sites close to estuaries like Still Bay, Witsand and Gouritsmond) may host highly connected populations. Eastward of Gouritsmond (along the south-east coast of South Africa) mostexposed sandy shores present low amounts of macroalgae (Anderson et

al., 2007; personal observation). Gouritsmond itself can therefore act as a natural boundary between the south-west and the south/south-east populations. Sampling at higher resolution along the south coast would certainly clarify the phylogeography of these populations.

Given that dispersal for sandhoppers is a sporadic event, highly dependent on the wrack-bed present on the shore (Dahl, 1946; Wildish, 1970) and the magnitude/direction of coastal currents (Persson, 2001), rafting is proposed as the main pathway for new colonisations. Sandhoppers, as many amphipods, carry juveniles for a relative long time period after hatching (Morritt and Spicer, 1996a, b, c; Morritt and Richardson, 1988; Thiel, 1999), allowing them to recruit directly onto the parental raft (Thiel, 1999). With such a strategy, juveniles and adults, feeding on the organic material available during rafting, are then massively and simultaneously washed ashore in a new suitable habitat (Persson, 2001).

In this study, *T. capensis* showed no significant genetic isolation by distance, as shown by Mantel tests performed for all the populations and within geographical distinct groups. This result disagrees with much of the works carried out on southern African invertebrates (Teske et al., 2007; 2011a), where direct developers often showed a pattern of isolation by distance. The results reported in the present study do, however fit with the rafting hypothesis, largely proposed to explain lack of isolation by distance and low differentiation among distant populations (Johannesson, 1988, reported in Thiel and Haye, 2006; Haye et al., 2012). Taxa showing rafting as dispersal mechanism displayed high levels of connectivity (both among adjacent and distant populations) and intermittent or frequent rafting may break the IBD pattern of genetic diversity that is otherwise expected (Thiel and Haye, 2006).

Gene flow among groups of distinct populations of *T. capensis* was of at least of one migrant per generation, the minimum number ensuring population connectivity (Mills and

Allendorf, 1996) and was present even between non-adjacent populations (as shown by the "Panmictic" model, although this was not well supported). These results suggest that, even though dispersal of sandhoppers is related to the amount of wrack along the south-west coast of South Africa, rafting could take place at even larger scales (from hundreds to thousands of km), allowing *T. capensis* to show connectivity among distant populations. The large amounts of kelp typically washed ashore along the west coast of South Africa (Anderson et al., 2007) could, in fact, explain the lack of isolation by distance within the S-W lineage. If rafting along the west coast of South Africa takes place frequently and intermittently as a consequence of the large amount of floating material available, separated populations of *T. capensis* should maintain high genetic connectivity through time.

Connectivity among populations within the same group (S-W) could be however a result of the inverse relationship between rafting routes (i.e. rafting distance) and colonization success, as reported in Thiel and Haye (2006). Within-lineage variability in the F*st* values (even though among interconnected populations) confirmed by the thermal physiology experiments carried out comparing individuals from Port Nolloth and Gansbaai, which showed differences in the metabolic response to the same environmental factor (temperature changes; see Chapter 4 for further details). In this case, if rafting becomes more sporadic or even episodic, these populations would experience genetic isolation which may lead to bottleneck or founder effect and, in the long-term, to speciation (Thiel and Haye, 2006; Teske et al., 2011a). Low to moderate gene flow is also present between the south and south-east groups, with a bidirectional migration taking place with relatively higher rates than along the west coast (Fig. 6b). Although this pattern is more difficult to explain in term of rafting, due to the fewer wrack-beds and less floating material found along these shores in comparison to the ones in the South and West (Anderson et al., 2007;

personal observation), this mechanism still remains the main way for sandhoppers to migrate. However, the presence of migration found in the south-east coasts, even with a lack of rafting material, could highlight an important aspect of the rafting hypothesis, which is the intrinsic dependency from currents and oceanographic features (Thiel and Haye, 2006). Given that, currents still play a fundamental role as major drivers in marine connectivity (Pineda et al., 2007). Teske et al. (2011a), reviewing most of the research carried out on coastal invertebrates and fishes along the south African coast, found a clear unidirectional, northward pattern of migration on the west coast, while a less clear, bidirectional pattern on the South-East coast. Interestingly, invertebrates showed bidirectional patterns of migration with more eastward than westward movement/migration (Teske et al., 2007), which is unexpected due to the Agulhas Current. Such a pattern has also been reported for the invasive mussels *Mytilus galloprovincialis* along the coast of South Africa (McQuaid and Phillips, 2000). These authors found a good match between the larval dispersal and wind patterns, suggesting that mussel larvae in their study region disperse like passive particles and that hydrographical conditions may have driven their eastward migration over time (McQuaid and Phillips, 2000). Hydrographical conditions causing eastward migrations of most of the invertebrates and some fish species of South-East coasts of South Africa have been ascribed to wind-driven alongshore currents (Roberts and van den Berg, 2005; Teske et al., 2008; 2011; Von der Heyden et al., 2008; but see Neethling et al., 2008 for contrasting pattern in fishes). *Talorchestia capensis* showed a bidirectional migration between the South and the South-East groups partially in agreement with the aforementioned works. The westward migration could be favoured by the net transport driven by the major Agulhas flow (offshore current), but also favoured inshore by the Natal pulse which occur regularly around Algoa Bay and St Francis Bay (Eastern Cape). The eastward migration could eventually take place by means of wind-

driven currents, which flow nearshore along the Tsitsikamma coast (Roberts and van den Berg, 2005). The same authors described a potential offshore displacement of about 40km, in concomitance of wind-driven upwelling events in a coastal area spanning from Tsitsikamma to approximately East London. Given that, the water flow could transport floating materials offshore, which are then caught by a westward flow, moving them towards that direction (Roberts and van den Berg, 2005). This hypothesis would explain the bidirectional gene flow of *T. capensis* along the south-east coast of South Africa, but comparisons must be made cautiously, because evidence for such patterns is not available. The low number of sampling sites between the South and the South-East populations included/available in this study, yield difficulty in identifying a phylogeographic break between the two lineages. The high number of mutations between the two lineages however shows a clear discontinuity, which could be located in the transition area between Algoa Bay and the Wild Coast, as suggested by several authors (reviewed by Teske et al., 2011a). Greater resolution in the selection of sampling sites is, however, recommended for future works dealing with phylogeographic discontinuities between the two lineages.

An important aspect to highlight is the increasing evidence that phylogeographic differences within traditionally-described taxa may underpin morphological cryptic speciation (Bickford et al., 2006), particularly common in marine invertebrates (Huelsken et al., 2013) and invasive species (Teske et al., 2011b). Recently it has been argued that phylogeographic splits can represent stages along a speciation continuum, with reproductive isolation between lineages of eco-morphologically defined species leading to cryptic speciation (Singhal and Moritz, 2013). Previous authors found a strong relationship between indices of reproductive isolation and the time divergence of eco-morphologically defined lineages of terrestrial rainforest skinks, suggesting cryptic speciation. *Talorchestia capensis* showed three defined lineages with high percentage of variation explained among

groups, suggesting that separation could be related to cryptic speciation. Additional work is however necessary to speculate on such evolutionary events.

The present work clarified the results of the biogeography (see Chapter 2), particularly in bringing more light on the gaps found in the spatial distribution of these animals (Baldanzi et al., 2013). For instance, a gap of approximately 600 km was found in the western region, and this study showed that haplotypes are shared between the northernmost (Port Nolloth and Kleinsee) and the southernmost populations of the same lineage. Furthermore, gene flow is enough to ensure connectivity among those populations. Gene flow calculated using coalescent-based methods (such as in the present study using the software MIGRATE-N) and mtDNA markers however, are likely to be strongly influenced by historical events and may not be able to detect or estimate recent rates of gene flow (Beerli, 2009). Historical events, such sea-level changes, temperature changes and glacial cycles (as causes of change of coastal topography), are crucial when investigating phylogeography and connectivity (Teske et al., 2007; Chakona et al., 2013). In the particular context of southern Africa, Teske et al. (2013b) proposed a model of range extension followed by divergence, to explain how climate change may have driven the appearance of new evolutionary lineages and speciation events across the Atlantic/Indian Ocean boundary (Teske et al., 2013b).

Nowadays, a large amount of work is done using multilocus data sets (combining several markers), which give a better representation of phylogeography and connectivity, especially when considering historical events and the effects of reproductive isolation on diverged lineages (Toews and Brelsford, 2012; Singhal and Moritz, 2013). “Mito-nuclear discordance” (i.e. conflicting geographic patterns between mitochondrial and nuclear genetic markers) is increasingly being reported when using multilocus analyses which include mtDNA and nuclear DNA (Teske et al., 2013a). Both markers may show the same

number of evolutionary lineages, but significant differences in the geographical extent of these, often due to sex biased asymmetries between individuals (Toews and Brelsford, 2012). Further studies dealing with phylogeography and connectivity among populations of *T. capensis* should incorporate the use of a multilocus data set, as it provides a better estimate of contemporary environmental conditions affecting the phylogeography of individuals (Teske et al., 2013a). Importantly, Next Generation Sequencing (NGS) techniques, able to scale-up the number of sequence reads per run (Metzker, 2010), will help phylogeographers to discover a high number of molecular genetic (DNA) markers in the genomes of non-model species. In the particular case of southern Africa, NGS and bioinformatics could help identifying gene regions that are under selection between provinces and also providing high resolution dataset on the divergence events across the Atlantic/Indian Ocean boundary (Teske et al., 2013b). Furthermore, using markers under selection will allow researchers to properly start to tease apart the influence of the environment and the different conditions on the biogeographic regions in term of organismal biology, physiology and evolution (Metzker, 2010).

In conclusion, this work contributes to the understanding of the phylogeography and connectivity of separated populations of *T. capensis*. The phylogeographic barriers which contributed to the highly defined population structure of *T. capensis* could be attributed to differences in the local environment rather than real geographic barriers. Indeed, the influence of local environmental conditions appeared to be an important factor shaping the biogeography and physiology of this South African sandhopper (see chapter 2 and chapter 4). Rafting could explain the low-moderate gene flow among distant and adjacent groups found in this study, but nearshore currents play certainly a clear role interconnecting ephemeral habitats with different local conditions and allowing *T. capensis* to show a wide range of distribution. It is however recommended further research to consider the

possibility that the clear structure of the populations reported in this study might underpin cryptic speciation.

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-Chapter 4-

Climatic variability shapes thermal plasticity in separated populations of the sandhoppers Talorchestia capensis (Amphipoda, Talitridae).

Introduction

It is widely recognised that temperature has a profound effect on biological functions at several hierarchical levels, ranging from molecules to ecosystems (Clarke, 2003; Somero, 2010). Thus, temperature is considered one of the main abiotic factors governing the distribution and abundance of species (Schulte et al., 2011; Bozinovic et al., 2011). A still current and controversial issue among eco-physiologists is why and when temperature scales with animal metabolism (i.e. resting metabolic rate, Clarke and Fraser, 2004). The Universal Temperature Dependence model (UTD, Gillooly et al., 2001; Brown et al., 2004) explains the temperature-metabolic rate relationship in a mechanistic way, where the kinetic energy of cellular processes alone (Boltzmann-Arrhenius kinetics) seem to drive the metabolic rate (Clarke and Fraser, 2004). Such models have been supported by a wide range of taxa (Savage et al., 2004), but criticisms of the UTD have been recently advanced, primarily because of its mechanistic approach (Clarke, 2004) and mathematical incorrectness (Kozlowski and Konarzewski, 2004), but also because of its relatively narrow thermal framework (Marshall and McQuaid, 2011). For example, in *Echinolittorina* snails (marine gastropods that experience high fluctuating temperatures during prolonged emersions), temperature scales with metabolic rate in an opposite way of that described by Boltzmann kinetics (Marshall and McQuaid, 2011). The authors suggested a complex mechanism behind the temperature-metabolic rate relationship, pointing-out the importance of energy balance in high temperature environments (Marshall and McQuaid, 2011).

The evolution of traits to suppress metabolism including trade-offs between costs, lifestyle and benefits, seems therefore to be a more realistic alternative to the energetically mechanistic model and to explain better the direct scaling between temperature and resting metabolic rate (Stillman 2003; Clarke, 2004; Calosi et al., 2008; Chown et al., 2010). In

the context of complex relationships between temperature and metabolism, a higher metabolic rate at high temperatures may imply wider "evolutionary options" at warmer environmental conditions than at low temperatures, but also inevitably higher costs for maintenance (Clarke, 2004). Several climate-based hypotheses have been proposed to explain the physiological variation in geographical ranges of species (see review by Pither, 2003). Particularly, the Climate Variability Hypothesis (CVH) states that positive relationship may exist between the range of thermal tolerance and the climatic variability experienced by taxa at higher latitudes (Spicer and Gaston, 1999; Bozinovic et al., 2011). Climatic models similarly focus on physiological traits and intrinsic properties of the species to predict their responses to climatic variables and, consequently, how physiology may affect the geographic ranges of species and populations (Spicer and Gaston, 1999; Addo-Bediako et al. 2000; Stillman 2003; Gaston, 2003; Calosi et al. 2007). Besides their intrinsic mechanistic assumptions, these models have the merit of incorporating phenotypic flexibility, which allows individuals to buffer fluctuating biotic and abiotic factors, and therefore increase performance and, possibly, fitness (Bozinovic et al., 2011).

In a context of climate change, the ability of organisms to cope with variation in temperature is fundamental, often determining evolutionary "winners and losers" (Stilman, 2003; Somero, 2010; Hoffmann and Sgrò, 2011). Such ability can be referred to as phenotypic plasticity and several authors agreed that its effects can theoretically lead to local adaptation of populations to their environment, if either little gene flow among populations or intense selective power, are kept constant (Sanford and Kelly, 2011). The broader the range of a species, the higher the probability that an isolated population (more likely, but not necessarily, at the edges of its spatial range) undergoes adaptation to local environmental conditions, especially in the case of direct developers with low dispersal (Bohonak, 1999; but see also Palumbi, 2004), and it may be reflected by differences in

physiology among populations. When investigating physiological differences within a species, a distinction between population adaptation and individual phenotypic plasticity is therefore critical, as it may lead to different predictions on the persistence of populations over time (Sanford and Kelly, 2011). Such distinction is however challenging and whether phenotypic plasticity or adaptation explains differences in thermal physiology, within or among species, is not yet well understood (Somero, 2010; Sanford and Kelly, 2011). Consequently, it is crucial to understand whether species are able to produce a variable number of thermal phenotypes (demonstrating phenotypic plasticity through different thermal tolerances) and how these are distributed and connected among populations (Sanford and Kelly, 2011). For instance, the authors reviewing several works showed that the distribution of phenotypes with different thermal tolerances within a species is important as it gives a more realistic representation of the sensitivity of that species to increasing temperature. That is, a species with high gene flow among its populations that has a homogeneous distribution of phenotypes is more likely to persist in extreme scenarios, because the thermal tolerance of each population is similar to that one of the species as a whole. Species with poor gene flow and a heterogeneous distribution of phenotypes is likely to be more sensitive because the thermal tolerances of separate populations are likely to be narrower than those of the species as a whole. An example is given by the tidepool copepod *Tigriopus californicus* that has a narrower distribution of thermal tolerance phenotypes within populations than the range of thermal tolerances found in the species as a whole (Kelly et al., 2012). Consequently, making predictions on the effect of climate change based only on the overall species thermal tolerance, and ignoring populations' differences, would fail to predict extinctions in locally adapted populations with a narrower range of tolerances (Kelly et al., 2012).

In order to make realistic predictions on the effects of climate change on shifts in species distribution, models should incorporate the ability of organisms to cope with different environments, and in so doing, include thermal adaptation and plasticity (Chevin et al., 2010). Undeniably though, climatic envelope models ("niche models"), which consider the "environmental force" as main driving factor of species distribution, may fail (by underestimating the thermal tolerance of species) to predict convincingly the effects of climate change on species persistence (Chevin, et al., 2010; Kelly et al., 2012). Recently, the importance of incorporating the effect of temperature variability into the study of thermal physiology of animals has emerged, especially when predictions of climate change are made (Schulte et al., 2011; Williams et al., 2012; Paajimans et al., 2013). Heterogeneous environmental conditions are likely to reduce the thermal sensitivity of ectotherms, a theory known as the Jensen's Inequality (Ruel and Ayres, 1999). The theory predicts that ectotherms experiencing high thermal fluctuations are more likely to show behavioural thermoregulation (behavioural plasticity) and lower Q_{10} values, the factor by which metabolic rate increases over 10°C increase in temperature (Ruel and Ayres, 1999; Fisher and Karl, 2010). Even though only little empirical work supports such predictions (Folguera et al., 2009; Williams et al., 2012; Paaijmans et al., 2013), it is increasingly obvious that it is necessary to consider both the means and the variability in temperatures in order to forecast the effects of climate change on species distribution and persistence (Paijmans et al., 2013).

In the present study, I investigate the thermal tolerance and metabolic rate (i.e. oxygen consumption) of separate populations of a supralittoral amphipods, *Talorchestia capensis* (Amphipoda, Talitridae), along the South African coast. Furthermore, I examined whether "historic" climatic variability (experienced over the past 23 years) may have modified the thermal sensitivity of *T. capensis*.

The sandhopper *T. capensis* is a supralittoral amphipod, widely and patchily distributed along South African sandy shores (Griffiths, 1976; Baldanzi et al., 2013), and its abundance seems to be mainly driven by the morphodynamic conditions of the shore (Baldanzi et al., 2013). Its distribution, however, encompasses three different biogeographic regions (defined by Harris et al., 2011) which are strongly affected by ocean currents with contrasting temperature regimes, suggesting that there may be differences in temperature physiology among populations (Baldanzi et al., 2013). The genetic population structure of *T. capensis* (based on mtDNA, see Chapter 2 for details) showed two separated lineages (South-West and South-East clades), with a main phylogeographic break on the south coast (Gouritsmond, see chapter 2 for details). A third lineage appears to be located on the south coast (see chapter 2 for details).

Therefore, I hypothesised that: 1) geographically and genetically separated populations differ in their thermal physiology, in terms of thermal tolerance (i.e. thermal limits) and performance (i.e. oxygen consumption) as consequence of thermal adaptation to local conditions; 2) the thermal sensitivity of separated populations has been modulated and influenced by the climatic conditions experienced over a relatively long time scale, resulting in "passive phenotypic plasticity" (Schulte et al., 2011). I envisaged that *T. capensis* would show strong intra-specific differences in its thermal physiology, which are related to the population genetics as well as its biogeography.

In particular, I expected differences between populations from distinct bioregions, as they have experienced contrasting climatic conditions for long periods. Furthermore, I expected that the physiological responses measured would reflect the historical temperatures experienced by the population, rather than the climatic conditions experienced by the individuals tested.

