Stress ecophysiology of polar terrestrial invertebrates and the impact of climate change

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ABSTRACT

Terrestrial invertebrates are small poikilothermic ectotherms and are thus susceptible to chronic cold, rapid temperature change and desiccation. In the polar regions, where buffered microhabitat temperatures regularly fall below 0°C and water availability is low, they are particularly vulnerable. However, polar terrestrial invertebrates successfully exist within these climes. Using whole organism experimental techniques, this thesis aims to further understand the capacity of invertebrates to tolerate different stressors, the underpinning physiological adaptations, and the potential impact of continued climate change. For just the second time in a freeze-tolerant polar insect, rapid cold hardening is observed. Acclimation to sub-lethal low temperatures is also demonstrated, through depression of the critical thermal minimum (CTmin) and chill coma temperature. Contrasting strategies of desiccation tolerance vs. resistance are noted, as well as evidence of cross-tolerance to temperature stress. At the opposite extreme, water submergence experiments confirm the first example of an amphibious terrestrial midge. A remarkable capacity to tolerate high temperatures, including those that may occur as a result of climate change, is also observed. This body of work underscores the physiological flexibility of polar invertebrates, which allows them to flourish in environments considered too extreme and inhospitable for most terrestrial species.

“To strive to seek to find and not to yield”

Alfred, Lord Tennyson, Ulysses, verse 3, line 70

“I must persevere”

Adelbert Steiner

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CHAPTER 1: INTRODUCTION

1.1. Overview

Stress has been defined by Grime (1989) as the "external constraints limiting the rates of resource acquisition, growth or reproduction of organisms". However, every living organism is limited by external constraints in this way. It is therefore important to delineate the extent to which stress occurs and thus of its significance to the organism. For the polar terrestrial invertebrates of the Arctic tundra (Strathdee and Bale, 1998) and the fellfields of the Antarctic (Block *et al.*, 2009), the significance of stress is great. Abiotic factors, such as temperature and water availability, impact negatively on their survival and development, and regulate polar ecosystems throughout the year (Block *et al.*, 2009; Hogg *et al.*, 2006).

The primary objective of this thesis is to build upon existing knowledge and clarify the capacity to which polar terrestrial invertebrates can tolerate and minimise stress. Specifically, four core objectives have been addressed:

1) Characterise the microhabitat conditions and stresses experienced on a diurnal and seasonal basis
2) Quantify the capacity of polar terrestrial invertebrates to cope with these stresses
3) Investigate the physiological adaptations that have evolved to minimise stress
4) Determine the impact of climate change for polar ecosystems and the invertebrates therein

By investigating invertebrates of the Arctic and Antarctic rather than those of lower latitudes, we are able to view the most extreme forms of adaptation shown in response

to stresses like low temperature. This information provides great value to the field of invertebrate ecophysiology. Not only because novel adaptations are discovered, but also because it is far easier to tease out what physiological mechanisms are effective in resisting and tolerating particular stresses, simply because they are more pronounced. Additionally, investigations into the stress biology of invertebrates may potentiate applications in cryopreservation (Katkov, 2006) and pest management (Bale, 2002).

1.2. Polar climate and biota

1.2.1. Geography

The Antarctic is conventionally described as being composed of three biogeographic zones - the sub-, maritime and continental Antarctic (Smith, 1984; Convey, 2013). The former envelops the Southern Ocean's major island groups, which include South Georgia, Prince Edward and Marion Islands, Îles Kerguelen, Îles Crozet, Maquarie Island and Heard and McDonald Islands. The maritime Antarctic encompasses the west Antarctic Peninsula and associated island archipelagoes (South Sandwich Islands, South Orkney Islands, South Shetland Islands and Bouvetøya), and the continental Antarctic constitutes the east Antarctic peninsula and the main body of the continent. Within these zones, the ice-free, and thus habitable, areas constitute just 0.33% of the 13.95 million km^2 of land (Convey *et al.*, 2009). Fellfield habitats make up much of this area and are largely barren landscapes consisting of rock/boulder fields, glacial till and patterned mineral substrata (Block *et al.*, 2009).

The Arctic, which is recognised as the area north of the Arctic circle (66^o 32^oN), is largely composed of the Arctic Ocean and is bordered by continental and other land masses, including Russia, Canada, Alaska, Greenland and Northern Scandinavia, as

well as their associated islands (Strathdee and Bale, 1998). The terrain of many of the terrestrial landscapes, especially in the High Arctic, bears a resemblance to the Antarctic fellfields, with rocks, boulders and glacial till giving rise to polar semi-deserts (Sjursen and Sømme, 2000; Coulson *et al.*, 1995a).

1.2.2. Climate

Air temperatures regularly fall below -10^{o}C during the winter in the maritime Antarctic and, in regions such as the continental Antarctic and High Arctic, frequently drop below -40^{o}C (Block *et al.*, 2009; Convey, 2013; Sformo *et al.*, 2010; Strathdee and Bale, 1998). Polar terrestrial invertebrates buffer these temperatures behaviourally to some extent through protection under snow, within the soil profile, or within cryptogamic vegetation such as mosses, lichens and algae (Bengtson *et al.*, 1974; Burn, 1986; Convey, 1996; Convey and Smith, 1997; Spaull, 1973). However, even within these microhabitats, they can still be subjected to sub-zero temperatures on a daily basis throughout the winter (Davey *et al.*, 1990; Block *et al.*, 2009; Strathdee and Bale, 1998). Microhabitat temperatures during the summer are also very low and rarely rise above 5^{o}C in the maritime and continental Antarctic, and slightly higher in