Material and Methods

Animal collection, maintenance and acclimation

Animals were collected from five geographically separated populations within the range of distribution of *T. capensis* (Fig.1): Port Nolloth (PN), Gansbaai (GB), Plettenbergbaai (PB), Port Alfred (PA), Mngazana (UM). GPS coordinates are reported in the Appendix. PN and UM are the western and eastern limits of the geographic range, respectively; PB is a site within the centre of the distribution; GB is included within the southern limit of distribution of the South-West lineage of *T. capensis*; PA represent the southern limit of the distribution of the South-East lineage. The sites were located in four separated biogeographic regions as defined by Lombard (2004) and are reported in Figure 1. Sites were sandy shores of similar morphodynamic type chosen in the basis of accessibility and the presence of adults.

Animals were collected in separate surveys from February 2013 to May 2013. One hundred adults of similar size were collected by hand from each site and placed in plastic cooler bags half filled with moist sand taken from the site of collection (ice blocks were used to buffer against abrupt changes in temperature). Gender was not considered at this stage. Immediately after collection, animals were transported by car to the laboratory (maximum travel time of 24 hours) and maintained in a controlled-environment room at 18°C with a 12h/12h photoperiod for a week before performing the experiments, in order to re-set metabolic stability (Calosi et al. 2007). During acclimation, animals were placed in small glass aquaria (20cm x 30xm) partially filled with sand from the site of collection. The sand was humidified daily with autoclaved seawater, again from the site of collection. Animals were fed at libitum using Kelp tablets (Norwegian seaweed, Weetol®) ground and mixed with sterilised seawater (1 ml of autoclaved seawater per tablet). Experiments were

performed in a thermostatically controlled room employing a water bath to ensure high thermal stability.

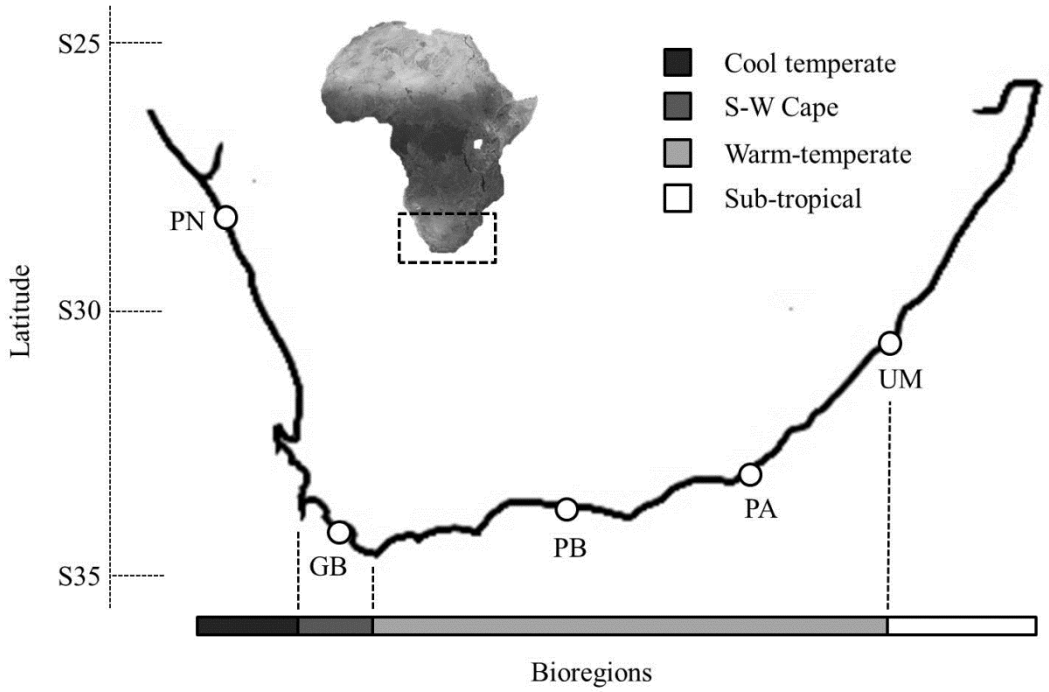

Fig.1: Map of the sites of collection.
PN: Port Nolloth; GB: Gansbaai; PB: Plettenbergbaai; PA: Port Alfred; UM: Mngazana. The edges and the extent of the Bioregions are reported on the bottom bar in greyscales. Top right legend report the names of the four Bioregion of interest.

Upper and Lower Critical Thermal Limits experiments

After acclimation, forty animals (similar size, random sex ratio) were collected from each aquarium and placed individually in 4ml plastic tubes. Twenty animals were used for the Upper Thermal Limit (UTL) and twenty for the Lower Thermal Limit (LTL). Size was checked using a microbalance, as there is a high correlation between size of sandhoppers and their upper thermal tolerance (Morritt and Ingolfsson, 2000). A small piece of 100% cotton was placed in the bottom of each tube and moistened by adding two drops of seawater to ensure enough humidity inside the tube, during the experiment. One drop of food (concentration as above) was also provided using a plastic Pasteur pipette. Both humidity and starvation can significantly affect the metabolism of the animal during long

term experiments (Terblanche et al., 2011). A small perforated patch of Paraffin film (Parafilm®) was used to close the tubes to avoid evaporation, while allowing for oxygen exchange into the tube. The tubes were then placed in a programmable water bath (GP 200, Grant Instruments®) filled with distilled water, ensuring that at least 75% of the tube length was submerged. To check that temperatures inside the tubes matched those of the water bath, a thermocouple probe was used and temperature recorded as close as possible to the animals, while avoiding contact with them. No correction for temperature was necessary. Tubes with animals were placed for 2h at 18°C in the dark before the start of ramping, following Morritt and Ingolfsson (2000) working on *Orchestia gammarellus.* The water bath was pre-set to increase or decrease at a rate of 1°C/h, as low rates of change in temperature are more likely to reflect natural conditions (Terblanche et al., 2011). Mortality was checked hourly by monitoring movement of the sensory antennules, mouthparts and other appendages. Animals which did not show any active movement were gently stimulated using the thermocouple probe. The thermal limits were calculated following a similar approach to that of Stillman and Somero (2000), using the LT50 method, the temperature at which 50% of animals from a sample die at which point the experiment was terminated.

Oxygen consumption experiments

Sandhoppers generally live in water-saturated sand, but do not experience immersion (Morritt, 1988). Also, their ability to osmo-regulate seems to be unaffected by the medium where they are acclimated (Morritt, 1988; Calosi et al., 2007); thus, acclimation and experiments were performed in air only.

After the acclimation period, 32 animals per population were selected as above and placed in individual respirometric chambers for measurement of oxygen consumption in air. Temperatures were increased/decreased at a rate of 1°C/h as in the experiments on thermal

limits as ramping rate has been shown to alter metabolism at the critical thermal limits (Terblanche et al., 2007). The temperature ramps used ranged between three degrees below and three above the LTL and UTL, respectively (values retrieved from the LT50 experiments) allowing the investigation of oxygen consumption within and beyond the sandhoppers' thermal limits. Oxygen consumption was measured using a portable Oxygen Meter (Fibox 3, Presens®) consisting of a temperature compensated fibre optical oxygen transmitter able to detect the air saturation of a closed environment through a sensor spot installed in the respirometric chamber. The respirometric chambers were made up of two ml glass vials with the sensor spot glued to the bottom (Oxygen Sensor Spots PSt3, Presens®). A metal stick-on adaptor was glued and silicone-sealed to the outside of the vial. The adaptor was linked to a polymer optical fibre that transferred the signal detected in the respirometric chambers to the Fibox and a computer that displayed percentage air saturation. On the opposite side of the vial, a 30cm long plastic tube (0.4 cm inner diameter) was attached and sealed using paraffin film (Parafilm®). The animals were gently placed inside the chamber, close to the sensor spot area (Fig.2). The entire system (vial and tube) was filled with toroid-shape glass beads (approximately 3mm, outer diameter), in order to minimise the volume of air inside the chambers. The reduction of volume allowed better detection of air saturation, since oxygen solubility is known to be high in air. This was confirmed in preliminary comparisons of sandhopper oxygen consumption with and without beads (n = 3, unpublished data).

An appreciable decrease of oxygen (between 5% and 10%) was detected in the closed chambers with beads after 3 hours. This method was therefore selected for the main experiments. The oxygen consumption experiments were performed in a programmable water bath as above. The chambers were completely submerged in distilled water, placed upside down, allowing the probe to be attached at the top of the chambers during

measurements (Fig.2). The free end of the pipes was place outside the water bath, in order to allow the percentage of air to increase again after each measurement (see below for details). A minimum of 60% air saturation was considered as a threshold for each chamber after each measurement; lower values could be lethal due to hypoxia (Schurmann and Steffensen, 1992). Sixteen replicates per temperature ramp were used.

The scheme for the temperature ramp is reported in Figure 3. After two hours of acclimation at 18°C in the dark, a first group of replicates experienced an increasing ramp of temperatures, while a second group experienced decreasing values. Oxygen consumption was recorded every three degrees in both increasing and decreasing ramping. For each selected temperature, two readings of air saturation were recorded. A time interval of three hours between the first and the second reading was selected. Between the two readings the tubes were clamped, creating a closed environment in which the decrease of oxygen at a specific temperature was due to the animal's respiration.

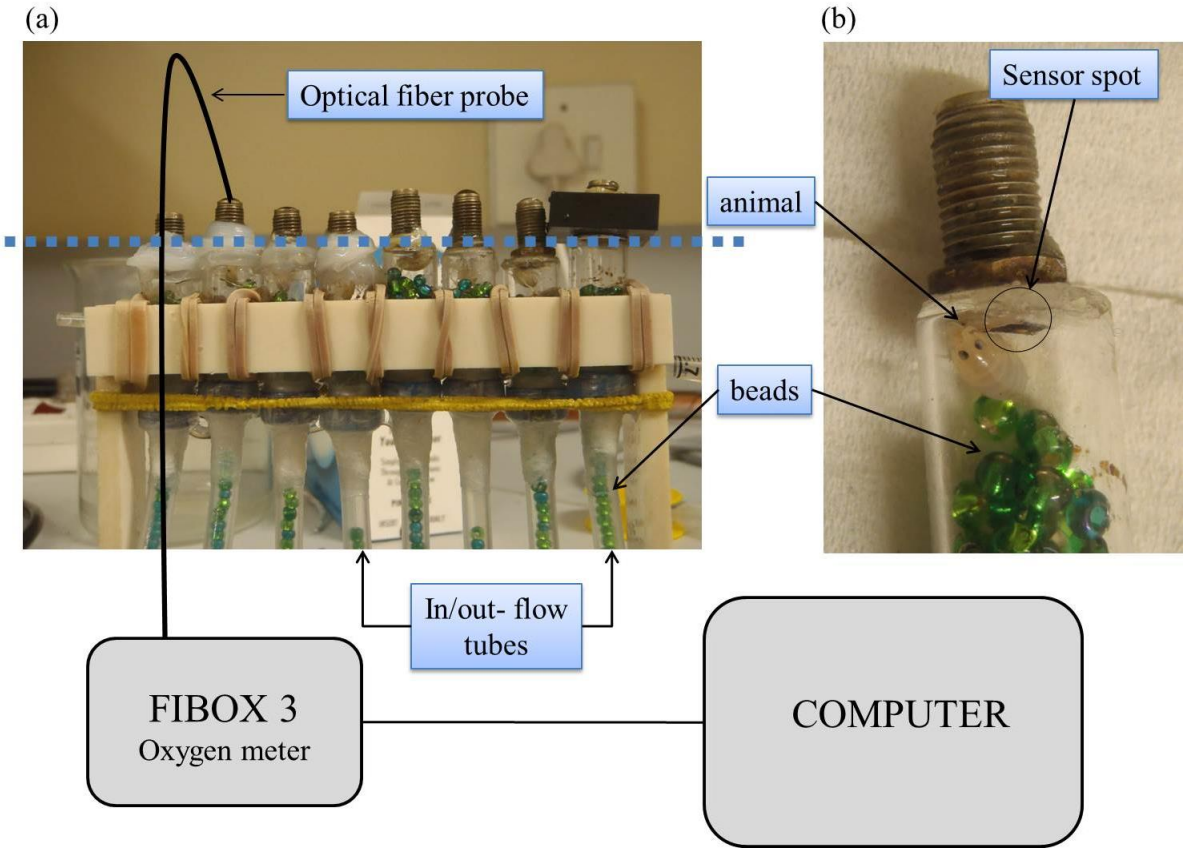

Fig.2: Schematic representation of the set up for the experiments on oxygen consumption.
(a) the respirometric chambers fully submerged in water bath (blue dot-dashed line shows the water level). The tubes were sealed at the bottom of the chambers, while its free end emerged from the water (not shown in the photo). The metal adaptor emerged to accommodate the optical fibre probe for the reading measurements. The probe transmitted the signal to the FIBOX 3 which transferred the reading to a computer. The final measurement was represented as percentage of air saturation. (b) zoom-in of the chamber showing the position of the sensor spot, animal and beads.

Readings consisted of one minute consecutive records of air saturation at a rate of one measurement per second (FIBOX default settings). Oxygen probes were calibrated prior to each experiment in air-saturated (100%) and oxygen-free distilled water, following the instruction manual. After each ramp, the vials and tubes were sterilised with 100% ethanol overnight to avoid growth of bacterial communities. To check for bacterial growth during ramping especially at high temperatures, sterilised control chambers with no animals were used in preliminary experiments. Oxygen was measured as above, at the following temperatures along the ramp: 0°C – 10°C – 20°C – 30°C – 40°C. An oxygen decrease of 0% - 0.5% was detected only at 40°C.

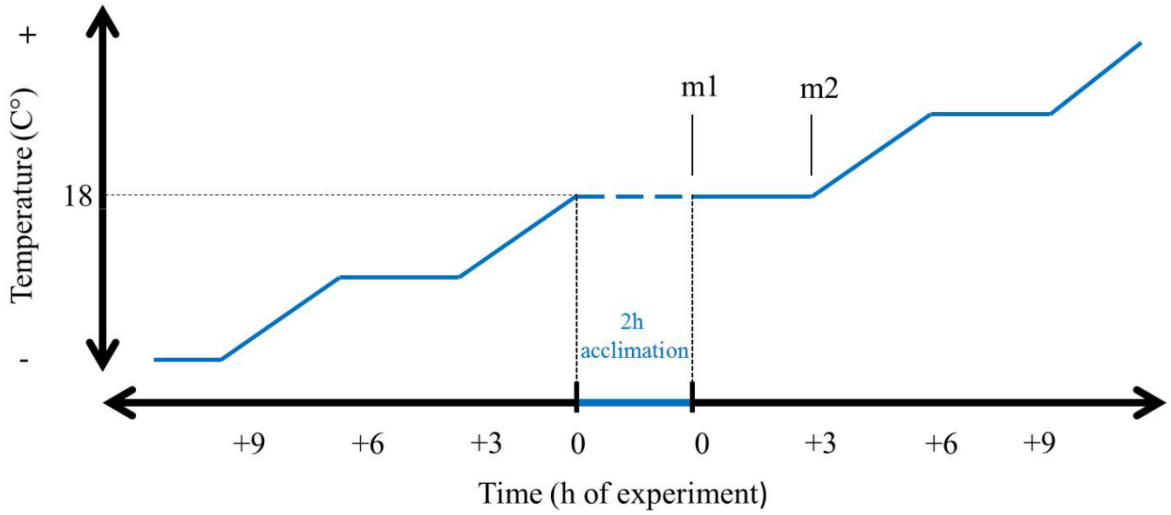

Fig.3: Schematic representation of the ramp of increasing/decreasing temperature.
The bottom axis indicates Time as hours of experiment after acclimation. m1 and m2 represent the first and the second reading, respectively, taken at 18°C. The readings were performed every three degrees along the temperature ramp (not shown). The left axis reports the temperature as increasing and decreasing values from time zero to the end of the experiment (not shown). + and – indicates increasing and decreasing temperatures.

Collection of climatic data

In order to evaluate whether the thermal sensitivity of the study populations was explained by their experience of climatic conditions, historical data of air temperature (aT), sea surface temperature (SST) and rain (Rain) were collected from meteorological stations for each site (South African weather service, www.weathersa.co.za). Historical data for Mngazana were not available over a long term period. Data were therefore collected from the closest location available (Port Edward, approximately 80km farther east, see Appendix for coordinates). The historical data ranged from January 1990 to June 2013 and were included: daily maximum and minimum air temperatures (°C), daily average of SST (°C) and daily Rain (mm).

Data analysis

At the end of the experiments on oxygen consumption, the total volume of air available for each animal in the chambers was calculated using the following formula:

$$V = (Vv + Vt) - Vb - Va$$

Where, Vv is the volume of the vials (2 ml), Vt is the inner volume of the plastic tube, Vb the total volume of the beads and Va is the volume of the animals. Vt was calculated assuming the tube as a cylinder, multiplying the internal area by the length of the tube (from the end attached to the chamber to the exact point where the tube was clamped during the experiment). Vb was assessed with the displacement method, using a 10 ml syringe half-filled with water, in which the beads were immersed (volume expressed in ml). Va was assessed with the same method, using a 1 ml syringe. Live mass of each animal was provided and weighed using a precision balance and expressed in mg. The percentage air saturation was converted to oxygen concentration [O_2] from values of oxygen partial pressure using temperature-dependent solubility coefficients for oxygen (Giomi and Pörtner, 2013). At each temperature, oxygen consumption was calculated as [O_2] (mmol) consumed by an animal for each minute. All values were then standardised per gram of animal and the final values expressed as $mmol*min^{-1}*g^{-1}$.

A Permutational Manova (PERMANOVA) was performed to evaluate the effect of population (fixed and orthogonal) and temperature (fixed and orthogonal) on oxygen consumption. Values above the UTL (collapsing points of the curves, see Fig. 3 in the results section) were excluded from the analysis. Since the same replicates were subjected to increasing or decreasing temperature ramps, data in each ramp were analysed as repeated measures and analysed separately. PERMANOVA was carried out on Euclidean distance resemblance matrix using 9999 permutations of the residuals under a reduced model. Analyses were carried out using PRIMER 6 & PERMANOVA + (Anderson et al.,

2003; Clarke and Warwick, 2001). In order to compare the overall trend of oxygen consumption among populations (the slopes of the curves), I performed analysis of covariance (ANCOVA, see below for details) excluding the values above and below the thermal limits, as extremes of the curves may alter the slopes. In fact, values exceeding the thermal limits do not give information on the performance within the thermal tolerance window, which was the aim of this study. Analyses were carried out on linearized data, using Arrhenius plots (natural logarithm of metabolic rate as a function of inverse temperature). The Arrhenius equation was calculated as:

$$\ln O_2 = \ln a - Ea/k \times 1/T$$

where, $\ln O_2$ is the natural logarithm of the oxygen consumption, a is a normalization constant, Ea is the activation energy (J mol^{-1}), k is the Boltzmann constant (8.31 J K^{-1} mol^{-1}), and T is the temperature (°K). To test the effect of population on oxygen consumption, ANCOVA was performed with temperature as covariate (continuous predictor) and population as factor (categorical predictor). Prior to the analyses, the parallelism assumption (i.e. interaction with the covariate) was tested using an ANCOVA homogeneity-of-slopes model. This test is necessary to accommodate the ANCOVA parallelism assumption that the slopes of the curves are homogeneous. If the ANCOVA homogeneity-of-slopes model was found to be non-significant, an analysis of covariance (ANCOVA) was performed removing the population x temperature interaction from the analyses. A post-hoc analysis (Tukey HDS test) was carried out on population effects to investigate different responses to temperature. Data were analysed using STATISTICA (ver. 10, StatSoft Inc.).

From the whole ramp of oxygen consumption rate, a temperature coefficient (Q_{10}) was calculated as follow:

$$Q_{10} = (R2/R1)\exp[10/(T2-T1)]$$

where, R1 and R2 are the rate of oxygen consumption calculated one step above the LTL and one step below the UTL of each population, respectively (note that one step corresponded to 3^{o}C in the ramp). T1 and T2 were the temperatures at which R1 and R2 were calculated. The Q_{10} is a factor by which the rate of a metabolic reaction increases for every 10-degree rise in temperature and is often used to compare temperature-dependent physiological responses, as the oxygen consumption in ectotherms is expected to increase with temperature (Clarke and Fraser, 2004). The Q_{10} is used here as a measurement of the "thermal sensitivity" of a population.

The climatic dataset of AirT and SST were monthly averaged. Differences between maximum and minimum daily values of AirT (showed as mean daily range, dAirT) were calculated and expressed in °C. The total monthly Rain dataset was averaged and expressed in mm of rain per month. Monthly averaged climate data were preferred over finer resolution of temperature variability as sandhoppers show behavioural buffering of extreme temperature (i.e. nocturnal activity and daytime burial). Data were plotted against Time (year). To assess climatic variability, a Coefficient Of Variation (COV) was calculated (multiplying the standard deviation by 100 and dividing by the mean) for ΔaT, SST and Rain and expressed in percentages. The COV measures the variability of a series of numbers and is used in the analysis of temperature trend datasets. COV also gives an indication of the reliability of the average: the higher the COV, the less reliable the average is (Hasanean, 2004). The COVs were linearly regressed with the Q_{10} of each populations, in order to investigate the thermal sensitivity of the animals to variability in climatic conditions experienced over the past 23 years. Data were analysed using SigmaPlot (Systat Software, San Jose, CA). For those variables which significantly correlated with Q_{10} we performed a more detailed analysis to assess the effect of predictability on this parameter.

A proxy of predictability in climatic conditions was assessed using exponential smoothing analysis. To fit the raw data, this statistical technique uses an exponentially weighted moving average which incorporates a seasonal adjustment factor calculated over a certain period (Holt, 2004). To recreate the normal seasonality we set this period at 12 months. Due to both the fixed seasonal factor and to the limited length of the historical data-set, climatic cyclic fluctuations acting over longer periods, such as the Southern Annular Mode (SAM, Marshall 2003) or the Indian Ocean Dipole (IOD, Saji et al., 1999), could not be analysed. We recognize this as a limitation of our statistical approach. In addition, exponential smoothing analysis requires the absence of gaps in the time-series to correctly fit the data. Thus, when 1-2 datum gaps were encountered (1-2 months gap), data interpolation from adjacent points was used to fill those gaps. However, when the lack of data extended over longer periods for a given site, we decided to break the historical data into smaller time series or subsets which were analysed separately. The monthly averaged raw data were linearly regressed to the smoothed, predicted values and the R^2 obtained was considered as a proxy of climatic predictability (R^2). In the case of those time series showing gaps, an ANCOVA with its a-priori tests (see above) was carried out on the raw data with subset as a factor and smoothed values as co-variable. In this way we checked that the linear fit between raw and smoothed values does not change between subsets, allowing data to be pooled. Finally, the R^2 values obtained for each location were used to fit Q_{10} estimates for each population by means of simple linear regression in order to investigate the thermal sensitivity of the animals to predictability in climatic conditions experienced over the past 23 years. Exponential smoothing analyses were performed using STATISTICA (ver. 10, StatSoft Inc.). Regressions were done using SigmaPlot (Systat Software, San Jose, CA).

Results

LT50 and Oxygen consumption

The UTL and the LTL for the five populations retrieved from the LT50 experiments are expressed in °C and ranked from the highest to the lowest values (UTL-LTL), as follow: Port Nolloth (0-39); Gansbaai (0-37); Port Alfred (5.5-36); Mngazana (5.5-36); Plettenbergbaai (5.5-33). MO_2 for the five populations are reported in Fig. 4. For simplicity, values of oxygen are shown in a single temperature ramp from the LTL to the UTL even if the analyses were separate for increasing and decreasing ramps. In general, in all populations MO_2 was significantly related to temperature, whether increasing or decreasing (as shown in Fig. 4 and Table 1 for the Arrhenius plots). The overall trends were similar among populations, with maximum oxygen consumption towards the UTL, whereas minimum values were towards the LTL (Fig. 4). The collapse of the curves at higher temperature values confirmed the UTL found for all populations in LT50 experiments (Fig.4). Considering the full ramp, from low to high temperature, the general trend can be described as a first region of linear slope at colder temperatures, a plateau at intermediate temperature (approximately from 18°C to 27°C) followed by a pseudo linear region, with a second smaller plateau at higher temperatures (from 30°C to 36°C) before the curve reached high stress values and collapsed. Taken separately, the decreasing ramps showed a more consistent trend within populations, with less variability around the mean values so that SD values for each temperature tended to be much smaller.

There were significant effects of population and temperature on MO_2 for both increasing (Population: *df=4; F=15.983; p<0.0001*; Temperature: *df=8 F=12.539; p<0.0001*) and decreasing ramp (Population: *df=4; F=6.6864; p<0.0001*; Temperature: *df=8 F=21.07; p<0.0001*), with no significant interaction. Results of the pair wise comparison tests, reporting significant groupings among populations, are reported in Figure 4. Increasing

ramps show a clear difference of PN from the other populations, which showed a south to east trend. The decreasing ramp showed a clearer "west-east" trend with PN and GB separated from the remaining populations.

The homogeneity of slopes analyses reported no significant interactions between population and temperature on the Arrhenius-linearized values of MO_2. The analysis of covariance showed a significant overall effect of population in response to temperature (ANCOVA, $F_{(4,190)}=5.925$, $p<0.0001$) (Fig. 5). The Tukey-HSD test revealed differences in the response of populations to temperature, with a general "west-east" trend (Fig. 4), less clear for the increasing ramp than for the decreasing one.

The Q_{10} values differed among all populations and are ranked as follow, from the highest to the lowest values: PB (Q_{10} = 2.58); GB (Q_{10} = 2.42); PN (Q_{10}= 2.02); UM (Q_{10}= 1.98); PA (Q_{10} = 1.70).

Climate data

The climatic data showed differences among all sites in term of variability (COV) and are ranked as follow, from the highest to the lowest values. dT: Port Alfred-Port Edward-Port Nolloth-Plettenbergbaai-Gansbaai. SST: Plettenbergbaai-Port Alfred-Port Nolloth-Gansbaai-Port Edward. Rain: Gansbaai-Port Nolloth-Port Alfred-Port Edward-Plettenbergbaai. Mean and standard deviation of dT, SST and Rain are reported for each location in Table 2, together with the appropriate Coefficients Of Variation. The analyses on climatic variability were carried out only for dT as significant regression between dT variability (COV) and Q_{10} was found to be significant (see below). Temperature predictability showed a clear west to east pattern, with the eastern locations (Port Alfred and Port Edward) being much more predictable than the others according to the goodness of fit to the smoothed values (Fig. 6). For Port Edward a 13-month gap was found (from October 2000 to December 2001, Fig. 6i) so dT historical data was divided into two

subsets and exponential smoothing analyses carried out independently for each one. Given that the homogeneity of slopes condition was preserved (p value of the interaction term subset*smoothed series=0.56), an ANCOVA was performed. Factor subset was not significant (p value=0.78), thus raw monthly dT averages from both subsets were analysed together to get an overall R^2 (Fig. 6j). Values of T predictability (R^2) are ranked as follow, from the lowest to the highest: PN (R^2 = 0.30); GB (R^2 = 0.31); PB (R^2= 0.34); PA (R^2= 0.79); PEd (R^2 = 0.87). In Fig. 6 are shown the results of the exponential smoothing analyses and the linear regression between the predicted and the raw data. The thermal sensitivity of the five populations (indicated as Q_{10} values) was negatively correlated with air temperature variability (r^2=0.78, $p<0.05$; Fig.7a). Similar trend was found for temperature predictability (r^2=0.52, p=0.174; Fig.7b) although no significant. No significant regressions were found between thermal sensitivity and COV of either SST or Rain (Table 3).

Table 1 Linear regression analyses on the Arrhenius plots for the five populations.
Arrhenius equation (lnO2 = ln a – Ea/k × 1/T). In the equations: O_2 = oxygen consumption; ln a = intercept and Ea/k = slope. In the table: n= number of samples; R^2 = coefficient of determination;*** = significant p-values (p<0.001); **= significant p-values (p<0.05). I=increasing ramps; D=decreasing ramps.

Population	n	Slope	Intercept	R^2	P-value
Port Nolloth					
I	85	3.376	14.088	0.238	***
D	47	12.647	46.133	0.455	***
Gansbaai					
I	44	4.709	18.983	0.391	***
D	48	8.958	33.425	0.648	***
Plettenbergbaai					
I	78	5.338	20.888	0.259	***
D	30	15.541	56.543	0.283	**
Port Alfred					
I	100	6.808	16.588	0.355	***
D	40	3.979	26.299	0.177	**
Mngazana					
I	63	4.058	16.861	0.271	***
D	31	14.275	52.262	0.392	***

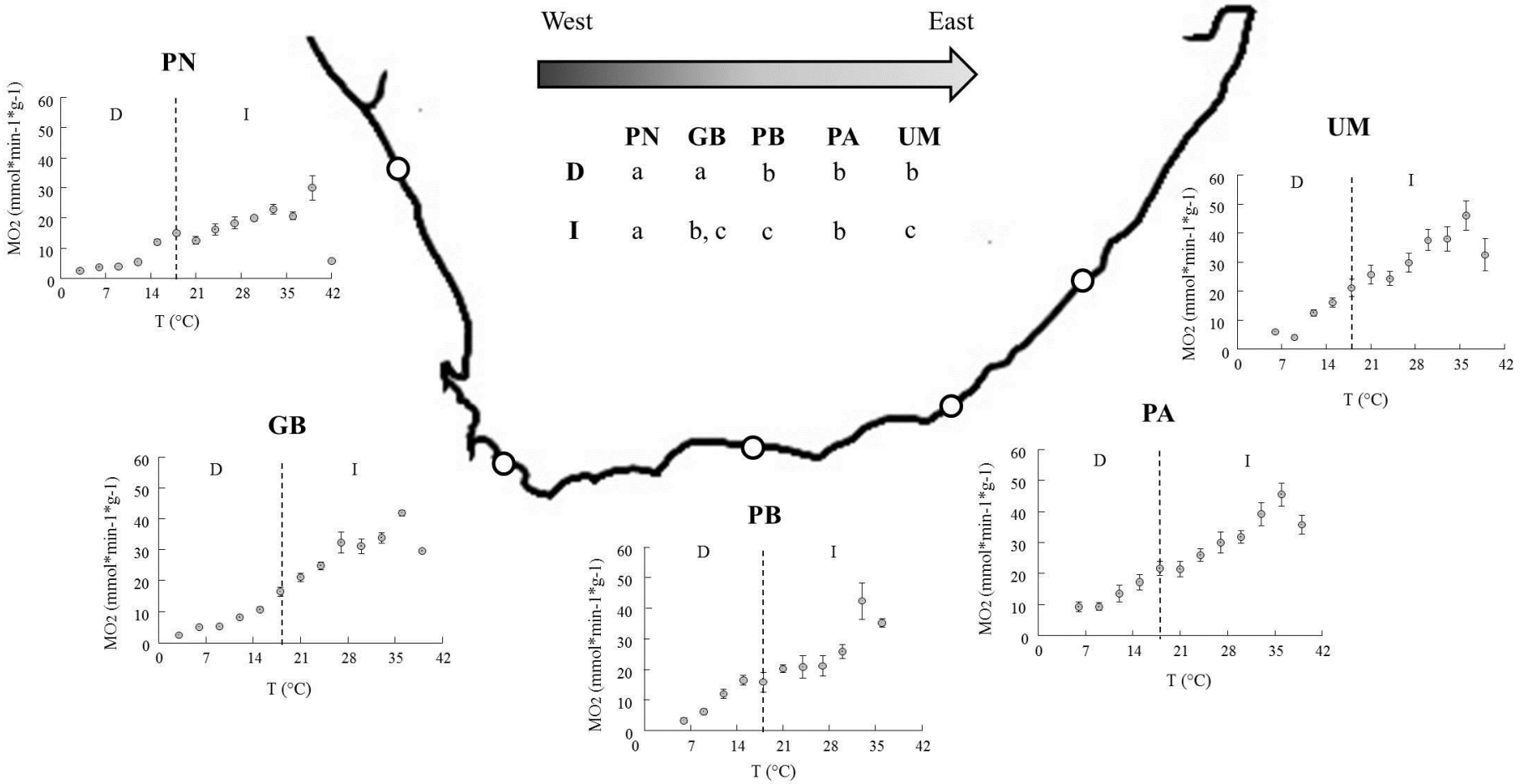

Fig. 4 Oxygen consumption curves of the five populations

Graphs are shown along the geographical gradient, from the west to east coasts of South African. The MO_2 for each population are reported as mean ± SE. The dashed lines divide decreasing ramps (D, from 15°C to 3°C) from increasing ramps (I, from 18°C to 42°C). The letters in the centre indicates the grouping on the base of the pair-wise comparison after performed PERMANOVA on the oxygen consumption values (see Material and Methods for details on the analysis). Letters are separate from analyses performed on the increasing (I) and decreasing ramps (D). The arrow shows the trend from west to east coasts as resulted from the pairwise tests.

Table 2 Mean, standard deviation, variability of dT, SST and Rain and predictability of dT for each location.
dT=mean monthly range (°C); SST=Sea Surface Temperature (°C); Rain=monthly rain (mm). T predictability is reported only for dT (see text in Material and Methods for details).

Location	dT	SST	Rain
Port Nolloth			
mean ± SD	8.26 ± 1.76	12.46 ± 1.32	5.90 ± 7.85
T variability (COV)	21.30	10.62	133.01
T predictability (R^2)	30		
Gansbaai			
mean ± SD	6.96 ± 0.60	15.89 ± 1.41	16.77 ± 28.38
T variability (COV)	8.69	8.85	169.19
T predictability (R^2)	31		
Plettenbergbaai			
mean ± SD	7.24 ± 0.96	16.35 ± 2.80	59.21 ± 43.52
T variability (COV)	13.30	17.12	73.50
T predictability (R^2)	34		
Port Alfred			
mean ± SD	9.39 ± 2.09	17.66 ± 1.99	53.35 ± 50.98
T variability (COV)	22.30	11.29	95.57
T predictability (R^2)	79		
Port Edward			
mean ± SD	6.34 ± 1.38	21.01 ± 1.66	98.72 ± 84.43
T variability (COV)	21.75	7.89	85.52
T predictability (R^2)	87		

Table 3 Linear regressions for the plots of the thermal sensitivity and climate variability/predictability.
Table shows the plots of Q_{10} against the values of COV for dT, SST, and Rain, respectively; the plots of Q_{10} against Tpred is also shown; *= significant p-value ($p<0.05$). r^2= coefficient of determination; n= number of samples.

Plot	n	Intercept	Slope	R^2	P-value
Q_{10} Vs dT	5	3.048	0.053	0.78	*
Q_{10} Vs T predictability	5	2.614	0.907	0.52	n.s
Q_{10} Vs SST	5	1.563	0.050	0.24	n.s
Q_{10} Vs Rain	5	2.012	0.00098	0.011	n.s

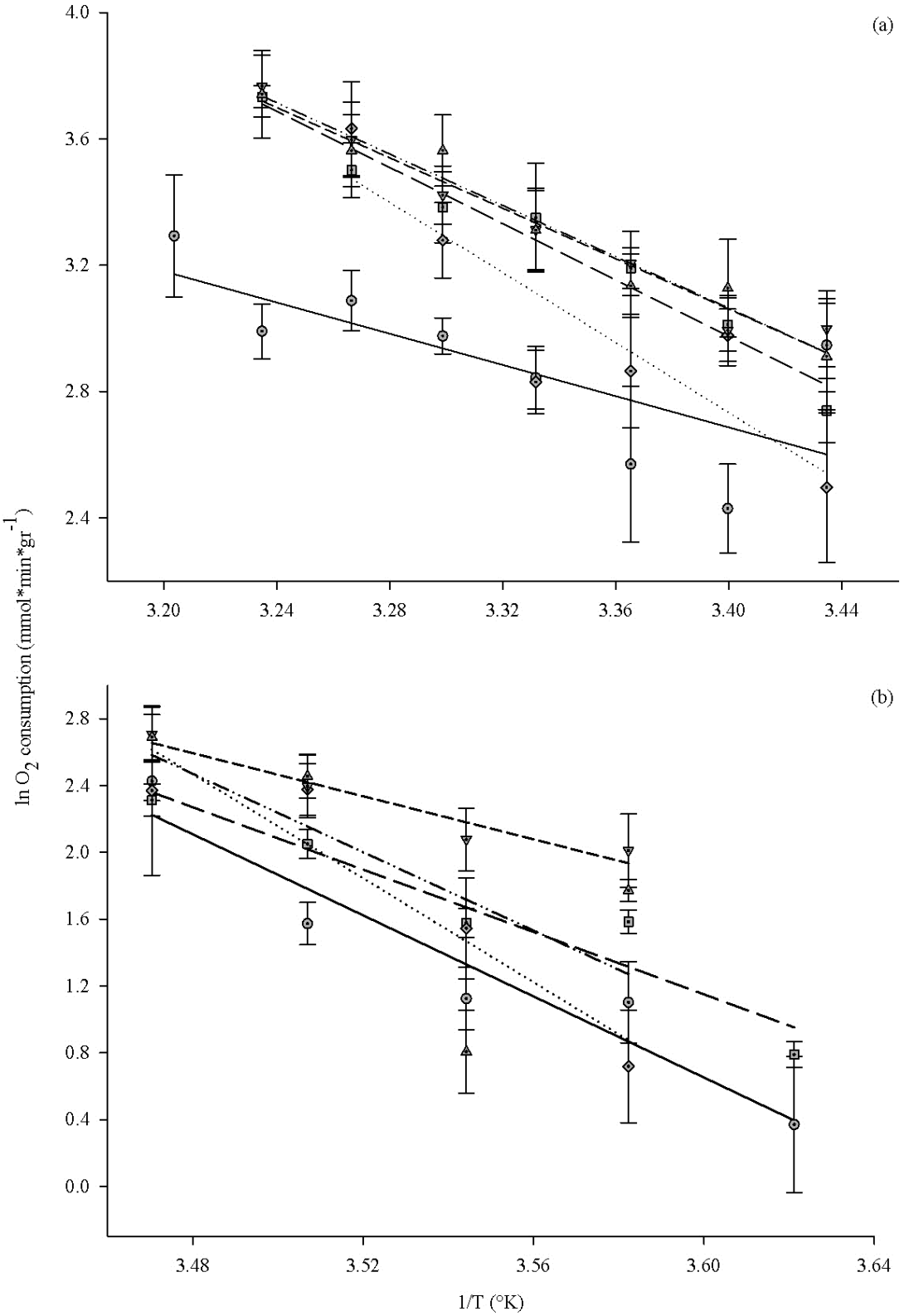

Fig 4 Arrhenius plots of O_2 consumption values (mean ± SE) for increasing (a.) and decreasing ramps (b.)

The figure shows the Arrhenius plots for the five populations. PN = circle; GB = square; PB = diamond; PA = down-triangle; UM = up-triangle. The lines represent the regressions, where PN = solid line; GB = long-dashed line; PB = dotted line; PA = short-dashed line; UM = double dot-dashed line.

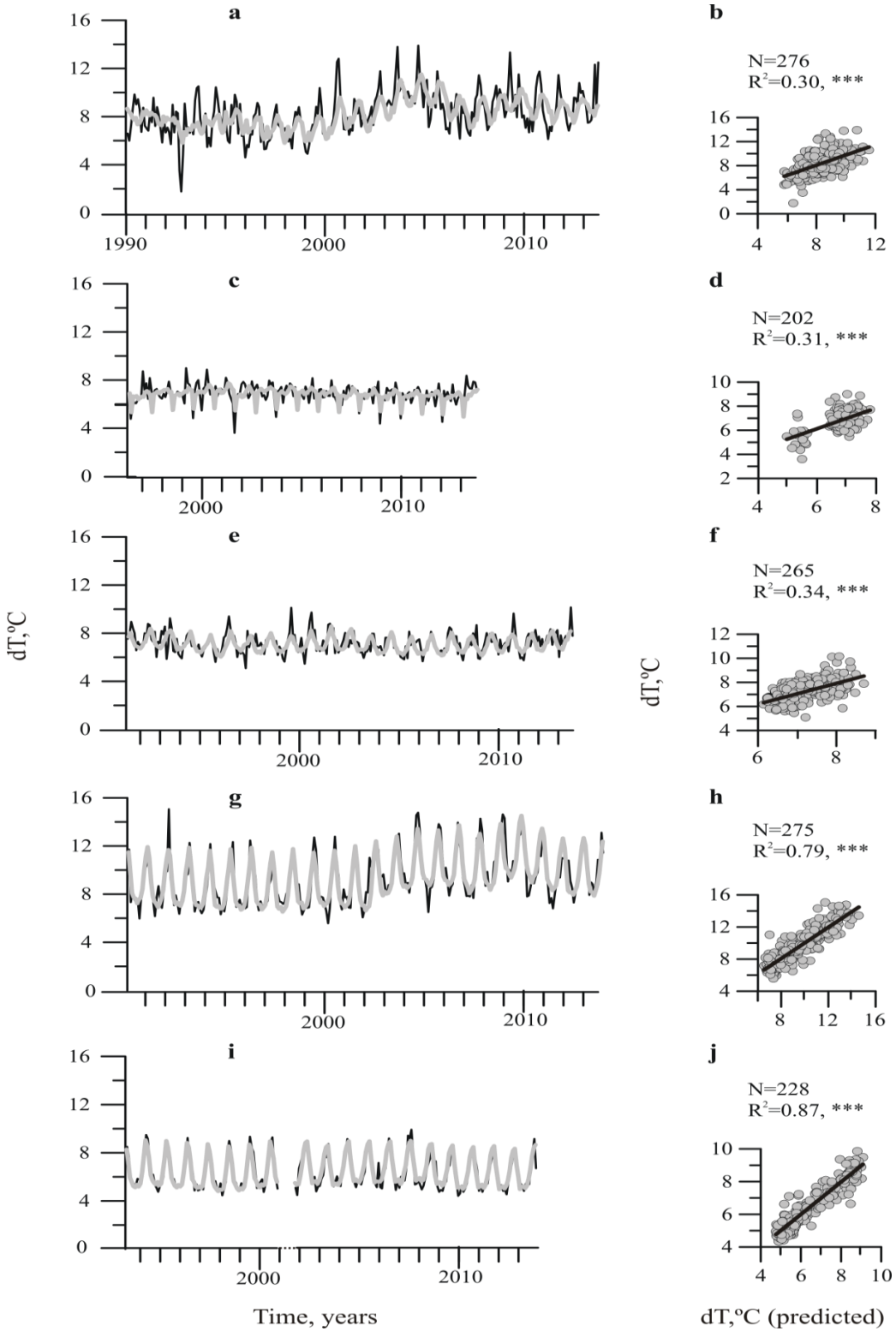

Fig. 6 Exponential smoothing analysis for each site and linear fit between rawdata, monthly means and predicted AirT.

Time series and linear fits are represented for PN (a and b, respectively), GBi (c and d), PB (e and f), PA (g and h) and PE (i and j). For the times series, black lines represent the raw data, while the grey lines the exponential smoothed values. In panel (i) the dotted line in the x axis show the gap within the time series (from October 2000 to December 2001). For the linear fits, the dark lines represent the least square fit. For each regression, the number of data points (N), the R^2 and their p-values are also shown. ***=p<0.001

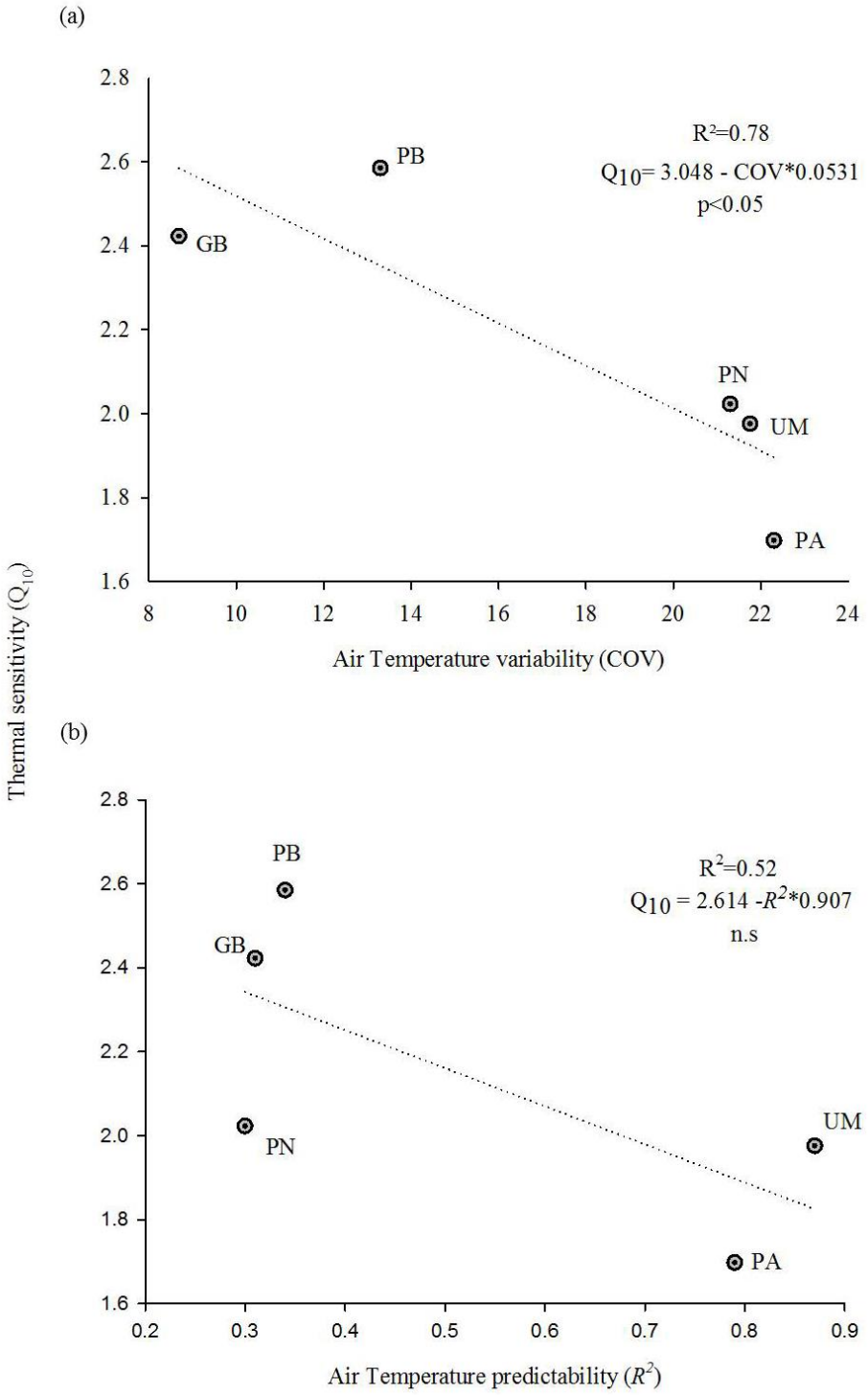

Fig. 7 Linear regression of Thermal sensitivity and Air Temperature variability (a) and predictability (b)

The Thermal sensitivity of the five populations (Q10 values) shows a negative relation with the temperature variability COV) and predictability (R^2) experienced over the past 23 year. R^2 values, linear equations and p values are reported in the figure. Legend: PN=Port Nolloth; GB=Gansbaai; PB=Plettenbergbaai; PA=Port Alfred; UM=Mngazana.

Discussion

The thermal tolerance (thermal breadth) and the metabolic rate (oxygen consumption) of *Talorchestia capensis* showed clear intra-specific differences among populations. Although, no replications were available for the LT50 experiments, the UTL and the LTL well matched the limits showed by the MO_2 curves, confirming that populations differed in their thermal breadth. In particular, the UTL for all populations is confirmed by the collapse of the metabolic performance beyond that point, suggesting the threshold of the critical temperature (Pörtner, 2010).

Combining the results of the LT50 experiments and the critical temperature threshold, results suggested that populations show a centre-margin pattern on the distribution of the thermal ranges. The data for air temperature retrieved from local stations showed that at the edges of the distribution (both Port Nolloth and Port Edwards) variability in air temperature (COV, see Table 4 and Fig. 6a) reach high value than at the centre (Plettenbergbaai). Thus, the effect of a more unstable environment at the edges of the distributional range of *T. capensis*, may have shaped its thermal range. Baldanzi et al. (2013), however, investigating centre-margin trends in the abundance, size and sex ratio of *T. capensis*, found weak support for the predictions of the Abundant Centre Hypothesis, suggesting that such mechanistic assumptions are more suitable for species with a continuous "North to South" range of distribution. Further analyses investigating thermal tolerance along the entire range of *T. capensis*, with a higher sampling resolution, could bring more light to the distribution of the thermal tolerance of this species.

In general, several hypotheses linking physiological properties (tolerance in particular) to species biogeography (range extent, position, and limits) have been proposed (for review, see Bozinovic et al., 2011). In particular, the Climatic Variability Hypothesis (CVH, Gaston, 2003) suggests that a positive relationship may exist between the range of thermal

tolerance and the climatic variability experienced by taxa at higher latitudes (Spicer and Gaston, 1999; Bozinovic et al., 2011). Although the CVH has been mainly demonstrated for congeneric species, the same concept has been proposed for conspecific populations at different latitudes along a thermal gradient (Hofmann and Watson, 1993; Hofmann, 2005). *T. capensis* showed differences in thermal tolerance across the entire range, thus supporting the CVH, but this pattern is probably driven more by the climatic differences experienced by the different populations, reflecting contrasting biogeographic conditions, rather than a direct effect of latitude.

While these mechanistic hypotheses seem to explain the geographic distribution of the thermal tolerance of *T. capensis*, MO_2 seem to follow a more complex pattern that may underpin the grouping among populations. I suggest that differences in the performance rates of *T. capensis* are possibly due to adaptation to local environmental conditions (local variability), driven by physiological acclimatisation and among-populations genetic heterogeneity. An interesting trend was the almost consistently higher slope of MO_2 for decreasing over increasing temperatures. Such consistency is difficult to explain, but presumably reflects differences in metabolisms during heating and cooling.

In general, populations show local adaptation when a certain phenotype is selected to cope with the environment, on average, such phenotypes will then persist if environmental conditions are unaltered (Kawecki and Ebert 2004). Thus, phenotypic selection acts to "diversify" populations, (i.e. expression of diverse or divergent genotypes) and favour local adaptation, while high gene flow tends to "homogenize" populations, resulting in similar phenotypes that are not locally adapted (Sanford and Kelly, 2011). Despite the fact that individuals from Port Nolloth and Gansbaai belong to the same genetic lineage, differences in local conditions may be pronounced, and explain the diverse physiology. For instance, Port Nolloth is characterised by upwelling (Xavier, 2007) and is geographically

situated within the cool temperate region (Namaqua Bioregion, as defined by Lombard, 2004), whereas Gansbaai is not influenced by coastal upwelling and is situated at the edge of a transition zone between the cool and the warm temperate bioregions (South-Western Cape bioregion, Lombard 2004). Individuals from Port Nolloth could be locally adapted to cope with environment characterized by upwelling, thus showing thermal physiology that is different from that of Gansbaai. In fact animals from Gansbaai showed similar physiological responses to those from Plettenbergbaai and the eastern populations. In this case, in evolutionary terms, climate-driven selection may have diversified the two populations, suggesting climatic rather than phylogenetic differences among populations. However, estimates of genetic diversity between Port Nolloth and Gansbaai suggested high within lineage variability (see Chapter 2 for details) which could also underpin the different physiology.

On the other hand, the comparable thermal physiology among the eastern populations (Port Alfred and Mngazana) is well supported by low within lineage variability in genetic diversity (see Chapter 2 for details). In evolutionary terms, the eastern populations may have developed a similar response to similar environment (confirmed by similar predictability of air temperature, Fig. 6 and Table 3) and no local adaptation has been selected. As a consequence they might be able to maintain a wide range of phenotypes to cope with environmental variability. The ability to adjust physiology to the environment is a form of plasticity that is a major component of intra- and inter-individual physiological variation (Spicer and Gaston, 1999; Ghalambor et al., 2007; Whitman and Agrawal, 2009) and it could be considered as the raw material for natural selection to act on (Pigliucci, 2001, 2005).

There was strong fluctuation in climatic variables over the last 23 years, with a clear geographical pattern. Several authors have demonstrated that physiological plasticity

depends upon daily and seasonal fluctuations of environmental variables (Bozinovic at al., 1990, 2003; Overgaard et al., 2006) and can be equally extended to the thermal limits and performances (Bozinovic et al., 2011). More interestingly, thermal variability can also have a significant impact on energy consumption by ectotherms and influence their thermal sensitivity over long time scales (Williams et al., 2012; Paaijmans et al., 2013). Jensen's Inequality Theory states that the mean value of metabolic rate over the accelerating portion of the curve will increase with increasing variance in temperature (Ruel and Ayres, 1999). Thus, organisms will need to reduce their thermal sensitivity (Q_{10}) and minimise the variance of their body temperature (Ruel and Ayres, 1999), to cope with high temperature fluctuations which would otherwise proportionally increase their energy requirements. In agreement with this theory, *T. capensis* showed decreased thermal sensitivity (Q_{10}) with increasing thermal variability and predictability of air temperature. (In particular, the low sensitivity to increasing temperature observed in Port Nolloth, could be explained as a depression of the metabolic response to increasing temperature due to adaptation to a variable, but unpredictable environment. Thermal variability and predictability have been demonstrated to affect several ectotherms, particularly inducing depression of the thermal sensitivity, reducing developmental time under warm conditions, and varying optimum and critical maximum temperatures (Nespolo et al., 2007; Williams et al., 2012; Paaijmans et al., 2013). Port Alfred and Mngazana had similar thermal performances, which seems to reflect the comparable temperature variability and predictability experienced over the past 23 years. Nevertheless these two populations responded differently from Port Nolloth which had similar variability in historical temperatures, possibly confirming that individual from eastern populations are more plastic in response to temperature and a stronger gene flow (compare to the western populations) may favour similar physiological performances.

The thermal sensitivity of a process can be considered as "passive" phenotypic plasticity, in the sense that it does not require a specific complex response by individuals (Ghalambor et al., 2007; Whitman and Agrawal, 2009), but it simply reflects a biophysical, rather than biochemical or physiological response to stimuli (Schulte et al., 2011). The present results suggest that the climatic variability and its predictability experienced over a relatively long time scale has influenced the plasticity of thermal responses in *T. capensis* when tested under laboratory conditions. Consequently, this "passive" plasticity to temperature (i.e. thermal sensitivity) showed by *T. capensis* could have been an initial a response to thermal variability that has been subsequently transmitted across generations, hence being selected as an important adaptation to local conditions. The process by which an environmentally induced phenotypic plasticity can become genetically fixed is called genetic assimilation and was firstly proposed by Waddington in 1942 (Waddington 1942, 1953a, 1953b). Several authors have demonstrated genetic assimilation in laboratory experiments (Sword 2002; Suzuki and Nijhout, 2006), but its occurrence in nature, and its role in evolution are still controversial (de Jong 2005; Pigliucci et al., 2006). Although the results do not prove genetic assimilation, they do show that the temperature variability, experienced during a relatively long time scale (i.e. multiple generations), is reflected in the thermal response to increasing temperature in these sandhoppers. The possibility that such "climatic memory" will persist over future generations of sandhoppers, would suggest that the effect of temperature fluctuation and predictability is fundamental when forecasting the effect of climate change on natural populations.

In conclusion, as recently proposed by several authors (Chevin et al., 2010; Williams et al., 2012; Paijmaans et al., 2013), when predictions on the effect of a warming climate over population persistence are made, temperature fluctuation, temperature predictability and

the importance of phenotypic plasticity as a rapid response to environment, should be incorporated, as they provide a more comprehensive and realistic scenario.

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-Chapter 5-

Temperature induced maternal effects on early ontogeny in the sandhopper Talorchestia capensis (Amphipoda, Talitridae)

Introduction

Phenotypic adaptation in natural populations is a combined effect of the genetic and environmental variation among individuals and it represents the raw material for natural selection to act on (Mousseau and Fox, 1998; Pigliucci, 2005). According to the classic laws of Mendelian inheritance, the phenotype of an individual has been envisioned as the result of its genotype plus the environmental effect experienced during its development, (Kirckpatrick and Lande, 1989; Mousseau and Fox, 1998). The effect of the phenotype of the mother or the environment that she experiences, however, may have a strong influence on the phenotype of the offspring beyond direct genetic transmission (Marshall and Uller, 2007). Such phenomena are known as Maternal Effects (MEs, hereafter), which represent a particular type of phenotypic plasticity and are increasingly considered to form important pathways for the evolution of organisms and ecological speciation (Agrawal, 2001; Räsänen and Kruuk, 2007; Marshall and Uller, 2007; Mousseau et al., 2009; Whitman and Agrawal, 2009; Fitzpatrick, 2012).

MEs occur widely in nature, with numerous studies showing the importance of a mothers' phenotype in buffering the effects of environmental stressors on her offspring (Galloway, 2005; Galloway and Etterson, 2007; Marshall and Uller, 2007; Mousseau et al., 2009). Of particular interest is the adaptive significance of MEs (positive effects on the fitness of the mother and/or offspring, Mousseau and Fox, 1998) and their implications for population dynamics (Plaistow et al., 2006; Plaistow and Benton, 2009), range expansion and colonisation (Duckworth, 2009), prey-predator interactions (Inchausti and Ginzburg, 2009), reproduction and life history traits (Marshall and Keough, 2004a,b; Marshall and Uller, 2007; Marshall et al., 2008) and evolution (Agrawal, 2001; Pigliucci, 2005; Galloway and Etterson., 2007; Ghalambor et al., 2007; Whitman and Agrawal, 2009; Pigliucci and Muller, 2010). Although MEs have been extensively studied, a great debate

remains on whether they show adaptive significance (Mousseau et al., 2009). Marshall and Uller (2007) suggest that MEs show adaptive significance when they enhance the fitness of the offspring and/or the mother. These authors proposed four different classes of MEs, based on their consequences for the offspring: 1) "anticipatory MEs", occurring when the fitness of the offspring is enhanced by the effect of the mothers' phenotype as a result of mothers' ability to "predict" the future environment of the offspring.

Mothers may be able to use the maternal environment as predictor of their offspring environment, as reported for seed beetles by Fox et al. (1999); 2) "selfish MEs", occurring when mothers are able to predict the future environment, but reduce the fitness of the offspring in order to enhance their own. For example, mothers can act selfishly when the "per offspring" investment may be of a high cost or mothers have the chance to reproduce repeatedly and under better conditions in the immediate future; 3) "bet-hedging MEs", occurring when mothers produce a range of offspring phenotypes, allowing them to cope with uncertain environments, which cannot be directly predicted by the mother. Example of this strategy are asynchrony in hatching time in birds (Laaksonen, 2004) and the within-brood variation in egg size found in several marine invertebrates (Marshall et al., 2008); 4) "transmissive MEs", occurring in the extreme, but not rare, case of a reduction of both maternal and offspring fitness (e.g. transmission of pathogens from mother to offspring, resulting in a maladaptation). The first three categories are considered to be adaptive and consequently to play an important role in the evolution of species (Marshall and Uller, 2007).

Offspring size (eggs are considered as offspring hereafter) is probably the most studied maternal effect in ecology because it has fitness consequences for both the offspring and the mother (Bernardo, 1996b), although selection seems to be skewed towards maternal fitness (Smith and Fretwell, 1974). Thus, mother and offspring may paradoxically compete

for fitness (Einum and Fleming, 2000). For instance, if more resources are invested in eggs, the performance of the offspring will be enhanced at the expense of the fecundity of the parent, whilst, if less energy is allocated to eggs, the fecundity of the parent will be favoured (Smith and Fretwell, 1974; Bernardo, 1996a). As a result, the offspring size/number trade-off, combined with the offspring size/fitness function, has attracted the interest of many evolutionary biologists and formed the basis of many life-history models (Smith and Fretwell, 1974; Marshall and Keough, 2007). Undeniably though, the ability of mothers to predict the relationship between offspring size, number and the optimal size produced to maximise fitness, is fundamental (Marshall et al., 2008).

In marine invertebrates, the planktonic offspring of indirect developers are potentially more dispersive than direct developers (see Thiel and Haye, 2006 for contrasting patterns) and experience several independent life stages prior to settlement. Given that the probability that the habitat of this offspring can be easily "predicted" by the mothers is reduced, as the maternal environment is likely to be different from the habitat of the offspring (Marshall et al., 2008). On the other hand, direct developers produce offspring with low dispersal and no larval stages, implying that the brood is released into the maternal environment. There is therefore the potential for mothers to assess environmental conditions and adaptively adjust the size and the number of their offspring (Fox and Mousseau 1998, Einum and Fleming 2002). The production of variably sized offspring is therefore favoured (i.e. bet-hedging MEs) when the offspring environment is unpredictable and we would then expect increased levels of variation *within broods* (Marshall et al., 2008). Similarly, if the offspring environment is predictable, we should see more variation among mothers than within broods (as reported mostly for direct developers, Marshall and Keough, 2007).

The environment therefore plays a fundamental and direct role in the plasticity of individuals, inducing mothers to adopt different strategies and investments which result in different MEs (Parichy and Kaplan, 1992). Moreover, the environment can either directly or indirectly affect the size and/or the number of the offspring (Kingsolver and Huey, 2008) and recent interest has addressed environmental temperature as a fundamental factor shaping life histories (Angilletta et al., 2006; Angilletta, 2009). Temperature has a profound effect on ectotherms and has complex relationships with body size (both adult and offspring) and fitness (Angilletta et al., 2003; Kingsolver and Huey, 2008). A common trend in the variation of offspring size across a wide range of taxa is the relationship between size of offspring and temperature, simply synthesised by the expression: "colder mothers produce larger eggs" (Blanckenhorn, 2000; Fischer et al., 2003a, b; Marshall et al., 2008; Bownds et al., 2010). The explanation for this pattern relies on the concept of an optimal size of offspring, indicating a perfect balance between female fecundity and offspring performance (McGinley et al., 1987). Since mothers tend to maximise their own fitness (Smith and Fretwell, 1974), they will produce large offspring (in low numbers) if the relationship between offspring size and performance is linear. Conversely, when this relationship is lacking, mothers should increase the number of eggs (at the expense of egg size), enhancing their own fecundity (Smith and Fretwell, 1974). Since temperature strongly affects the relationship between offspring size and performance, described by the Temperature-Size Rule (Laptikhovsky, 2006), mothers should adjust the size/number of their offspring accordingly (McGinley et al., 1987). In the literature, there is increasing evidence of temperature-induced maternal effects on offspring size and performance in a wide variety of taxa (Blanckenhorn, 2000; Fischer et al., 2003a, b; Angilletta et al., 2006; Bownds et al., 2010; Liefting et al., 2010; Burgess and Marshall, 2011; Lorioux et al., 2012; Sun and Niu, 2012).

Marine invertebrates are useful in the study of variation in egg size (Marshall and Keough, 2007), although most of attention has been addressed to indirect rather than direct developers (Marshall et al., 2008; Collin, 2010; Collin and Salazar, 2010; Collin 2012). Sandhoppers are direct developing amphipods, with the juveniles being released into the maternal environment after a period of active parental care (Morritt and Spicer, 1996a, b, c; Morritt and Richardson, 1988; Thiel, 1999). Parental care is a key mechanism that has allowed talitrids to colonise the supralittoral and terrestrial environments successfully, and take their evolutionary departure from the truly marine environment (Spicer et al., 1987). This unique aspect makes sandhoppers an ideal model organism to study maternal effects, and the maternal-mediated influence of temperature on the offspring size.

In the present work, I investigated temperature induced MEs on the offspring size of the South African sandhopper *Talorchestia capensis* (Order Amphipoda, Family Talitriadae). In particular, I focused on the effect of temperature on the reproductive strategies of the mothers, investigating *within brood* and *among female* variation in the size of eggs. To understand the causal relationships among temperature, egg quality (i.e. density of the eggs) and fecundity (i.e. number of eggs), I tested the prediction of optimality models, using an information theoretic approach, following Angilletta et al. (2006). The models predicted a positive effect of temperature on egg quality (density) and fecundity (the number of eggs per female), as well as its indirect effect through maternal size, due to energy acquisition and/or parental care (Angilletta et al., 2006). I predicted that an "invariant" strategy (*sensu* Marshall et al., 2008) would be adopted by sandhoppers in response to temperature, due to their direct developmental mode, with no significant *within brood* variation in the size of the eggs. Furthermore, temperature is predicted to have an indirect effect on maternal fecundity and egg quality, as a result of the direct effects on the mothers during early ontogeny.

Material and Methods

Animal collection

Animals were collected from a single site on the south-east coast of South Africa (Port Alfred, S33.89336; E26.29815), during two separate surveys (February 2012 and 2013). The site was chosen because it hosts the most abundant population of *T. capensis* in the south-east coast and is easily accessible. The month of collection (February) was chosen to maximise the collection of females carrying eggs when the proportion of ovigerous females of *T. capensis* is expected to be highest (Van Senus, 1988), although females carrying eggs are present throughout the year (Van Senus, 1998; SB, pers. obs.). The animals were hand-picked from their habitat and placed in a plastic cooler bag half filled with moist sand taken from the site of collection. Prior to collection, animals were sexed, and the females visually checked for the presence of eggs. Males and females carrying eggs were then separated and placed in two different containers. A total of 90 females and 90 males were collected. Immediately after collection, animals were transported to the laboratory, keeping to a minimum any stress due to abrupt changes in temperature. In the laboratory, animals were acclimated under a 12/12h photoperiod in a controlled environment room at 18°C for at least 48 hours.. This temperature is the annual average air value in Port Alfred for 2012 and 2013 (data provided by the South African weather service, www.weathersa.co.za).

Since sandhoppers in nature burrow underneath the moist organic matter present on the shore to avoid daytime desiccation and predation (Williams, 1995), macroalgae found on the shore were also haphazardly collected and added to the breeding chambers to recreate natural conditions. Sand and seawater from the original site of collection were provided regularly during the course of the experiments and replaced whenever necessary. Animals

were maintained in the moist sand with wet macroalgae and fed *ad libitum* (see below for details on food regime).

Animal maintenance and experimental settings

After the acclimation time, animals were equally divided among three separate rooms at different constant incubation temperatures: 13°C, 18°C and 23°C, representing the three experimental temperatures. Acclimation temperature has been shown to be critical when designing mesocosm experiments (Terreblanche et al, 2007). In particular, exposure to fluctuating temperatures around different means may lead to different results compared to constant incubation temperatures (Paaijmans et al., 2013). I recognize this as a limitation to the results and the possible effects of temperature fluctuations on the MEs are reported in the Discussion. Prior to the start of the experiments, females were gently de-brooded, as described by Morritt and Spicer (1996a), avoiding damage and minimising manipulation stress. To ensure that no stress occurred after de-brooding, females were checked and their locomotive activity monitored. The eggs removed were preserved in 70% ethanol prior to 24h fixation in 10% formalin, for subsequent analyses of the embryonic stage (following Wilhelm and Schindler, 2000 and Cunha et al., 2000). This preliminary investigation provided important insights on the embryological stages of the eggs of *T. capensis*, as no previous studies are available for *T. capensis*. Subsequently, female length was measured using a stereomicroscope, from the base of the antenna to the telson and the length recorded as Female Size (Pavesi and DeMatthaeis, 2009). Females and males were then coupled and reared (n = 30 couples) in square plastic containers (30 containers, 10X10cm wide), partially filled with approximately 2-3 cm of moist sand from the site of collection. During the experiments, sandhoppers were fed *ad libitum* using Kelp tablets (Norwegian seaweed, Weetol®) ground and mixed with sterilised seawater (1 ml of autoclaved seawater per tablet). When needed, the food was replaced with a fresh mixture. To reduce mortality

due to contamination from faecal residue, the sand was replaced twice a week. To preserve humidity, pre-autoclaved seawater (1 hour at 120 °C) from the site of collection was daily sprayed in each box and a perforated plastic lid limited evaporation, while allowing air exchange. These experiments did not take into account the effect of salinity on the reproduction of *T. capensis*, although it has a demonstrated effect on the reproduction of *Orchestia gammarellus* (Amphipoda, Talitridae), with mothers being able to control the osmotic environment of developing embryos (Morritt and Spicer, 1996c). A daily input of fresh seawater (35‰) and a partial replacement of sand however, ensured that salinity variation within the box was minimal, hence any possible effect of salinity would affect all the animals likewise, excluding any confounding effects.

Fig 1. Mating Couple of *T. capensis* The photograph shows the male and the female of *T. capensis* in the typical mating position. The male grasps the female using the enlarged 2nd gnathopod (also used for display during the pre-copula). This position allows the male to deposit the sperm in the female oviduct and is maintained for about 60 minutes (Van Senus, 1988 and pers obs). This is the only documented mating couple observed during the experiments. It was indeed difficult to observe mating without disturbing the couple, as it usually takes place in the dark, when sandhoppers move to the surface in search of a mate. Little information is available for natural populations of *T. capensis* (but see the unpublished MSc thesis of Muir, 1977). The photograph has been taken by myself during the experiments.

Experimental procedures, egg collection, staging and measurements

The experiments began when the animals were coupled and incubated at three different temperatures (see above). In talitrid species, copulation happens only after ecdysis (Williamson, 1951; Van Senus, 1988), when females show a more flexible exoskeleton which can be easily grasped by the male in order to deposit the sperm in to the female's brood (Van Senus, 1998). After copulation, which lasts up to 60 minutes in *T. capensis* (Van Senus, 1988; SB, personal observation), the male releases the female and approximately 4 weeks later, the juveniles appear in the female's brood (Van Senus, 1998). While mating was observed often during the course of the experiments (Fig.1), it was not possible to determine the exact time of copulation, which is required to calculate the embryos developmental time. It was in fact difficult to find moulting females (a sign of imminent mating) and to monitor their activity to record the first appearance of eggs. The rearing of animals as separate couples, eased, however, the retrieving of individual information on reproductive traits, although reducing the probability of mating, compared to a mass-rearing situation. In order to minimise the stress induced by a constant monitoring of the sand in search of moulting females (which can disturb the female and negatively affect mating), I opted not to consider the embryos' developmental time in this study. Males and females were however monitored weekly. A fine wooden stick was used to gently move the sand in search of animals. Males, easily detected by the longer antennae, were observed and their general condition (i.e. locomotory activity) visually checked. Any reduced locomotory activity, was noted and replacements were made if animals were found to be dead (on 5 occasions animals were found dead). Females were gently picked up and visually checked for the presence of eggs. If eggs were observed within the brood, a fine brush was used to gently remove the egg mass without damaging the female oostegites or the eggs. An ovigerous female is easily detectable since it shows noticeable slow activity, a broadening of the abdomen and a typical bent-forward posture

(pers. obs.). Such observations were fundamental to avoiding egg loss during female inspections. Females tend to release eggs when stressed (e.g. picked up from the sand), simply flexing their body and using the appendices to pull the eggs out (Muir, 1977, unpubl.; pers. obs.). Thus, reducing the time spent checking for the presence of eggs, by simply observing the behaviour and morphology of females, minimised possible errors in estimating the number of eggs per female due to egg loss. After removal, eggs were immediately fixed in 10% formalin for 24 hours and stored in 70% ethanol. This fixation protocol does not affect the shape of the egg (e.g. shrinking due to ethanol/formalin) and is largely used in standard procedures (Frimpong and Henebry, 2012). Under a stereo microscope set to 16x magnification, equipped with a recording camera (Olympus, SZ61 with CMOS colour camera CX30), the number of eggs was counted and the developmental stage of each egg determined following Cunha et al. (2000): Early stages (E), Medium stages (M) and Late stages (L). Stages are shown in Figure 2.

In this chapter, size is considered with its broad meaning and includes measurements of volume, weight or density. Images of each egg were analysed using Image-J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/), to estimate the volume and are shown in Figure 2. After calibration, the ruler tool was used to measure the length and the width of each egg. Since eggs were oval-shaped, the volume (v) was calculated following the equation of a prolate-spheroid (Beyer, 1987, cited in Wilhelm and Schindler, 2000):

$$v = 4/3 \times \pi \times r_1 \times r_2 \times r_3$$

Where, v is the volume (mm^3), r1 is half the length, r2 is half the width and r3 is half the height (width and length were assumed to be equal). All the photographed eggs were then dried at 60°C for 24h in order to measure the dry mass, following Wilhelm and Schindler

(2000). The eggs were weighed using a precision balance to the nearest 0.1 µg. The dry mass of the eggs is reported as egg Weight (w) and expressed in mg. A measure of the Density (d) of the egg was calculated by dividing the egg dry mass by the volume and expressed in $mg.mm^{-3}$. Density can give a better estimate of egg condition than volume or mass, as it provides a powerful index of nutrient storage (lipids), structure (proteins) and reproductive success (Moya-Laraño et al., 2008). Thus, density was used here as a proxy for egg quality.

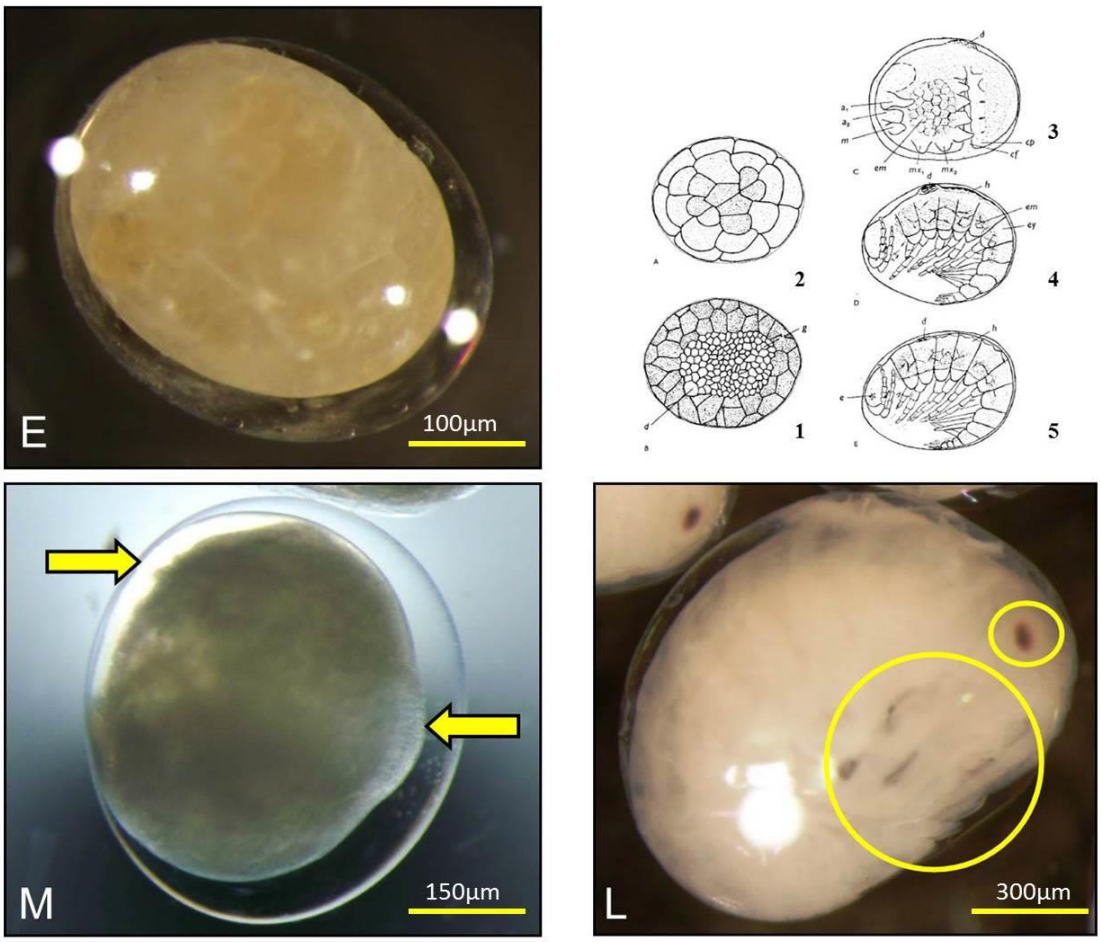

Fig.2 Developmental stages of *T. capensis* embryos
Stages of embryonic development following Cunha et al. (2000). Stages described by Sheader and Chia (1970) are reported for comparison in the upper right quadrant. Early stages (E) showed a homogeneous mass of yolk (corresponding to 1st and 2nd stages of Sheader and Chia, 1970); Medium stages (M) represent the beginning of gastrulation (corresponding to the 3rd stage of Sheader and Chia, 1970), showing the dorsal organ rudiment (right arrow) and the invagination of the caudal furrow (left arrow); Late stages (L) showed the formation of a rudimentary eye, appendages and antenna (corresponding to the 4th and 5th stages of Sheader and Chia, 1970).

In order to investigate whether females produce offspring of similar size (invariant strategy, *sensu* Marshall et al., 2008) a Coefficient Of Variation (COV) of egg volume, weight and density was calculated for each clutch, using the following equation (see Marshall et al., 2008 for eggs of marine invertebrates):

$$COV = (SD/mean) \times 100$$

Where SD is the Standard Deviation of egg volume, weight or density and mean is the average of egg volume, weight or density.

Data analysis

To evaluate the effect of temperature (fixed and orthogonal), stage (fixed and orthogonal) and year (random and orthogonal) on the *within-brood* variability, three separate Permutational Anovas (PERMANOVA), were performed on the values of COV for egg volume, weight and density respectively. To show *within-brood* variability, an MDS ordination of the data cloud was performed, prior to a hierarchical cluster analysis of a Bray Curtis similarity matrix of the square root transformed data. Groupings were based on a threshold of 82% of similarity (Clarke and Gorley, 2006). To evaluate the effect of temperature (fixed and orthogonal), year (random and orthogonal) and female (random and nested in temperature) on *among-female* variability on the size of the egg, three separate Permutational Anovas (PERMANOVA) were performed on volume, weight and density respectively. To identify possible changes in the maternal effect between stages, separate analyses were conducted on early and medium stages. Medium stages were only available in 2012, thus analyses were conducted with temperature (fixed and orthogonal) and female (random and nested in temperature) as factors. If the effect of year was not significant, analyses were conducted by pooling years and excluding the factor year, with temperature (fixed and orthogonal) and female (random and nested in temperature) as factors. To

further explore the effect of year with a possible bias towards number of samples (i.e. number of eggs), a PERMANOVA was performed separately for 2012 and 2013 with temperature (fixed and orthogonal) and female (random and nested in temperature) as factors. All PERMANOVAs were performed on a Euclidean distance resemblance matrix, using 9999 permutations of residuals under a reduced model. All analyses were done using PRIMER 6 & PERMANOVA + (Anderson et al., 2008; Clarke and Warwick, 2001). In order to investigate the relationships among temperature, maternal size and reproductive traits (egg quality and fecundity) I used an information-theoretic approach to identify the model that best fitted the available data, following Angilletta et al. (2006). The optimality models predicted direct and indirect effects of temperature on the quality of eggs (egg size) and fecundity (egg number) based on the theory of reproductive allocation (see reviews by Bernardo, 1996a, and Roff, 2002). The theory predicts an optimal egg size which is the result of a balance between offspring fitness (greater allocation of resources should enhance quality of the offspring) and parental fitness (lesser allocation of resources should enhance fecundity of the parents). Three main models were tested, following Angilletta et al. (2006) and are shown schematically in Figure 3: a temperature effect (direct effect of temperature on egg quality, which affects fecundity), a maternal acquisition effect (indirect effect of temperature on both egg quality and fecundity through maternal size) and a parental care effect (indirect effect of temperature on egg quality which enhances fecundity). A simultaneous effect of the three main conditions was also tested, resulting in three additional models (see Fig. 3). The reproductive traits analysed were egg density (averaged per female), as a proxy of egg quality (as it provides a good estimate of body condition and energy storage; Moya-Laraño et al., 2008) and egg number, as proxy of fecundity. If the effect of year was not significant, models were tested by pooling the data from 2012 and 2013. Early stages only were tested, as they are particularly subjected to

influences from the environment, and also provide a powerful index of the "per-offspring" maternal investment (Parichy and Kaplan, 1992; Yan et al., 2011; Lorioux et al., 2012). Temperature should have a positive effect on egg quality (i.e. density) as density is inversely proportional to volume and also a positive effect on fecundity as number of eggs is predicted to be negatively correlated with their volume. As density is here calculated as the mass of the eggs divided by their volume, any effect of temperature on the volume of the eggs (see the Temperature-Size Rule of Rass, Laptikhovsky, 2006) should inversely affect its density. To evaluate the competing models (Fig.3), I performed a path analysis of the raw data. Path analysis is a statistical technique used to examine causal relationships between two or more variables and, in ecological studies, can be performed to understand comparative strengths of direct and indirect relationships among a set of variables (Shipley, 1997; 1999). Analyses were performed with AMOS 5.0 (SpSS, Chicago), following the approach of Angilletta et al. (2006). The software used a maximum likelihood method to obtain path coefficients and a χ^2 estimate of the lack of fit for each model. An information-theoretic approach, using the second order Akaike Information Criterion (AICc), was applied to each model, following the formula proposed by Burnham and Anderson (2002) for relatively small sample sizes:

$$AICc = \chi^2 + 2K + 2K(K+1)/(N-K-1)$$

Where χ^2 is the goodness of fit, K the number of estimated parameters, and N is the number of samples (i.e. females). This approach produces estimates of the expected Kullback-Leibler information (the information lost when a particular model is used to approximate truth), hence enabling one to rank models based on their power to explain relationships among variables, when these relationships are unknown (Burnham and Anderson, 2002). Values of AICc were rescaled as simple differences between the value of AICc for each model and that of the model with the lowest value (ΔAICc). The models were therefore

ranked by their values of ΔAICc. Akaike weights (Aw) were used to assess which model was most likely to be the best (the Akaike weight is the normalized likelihood that a model fits the data better than any other model in the set; see Burnham and Anderson, 2002). Models were supported if their ΔAICc was less than 2.0 (Burnham and Anderson 2002; Angilletta et al., 2006).

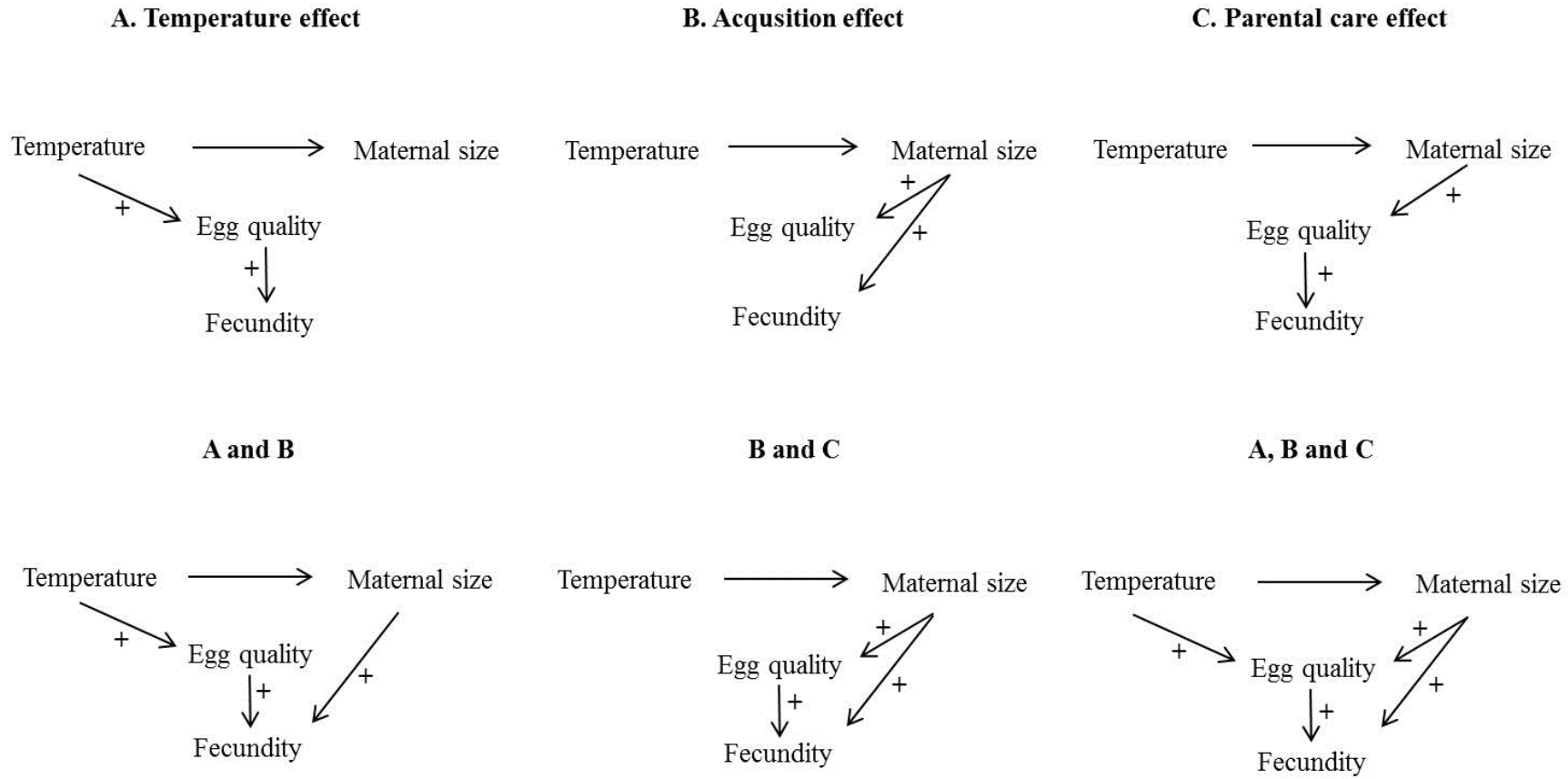

Fig. 3 Path models of direct and indirect effect of temperature on reproductive output.
In the first panel are shown the three main statistical models (modified from Angilletta et al., 2006): A. Temperature effect (direct), B. Acquisition effect (indirect), C. Parental care effect (indirect). The remaining models represent a combination of the simultaneous effects of the first three. The arrows represent the paths (causal relationships) among variables. Predicted positive relationships among variables are reported (+).

Results

Sixty seven females (out of a total of ninety used in the experiments) were able to mate and produce eggs. The results are reported for early and medium stages only, as eggs did not reach the late stage at the lowest incubation temperature (13°C). The analyses performed to evaluate the *within-brood* effect of temperature showed no significant effects for all three traits, volume, weight or density. Nor were there significant effects of Year or Stage on any of the three traits (Table 1). Figure 4 shows the MDS displaying the absence of groupings based on temperature. In the analysis of *among-female* variability, temperature had a significant effect on density (after removing year because its effect was not significant, see Table 2) and a not-significant effect ($p=0.0506$) on volume (Table 2). No significant effect of temperature was found for weight for any stage (Table 2). A strong, significant effect of female is reported for all the analysed traits (Table 2 and 3). Egg volume was significantly different between 2012 and 2013, although the interaction between year and temperature was not significant (Table 2). There was an effect of temperature on volume and density in 2013 but not in 2012 probably detected because of the greater number of eggs available in 2013 (Table 3). Consequently, analyses were performed to test whether the absence of a significant effect of temperature in 2012 was due to the unbalanced number of samples between years. The analysis revealed a strong and significant effect of temperature on volume ($p<0.001$) and density ($p<0.0001$) for early stages in 2013 (Table 3 and Fig. 5). In the path analyses, standardised coefficients showed consistency with the prediction on the causal relationship between temperature, female size and egg quality and fecundity (as indicated by the signs in Figure 6, reporting the standardised path coefficient). The model that best described the empirical data for early stages was the one derived from simultaneous interaction of temperature and maternal size ("A and B" model), as reported in Table 4, where models were

scaled on the base of their ΔAICc. In particular, in the model "A and B", temperature seemed to affect positively egg quality (i.e. density), while maternal size influenced the fecundity (i.e. egg number). The other models showed a ΔAICc greater than 2.0, thus these models were not supported by the available data.

Table 1 PERMANOVA performed to investigate the effect of temperature, year and stage on the *within-brood* variability of egg size. he table reports the analyses on COV for volume (COVv), density (COVd) and weight (COVw).n.s = not significant. All interactions were no significant and omitted from the table below.

Egg size	df	MS	Pseudo-F	p-value
COVv				
Year	1	311.9	3.598	n.s
Temperature	2	18.24	2.557	n.s
Stage	1	55.14	0.643	n.s
Year*Teamperature	2	24.68	0.247	n.s
Year*Stage	1	84.10	0.842	n.s
Temperature*Stage	2	28.60	0.286	n.s
RES	43	99.79	-	-
COVd				
Year	1	477.6	9.478	n.s
Temperature	2	40.28	0.546	n.s
Stage	1	184.7	3.116	n.s
Year*Teamperature	2	251.4	1.732	n.s
Year*Stage	1	48.86	0.336	n.s
Temperature*Stage	2	146.1	1.006	n.s
RES	43	145.1	-	-
COVw				
Year	1	215.9	19.19	n.s
Temperature	2	29.2	2.721	n.s
Stage	1	13.61	0.701	n.s
Year*Teamperature	2	9.657	0.108	n.s
Year*Stage	1	10.97	0.123	n.s
Temperature*Stage	2	35.83	0.403	n.s
RES	43	85.81	-	-

Table 2 PERMANOVA performed to investigate the effect of temperature, year and female on the *among-female* variability of egg size. The results are reported for each factor. "temperature*year" represents the interaction between the two factors. The factor year is not reported for density and weight (see material and methods). **= p<0.001; *** = p<0.0001; n.s = not significant .

Egg size	df	MS	Pseudo-F	p-value
volume				
year	1	0.852	8.956	**
temperature	2	0.282	16.68	n.s
female	31	0.129	5.826	***
temperature*year	2	0.017	0.176	n.s
RES	251	0.002	-	-
density				
temperature	2	0.043	9.602	***
female	34	0.006	3.806	***
RES	251	0.001	-	-
weight				
temperature	2	0.007	1.209	n.s
female	34	0.007	8.205	***
RES	251	0.000	-	-

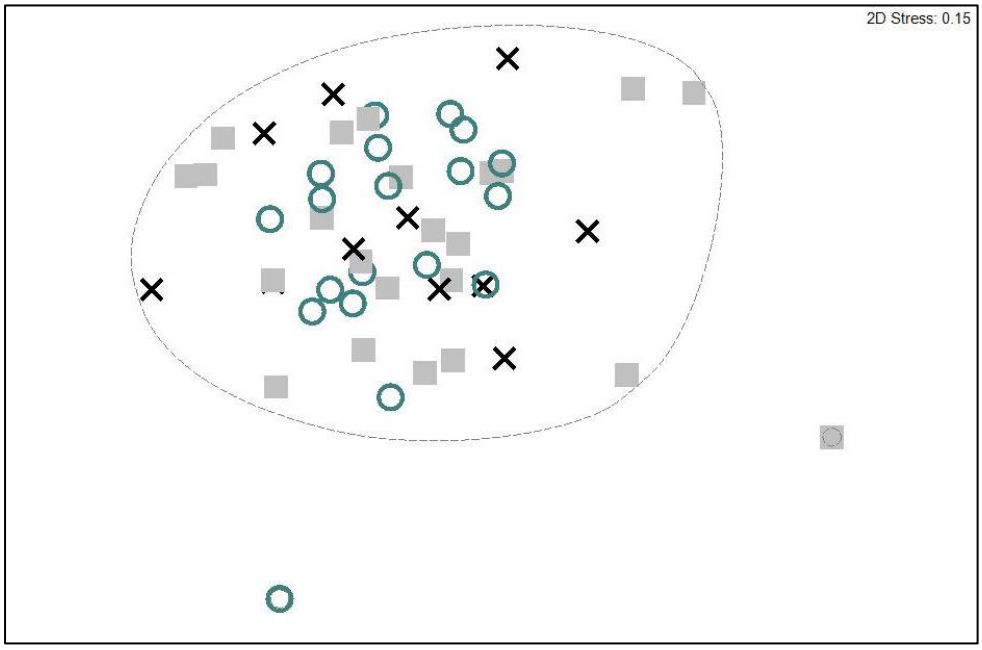

Fig 4. MDS plot of the COV values of volume, weight and density
The multidimensional scaling shows the distribution of the data cloud for COVv, COVw and COVd. Light grey squares represent incubation temperature of 13°C; dark grey circles, 18°C; black crosses, 23°C. The grey dashed lines represent the grouping based on hierarchical cluster analyses (see methods for details). Data were square root transformed prior to developing the Bray-Curtis resemblance matrix. The similarity within groups is 82%.

Table 3. PERMANOVA to account for temporal variation in the effect of temperature on three egg parameters

The table reports the values of volume, density and weight (mean ± SD) for the three incubation temperatures. Analyses were performed separately for early and medium stages and year. N=number of eggs; **=significant p-values (p<0.001); *** = significant p-values (p<0.0001); n.s = not significant p-values.

Egg size	13°C	18°C	23°C	df	n	MS	Pseudo-F	p-value
volume								
Te *early 2012*	0.98±0.12	0.90±0.17	0.82±0.08	2	78	0.092	0.846	n.s
Fe(Te) *early 2012*	-	-	-	8	78	0.133	14.25	***
RES	-	-	-	67	-	0.009	-	-
Te *medium 2012*	1.29±0.57	1.40±0.34	1.44±0.35	2	115	0.158	0.104	n.s
Fe(Te) *medium 2012*	-	-	-	10	115	1.589	45.50	***
RES	-	-	-	102	-	0.004	-	-
T *early 2013*	1.13±0.14[a]	1.06±0.08[a,b]	0.96±0.14[b]	2	210	0.473	4.498	**
F(T) *early n2013*	-	-	-	23	210	0.127	4.475	***
RES	-	-	-	184	-	0.027	-	-
density								
Te early 2012	0.17±0.03	0.19±0.02	0.18±0.00	2	78	0.012	1.210	n.s
Fe(Te) early 2012	-	-	-	8	78	0.004	3.444	***
RES	-	-	-	67	-	0.001	-	-
Te medium 2012	0.14±0.05	0.13±0.03	0.10±0.03	2	115	0.002	0.589	n.s
Fe(Te) medium 2012	-	-	-	10	115	0.010	18.88	***
RES	-	-	-	102	-	0.001	-	-
Te early 2013	0.15±0.02[a]	0.18±0.04[a,b]	0.21±0.03[b]	2	210	0.067	12.85	***
Fe(Te) early n2013	-	-	-	23	210	0.006	3.853	***
RES	-	-	-	184	-	0.001	-	-
weight								
Te early 2012	0.17±0.02	0.17±0.02	0.15±0.01	2	78	0.001	0.779	n.s
Fe(Te) early 2012	-	-	-	8	78	0.002	4.215	***
RES	-	-	-	67	-	0.001	-	-
Te medium 2012	0.16±0.03	0.17±0.03	0.14±0.01	2	115	0.011	1.482	n.s
Fe(Te) medium 2012	-	-	-	10	115	0.007	10.41	***
RES	-	-	-	102	-	0.007	-	-
Te early 2013	0.17±0.04	0.19±0.04	0.20±0.02	2	210	0.017	2.512	n.s
Fe(Te) early n2013	-	-	-	23	210	0.008	9.039	***
RES	-	-	-	184	-	0.001	-	

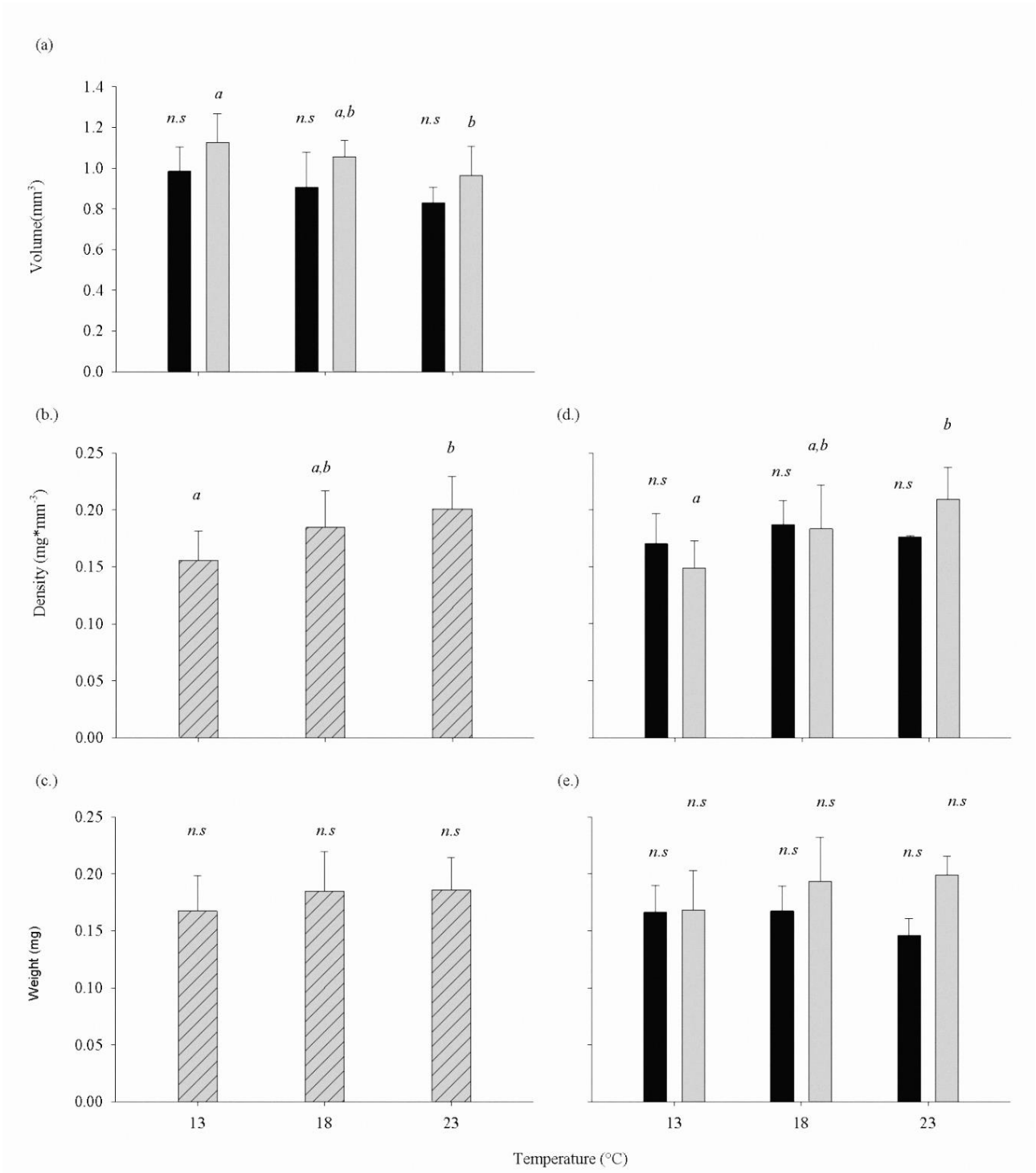

Fig. 5. Effect of temperature on volume, density and weight (mean±SD)
The bars show the different effect of temperature on volume (a.), density (b. and d.) and weight (c. and e.). The black bars represent data from 2012, solid grey bars are from 2013. For density (b. and d.) and weight (c. and e.) data are shown both pooled (b. and c.) and separated by years of collection (d. and e.). Letters on top of the bars represent the groupings based on the pair wise analyses for the factor temperature (see material and methods). n.s=not significant differences.

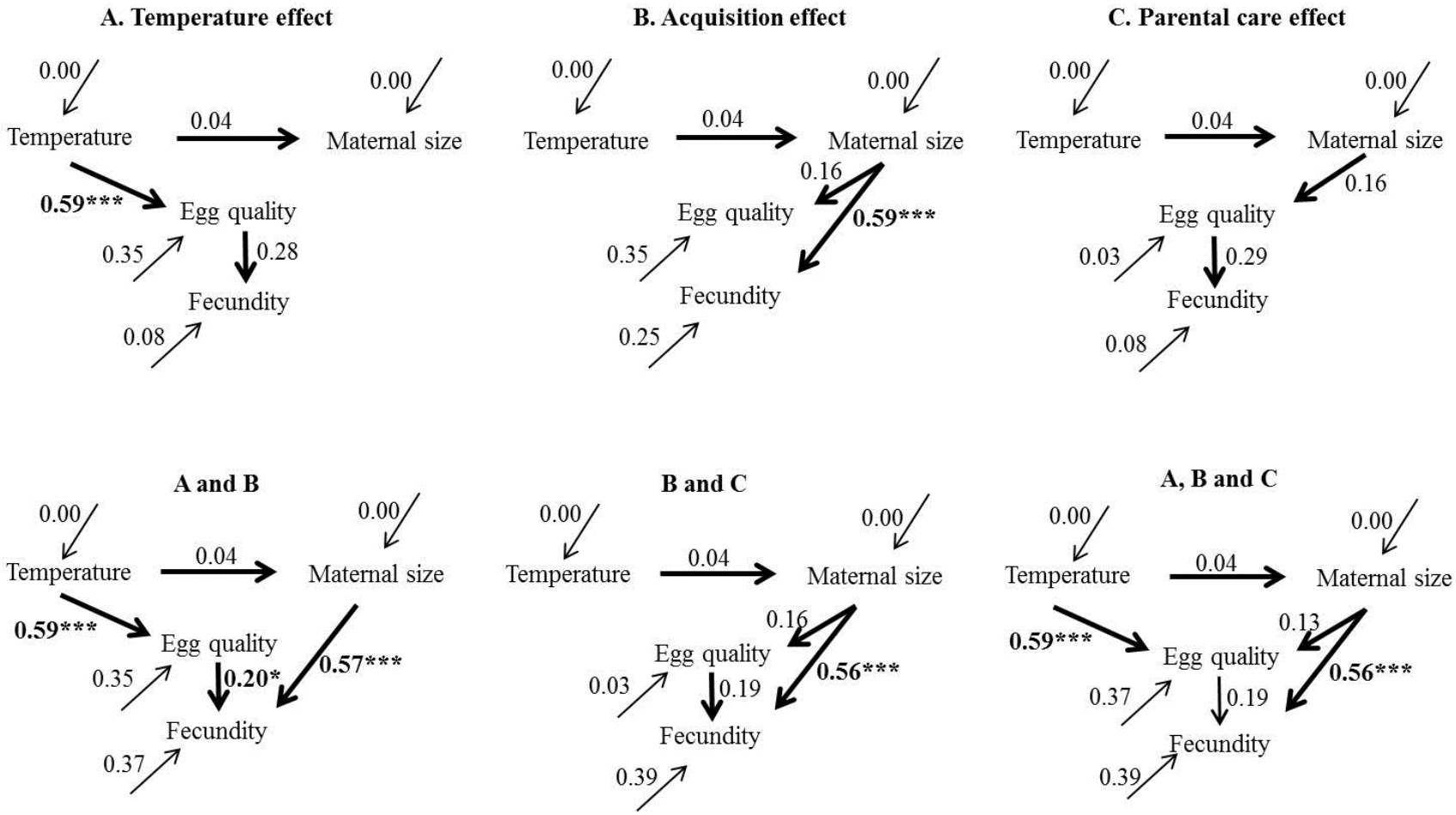

Fig 6. Path models with coefficients for the direct and indirect effect of temperature on reproduction
Standardized coefficients are provided for each path (solid arrows). Values in bold are significant (*=p<0.05; ***=p<0.001). Unexplained residual variance for each variable is also reported (dotted arrows). The model "A and B" is the most supported based on its ΔAICc (see table 4).

Table 4. Scaling of the path models for direct and indirect effects of temperature on reproduction
Each model is scaled based on the ΔAICc (see Material and Methods). $\chi 2$ = goodness of fit; K = number of estimated parameters; AICc = Akaike Information Criterion (see text for formula); ΔAICc = differential AICc; Aw = Akaike weight. In bold are reported the ΔAICc and the Aw for the model that best fitted the data.

Model	**$\chi 2$**	**K**	**AICc**	**ΔAICc**	**Aw**
Temperature and acquisition effects (A and B)	1.09	8	22.43	**0**	**0.7817**
Temperature and acquisition and parental care effect (A, B and C)	0.11	9	25.04	2.61	0.2119
Temperature effect (A)	15.35	7	33.35	10.92	0.0033
Acquisition effect (B)	17.11	7	35.11	12.68	0.0014
Acquisition and parental care effects (B and C)	15.13	8	36.46	14.03	0.0007
Parental care effects (C)	29.38	7	47.38	24.96	0.0001

Discussion

Talorchestia capensis showed an "invariant" reproductive strategy (*sensu* Marshall et al., 2008) in response to temperature, confirming the hypothesis of no *within brood* variability in offspring size to different environments (Table 1 and Fig 4). This can be interpreted as meaning that females of *T. capensis* consider their environment to be a good "predictor" of the thermal environment of their offspring and so produced a single phenotype (i.e. size) of egg. The same type of response/pattern has been confirmed in other direct developers, where offspring show less *within brood* variation and more *among female* variation, than do the offspring of indirect developers. This is because mothers of direct developers should be able to predict the conditions that will be experienced by their offspring and produce progeny of optimal, and comparable, size (Marshall et al., 2008).

The high *among female* variability showed by *T. capensis* in this study seems to confirm the prediction of Marshall et al. (2008). *Among-female* variability in offspring size is a common trait/pattern in direct developers as reported by Marshall and Keough (2007) in a review of 102 marine invertebrates. Direct developers show greater within species variation in offspring size than do species with indirect development, after accounting for proportional differences in egg size between the two categories of developers (Marshall and Keough, 2007). Although this study shows that mothers of *T. capensis* adopted an invariant strategy, I cannot exclude the possibility that *T. capensis* would produce variable sizes within broods if temperature changed unpredictably. Bet-hedging MEs are common plastic responses of mothers living in unpredictable environments (Marshall et al., 2008; Crean and Marshall, 2009; Burgess and Marshall, 2011) and different bet-hedging strategies have been proposed (Marshall and Uller, 2007; Olofsson et al., 2009). Further studies should incorporate temperature unpredictability (i.e. variability in temperature during incubation, instead of a constant regime), to see if

mothers of direct developers respond to short term environmental variability/uncertainty and shift strategy during the developmental time of their offspring.

The results, however, still demonstrated that *T. capensis* shows a plastic response to temperature, producing eggs of different sizes (seen in egg density in both years and in egg volume in 2013) depending on the incubation temperature. Particularly in 2013, females produced significantly larger eggs at colder temperatures. This result may be an artefact due to the greater number of eggs available for a single stage. Larger eggs at colder temperatures is a common relationship in a wide range of taxa (Fischer et al., 2003a; Bownds et al., 2010; Liefting et al., 2010; Burgess and Marshall, 2011; Sun and Niu, 2012; but also see Blanckenhorn, 2000; Stillwell et al., 2008 for contrasting patterns). There are, however, still debates on whether such variation in offspring size is a direct adaptive response to temperature changes (Angilletta et al., 2003; Fischer et al., 2003a Angilletta, 2009) or an indirect effect of temperature imposed by a physiological constraint (Blanckenhorn, 2000). It is certainly clear that the temperature mediated maternal effect reported in the present study, can only be considered adaptive if selection acts differently at different incubation temperatures and this could be maintained over multiple generations (Bownds et al., 2010; Burgess and Marshall, 2011). To address the adaptive significance of temperature-induced maternal effects on offspring size, it is necessary to evaluate the fitness resulting from the size of the eggs (e.g. metabolic response of offspring from different temperature) and to be able to manipulate maternal and offspring environments (Burgess and Marshall, 2011). Although this study did not explicitly investigate the adaptive aspect of the temperature-mediated maternal effects, the results provide a first step towards understanding the adaptive significance of temperature-induced maternal effects in sandhoppers. Further studies on *T. capensis* should explicitly include fitness estimates and control over the environment through multiple generations (transgenerational phenotypic plasticity) and/or multiple reproductive

bouts. For example, clutch-frequency (i.e. number of reproductive bouts) can influence the trade-off in offspring size/offspring number in different populations of lizards (Wang et al., 2011).

An important result in the present study is the hierarchical difference in the variation of egg size. For instance, *T. capensis* showed intra-individual (within-brood) homogeneity in egg size, but high inter individual variability, regardless of temperature so that female identity was highly significant. A possible explanation for this hierarchical difference in the size of eggs is individual specialisation (Bolnick et al., 2003), leading to among-female differences in optimal egg sizes (Einum and Fleming 2002). Niche variation within populations may be the source of such individuality, decreasing the level of intra-population competition (Bolnick et al., 2003). Consequently, under constant environmental conditions (such as the temperature treatment in this study), heterogeneity within populations may buffer populations against temporal variation in environmental conditions relative to more homogenous populations where all individuals are affected similarly by environmental factors (Philippi and Seger, 1989; Einum and Fleming, 2004). From this perspective, individuals gain strategic importance, in that differences among individuals may increase as population stability decreases (Bolnick et al., 2003). Olofsson and co-authors (2009) in fact proposed within-population variability as an adaptive strategy resulting from a combination of different forms of bet-hedging, including the "adaptive coin-flipping" strategy (Cooper and Kaplan, 1992). An individual with an "adaptive coin-flipping" strategy will *flip a coin* every time it has to invest in offspring, and chose the right behaviour to accord with the environmental conditions. The probability that an individual will use a specific strategy will therefore be adaptive and will match the probability for that kind of environment (Olofsson et al., 2009). If the same strategy is highly represented in the population, it can be successful even in cases when some individuals "flipped the coin in the wrong way" (Cooper and Kaplan, 1992). My

personal observations in the field may confirm the bet-hedging strategy at the population level. During the repeated surveys in the field and laboratory experiments, asynchrony in hatching times (a function of the embryonic development time) was commonly observed. Asynchrony in the hatching time has been proposed as a bet-hedging parental strategy in birds to produce phenotypic variation in the offspring through asymmetric sibling competition (Laaksonen, 2004). As far I am aware of, no work has proposed or reported this strategy for marine invertebrates. Further studies, dealing with this specific topic, should test the so called "offspring diversity hypothesis" (Laarksonen, 2004) in marine invertebrates and its adaptive significance.

The results of the present study showed that density was an important proxy of egg quality. Density (here calculated as the dry mass divided by volume) was significantly different between 13°C and 23°C for both 2012 and 2103, showing stronger effects than volume and weight. Density has been proposed as a particularly efficient estimate of body condition, if integrated with the more classic estimates of condition, such as volume and mass, and is an especially good indicator of quality of stored nutrients (Moya-Laraño et al., 2008). Additionally, eggs with higher density should have more protein stored, while lower density suggests more lipid storage (Geister et al., 2009). In the present study, the density of the eggs from females incubated at the lower temperature (13°C) showed lower values than those at the higher temperature (23°C), suggesting that *T. capensis* invested more in lipids at low temperatures and more in proteins at high temperatures. This differences in investment (different MEs mediated by temperature) can be explained as the necessary allocation of more energy at low temperature (lipids are a source of energy during development) in order to cope with the slow developmental time at low temperatures (Geister et al., 2009). Conversely, fewer lipids were needed at 23°C, when proteins played an important role as an energy resource for growth and somatic maintenance at a faster developmental rate (Jarošík

et al., 2004). In the context of a temperature-driven balance between energy and structural investment per egg, *T. capensis* showed an adaptive maternal effect in the sense that mothers adjusted the offspring phenotype, buffering the different environmental conditions perceived during early ontogeny. Such maternal effects would evolve and pass across generations if the environment that the mothers experienced was still predictable (Mousseau and Fox, 1998a; Bateson and Glickman, 2012). The present work, however, did not focus on the effects of temperature on the biochemical composition of eggs, as several authors reported for arthropods (Fischer et al., 2003a, b; Geister et al., 2009; for a comprehensive review, see Moran and McAlister, 2009).

The overall effects of temperature on eggs quality and quantity were addressed using a modelling approach that involved an information-theoretic method. The results of this analysis indicated that the reproductive traits of *T. capensis* seem to be affected by both temperature and maternal size (Table 4). In particular, the model that best fitted the available data showed a direct effect of temperature on the quality of the eggs (i.e. density) and a concomitant effect of maternal size on the fecundity (i.e. number of eggs). This is a confirmation of the maternal effect mediated by temperature, in the sense that mothers may adjust the number of eggs they produce (enhancing their own fitness) to balance the physiological consequences of temperature that result in smaller egg sizes at higher temperatures, as explained by the Temperature-Size rule (Laptikhovsky, 2006). The time-fecundity Hypothesis of Vance (1973), (reported in Moran and McAlister , 2009, and references therein), predicts that allocating resources to produce large eggs at low temperatures is energetically costly for the mothers so that they react by reducing the numbers of eggs. This is in agreement with the theory that selection usually tends to maximise maternal fitness (Smith and Fretwell, 1974), but can also be seen as a mere constraint, as maternal brood volume may limit the number of eggs (Du et al., 2005). In the

current study, the fact that maternal size is causally related to fecundity (i.e. number of eggs) can be considered as a maternal effect and several authors have stressed the existence of complex dynamics beyond the classic egg size/number trade off (Brown and Shine, 2009). For instance, in snakes exposed to constant water availability, the amount of water taken up by an individual egg depends upon the number of adjacent eggs. Clutch size operates therefore through a range of alternative pathways that go beyond the simple trade-off between size and number and could stand alone as a good estimate of maternal effects (Brown and Shine, 2009). Consequently, one can speculate that the influence of *T. capensis* on clutch size (through maternal size), could differ depending on the temperature regime. The importance of egg number, coupled with changes in temperature, has been reported to affect the oxygen concentration in artificial egg masses, where dense packing of clutches (i.e. high number of eggs per clutch) would affect oxygen consumption if temperature changes (Moran and Woods, 2006). Although these authors demonstrated that interactions among factors influenced oxygen levels in egg masses under laboratory conditions, they also highlighted the importance of complex interactions to natural populations, encouraging the development of experimental and field comparisons (Moran and Woods, 2006).

In conclusion, this study documented for the first time phenotypic plasticity in the size of the offspring in sandhoppers. Under laboratory conditions, sandhoppers showed plasticity through maternal effects mediated by changes in temperature. The effect of temperature on offspring size was clear, particularly in the case of egg density. This result confirms that colder mothers produce larger eggs (Blanckenhorn, 2000; Fischer et al., 2003a, b; Marshall et al., 2008; Bownds et al., 2010), and indicates that density is a good proxy for egg quality, providing information on resource allocation and maternal provisioning. Further investigations on offspring size and fitness should couple measurements of egg density with biochemical analyses of egg composition. The reproductive strategy that sandhoppers

adopted under laboratory conditions, seem to reflect their developmental mode. The experiments were ran under constant temperatures, however, and a strategy of *within brood* bet-hedging, could be selected if temperature or the general environment, change unpredictably. This study provided an important contribution towards the integration of experimental and theoretical approaches, highlighting the value of an information theoretic approach combined with path analyses (Angilletta et al., 2006).

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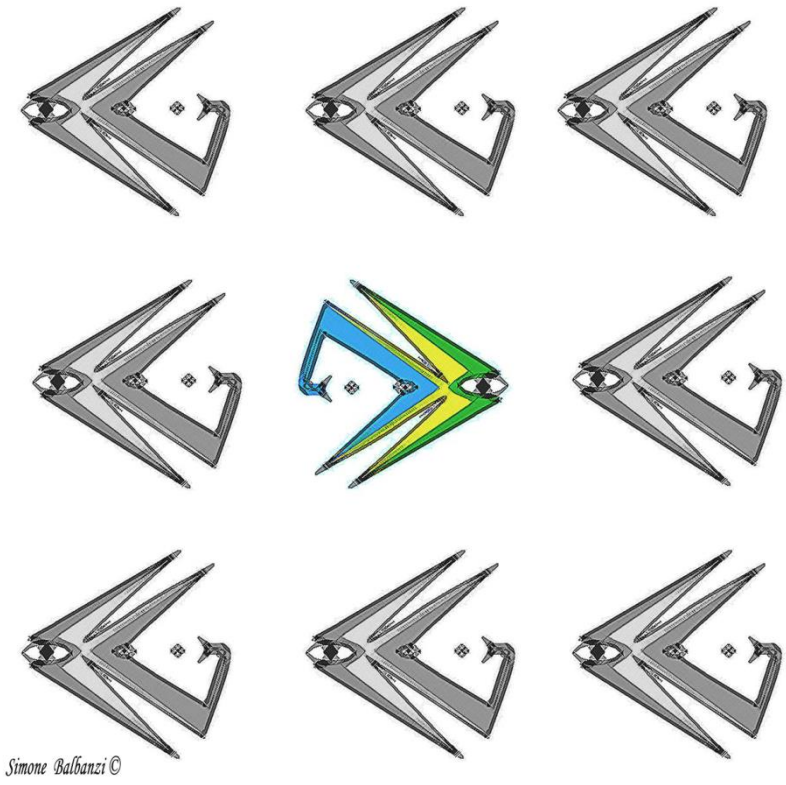
-Chapter 6-

Synthesis



"…variation itself is nature's only irreducible essence […] I had to place myself amidst the variation".

Stephen Jay Gould

Individuals can change their morphology, spatial ecology, physiology, behaviour, or life history in response to changing environmental conditions. Such plasticity is universal among organisms and is derived from the fact that environments vary (Agrawal, 2001). These environmental variations, whether temporal, spatial, predictable or unpredictable, are challenging because they can destabilise homeostasis, metabolism and development, hence disrupting the match between an organism's phenotype and the environment and overall resulting in lower individual fitness (Agrawal, 2001).

Organisms buffer environmental variation through adaptive variation in two ways: between- and within-generation variation (Meyers and Bull 2002, DeWitt and Langerhans 2004). The former is mostly genetic and can result in adaptive changes in a population. Between-generation variation is based on natural selection acting on heritable variation caused by mutation, recombination, genetic drift, etc. Conversely, within-generation variation is a non-genetic plastic response occurring at the individual level, and it allows individuals to adjust promptly to environmental variation (Whitman and Agrawal, 2009). From the perspective of phenotypic evolution, plastic *within generation variation* facilitates genetic *between generation variation*, resulting in an adaptive and genetically fixed response to the environment (Pigliucci and Murren, 2003; Pigliucci and Müller, 2010). The environment can be therefore considered as the 'cradle' of evolutionary and developmental processes, and studying its influence over the geographical distribution, ecophysiology and life history of a species is fundamental (Brown, 1984; Sagarin et al., 2006; Ghalambor et al., 2007; Fernàndez et al., 2009; Bozinovic et al., 2011). Considering the importance of environmental variation in inducing phenotypic variability in animals (Pigliucci, 2001), which represents a potential rapid response to climate change (Franks and Hoffmann, 2012), the present study contributes to a better understanding of such process on southern African sandhoppers.

The multi-disciplinary approach undertaken, comprising spatial biogeography, phylogeny, ecophysiology and reproductive biology, has shown the important effect of the environment (focussing mainly on temperature effects) on sandhoppers. Several hierarchical levels were investigated, from species to individuals, through population comparisons, showing that the environment has a clear and substantial influence on the biogeography, ecophysiology and reproductive plasticity of sandhoppers. Primarily, environmental domains (mainly the morphodynamic state of the shores, but also temperature and salinity) were shown to drive the abundance of the two study species, limiting their spatial distribution. Interestingly, a test of a possible 'centre to margin' pattern of abundance of sandhoppers (ACH, Abundant Centre Hypothesis, [Brown, 1984; Sagarin and Gaines, 2002a]) substantially failed to explain the spatial distribution of both species (see Chapter2 and Baldanzi et al., 2013). This could be due to an intrinsic constraint of the ACH, which assumes that sites close to one another should provide similar environmental conditions (Brown, 1984). In nature, such patterns are unlikely, and the present study confirmed how natural variation among sites is the driving force for the abundance of sandhoppers, obscuring the expected deterioration in environmental quality from centre to edge and limiting their geographical range. In fact, as several authors have highlighted, sandy shores are variable environments with different physical characteristics, meaning that a single stretch of sandy shore can show considerable environmental variation along its length (Lercari and Defeo, 2006; Defeo et al., 2009; Defeo and McLachlan 2011). The importance of the environment in shaping the ability of sandhoppers to colonise new habitats, influencing their range of expansion and persistence, is therefore clear. The present study, investigating environmental forces interconnected with the abundance of sandhoppers, also highlighted the importance of analysing field data to find variables and potential mechanisms that define species range limits.

Among the environmental variables, temperature was considered important for *Talorchestia capensis*, mainly because this sandhopper exhibit a widespread range of distribution, involving a high degree of variation in environmental temperatures along southern African coasts, making it a good candidate to test metabolic responses to changing temperatures. The thermal sensitivity of separate populations of *T. capensis* was found to be negatively and significantly correlated with air temperature variability recorded over the past 23 years. A similar trend was also found for the predictability of air temperature (see Chapter 4 for the calculation of temperature predictability), although this effect was not significant. *T. capensis* therefore showed a plastic thermal response, tested under laboratory conditions, which has been shaped by historical climatic variability, suggesting a sort of "climatic memory" transferred across generations. This finding is an important confirmation of how environmental conditions, particularly environmental variability rather than the average itself, are fundamental drivers of metabolic responses and therefore being crucial to determining physiological influences on the spatial distribution of *T. capensis*. In evolutionary terms, this study corroborates the idea of phenotypic plasticity as a possible start-up process for phenotypic evolution, meaning that the physiological differences shown by *T. capensis* (i.e. different thermal sensitivities) to the same temperature treatment could be a result of genetic accommodation of that trait. The possibility that three separate groups of populations of *T. capensis* represent in fact three cryptic species (see Chapter 3 for details), could be a confirmation that the environmentally-shaped physiological responses to different temperature regimes favour cryptic speciation. One of the main results reported in Chapter 4 however, is the similar thermal sensitivity (i.e. similar plastic response) of individuals from Gansbaai (western lineage/cryptic group) and the two eastern populations of Port Alfred and Mngazana, in responding to increasing and decreasing temperatures. If the two populations are in fact two different (crypto) species, the plastic response to identical temperature

treatments still underlines the importance of plasticity as a driver of speciation, where cryptic species experiencing different environments were similarly plastic. It is however complicated and potentially erroneous, to conclude that plasticity alone underpinned, or even explained, the variation in response to temperature among separated populations (or potentially cryptic species) of *T. capensis*. The high genetic variability among geographically separated groups, and intra-population variability reported in Chapter 3, could have itself driven the different responses to the same temperature treatment. The genetic variability among individuals within a single population, could in fact explain alone the high variance around the mean values of oxygen consumption reported especially for increasing temperatures (see Chapter 4).

Inter-individual variation was also reported for the maternal phenotypic adjustment of egg size in response to different temperature regimes (see Chapter 5). Females of *T. capensis* were able to adjust the quality and the quantity of the eggs within their brood in a similar way in response to temperature (producing larger eggs in colder environment), but they did so with high variability among individuals. This fundamental result suggests that the environmentally induced-, potentially transgenerational mechanisms by which mothers adjust their offspring phenotype are similar in their response to the environment, but individuality produced phenotypic variability at the population level. This phenotypic variability in the maternal effects at the population level could be a potential explanation of the relatively widespread distribution of *T. capensis*, (according with the high colonisation ability of direct developers in general, Thiel, 1999), providing a wide range of evolutionary possibility for a newly established population in a new habitat, or after an abrupt change in environmental conditions. A stimulating result was the absence of within brood variability in the size of the egg (see Chapter 5). It is however possible that *T. capensis* would produce variable egg sizes if temperature varies daily, adopting a bet-hedging strategy (Marshall and Uller, 2007).

Additionally, temporal scale and reproductive bouts play an important role on the reproductive plasticity (Olofsson et al., 2009; Collin, 2010) which was not investigated in this study. For instance, *T. capensis* could adopt the so called "adaptive coin-flipping" strategy (Kaplan and Cooper, 1984) which allows individuals to 'flip a coin' every time they invest in reproduction by determining whether to behave according to a changing environment (Kaplan and Cooper, 1984). The probability of the individual using a specific strategy evolves to match the probability for that kind of environment. At the population level, this strategy could be successful even if a specific individual makes the wrong choice at a certain time (Olofsson et al., 2009). An investigation of how phenotypic changes appear across multiple generations would clarify if an environmentally induced maternal effect is transmitted from mothers to offspring, helping us to understand the adaptive significance of maternal effects in evolution. In this view, an important contribution could come from the new field of ecological epigenetics, which studies the interaction between environment and genome without involving any alteration of the DNA sequence itself. Several DNA modifications, including DNA methylation, are environmentally inducible (Richards, 2006), thus investigating epigenetic modifications induced by environmental changes, and potentially their persistence through several generations, could be the key to clarify how animals respond rapidly to the environment (Ho and Burggren, 2010). In a general sense, phenotypic plasticity (such as maternal effects and thermal plasticity) may result from underlying epigenetic mechanisms that cause persistent phenotypic effects, either ontogenetic or transgenerational (Richards et al., 2010).

Concluding remarks

The overall study investigated the complex dynamics behind the effect of the environment on the spatial distribution, thermal physiology and reproductive plasticity of southern African sandhoppers. An investigation of the phylogeny and phylogeography of *T. capensis* also

provides an important base study supporting the main core of the thesis. In a general sense, given the importance of understanding rapid responses of organisms to climate change and considering the fundamental role played by phenotypic plasticity in evolution, the study revealed the importance of individual plasticity and variability in response to the environment. Particularly, studying the thermal physiology of separated populations and understanding the within-population reproductive plasticity in response to temperature, helped to clarify how differences among individual responses have important consequences at the population level and are possibly the explanation for the widespread distribution shown by *T. capensis.* Such individual variability could be the result of local environmental discontinuities and high variability that sandy shores naturally show, even within a single stretch of beach (Lercari and Defeo, 2006; Defeo et al., 2009; Defeo and McLachlan, 2011). In such a spatially and temporally variable environment, individuality is important because it creates variability in the expression of a wide range of phenotypes at the population level, allowing selection to favour even the most (apparently) unfitted, but potentially successful, phenotypes. Variability of the environment is therefore the core of phenotypic evolution, being the spark that initiates a new evolutionary line through the selection of a plastic phenotypic trait. In this sense, "evolution would occur not by the *survival of the fittest*, but by the *survival of the non-unfit*" (Guerrero-Bosagna, 2012, p. 296). In other words, inquiring *how* an expressed phenotypic trait has been shaped by the environment (focussing on the proximate causes), rather than asking *what* that trait has been selected *for* (an ultimate, finalist investigation), would give a more measurable, comprehensive, persuasive and fascinating explanation of one of the most intriguing aspects of nature, the evolution of species.

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APPENDIX

Table reporting the locations sampled for the entire study. The table also report the geographical coordinates in decimal degrees, the Bioregion of interest (following Lombard, 2004) and the number of the chapters where the location is mentioned.

Site	Coordinate	Bio-region	Chapter
Swakopmund	S22.67410 E14.52784	Cool-temperate Namaqua	2
Luderitz	S26.64583 E15.15388	Cool-temperate Namaqua	2
Port Nolloth	S29.28002 E16.87979	Cool-temperate Namaqua	2,3,4
Kleinsee	S29.32048 E16.96740	Cool-temperate Namaqua	3
Hodenklipbaai	S30.31644 E17.27566	Cool-temperate Namaqua	2
Groenrivier	S30.59690 E17.44224	Cool-temperate Namaqua	2
Doringbaai	S31.74402 E18.22239	Cool-temperate Namaqua	2
C. Columbine	S32.87481 E17.88049	Cool-temperate Namaqua	2
Yzerfontein	S33.34021 E18.16109	Cool-temperate Namaqua	2
Blouberstand	S33.85028 E18.48831	Cool-temperate Namaqua	2
Muizenberg	S34.09897 E18.49471	S-W Cape	2
Pringle Bay	S34.33435 E18.82616	S-W Cape	2
Kleinmonde	S34.34219 E19.04712	S-W Cape	2
Mosselrivier	S34.41525 E19.28987	S-W Cape	2
Franskraal	S34.61053 E19.42179	S-W Cape	2
Gansbaai	S34.56361 E19.35678	S-W Cape	3,4
Betty’s Bay	S34.35863 E18.90888	S-W Cape	3
Pearly Beach	S34.66928 E19.51424	S-W Cape	2
Struiss Bay	S34.78577 E20.04620	Warm-temperateAgulhas	2
Still Bay	S34.37306 E21.42896	Warm-temperate Agulhas	2,3
Mossel Bay (Glentana)	S34.05003 E22.30704	Warm-temperate Agulhas	2, 3
Knysna	S34.08062 E22.97628	Warm-temperate Agulhas	2

Site	Coordinate	Bio-region	Chapter
Plettenbergbaai	S34.05241 E22.37763	Warm-temperate Agulhas	4
Jeffreys Bay	S33.96930 E25.01428	Warm-temperate Agulhas	2
Sundays River	S33.78453 E25.36834	Warm-temperate Agulhas	2
Port Alfred	S33.89336 E26.29815	Warm-temperate Agulhas	2,3,4,5
Fish Mouth	S33.90128 E26.56976	Warm-temperate Agulhas	3
Kidd's Beach	S33.92725 E27.87636	Warm-temperate Agulhas	2
Kei Mouth	S32.68138 E28.38368	Warm-temperate Agulhas	2,3
Mngazana	S31.6836 2 E29.4372	Subtropical Natal	3,4
Port St. Johns	S31.62035 E29.55922	Subtropical Natal	2,3
Port Edward	S31.03222 E30.23677	Subtropical Natal	2,4
Clansthal	S30.24457 E30.78153	Subtropical Natal	2
Ballito	S29.61103 E33.59488	Subtropical Natal	2
Richard's Bay	S29.09553 E32.44105	Subtropical Natal	2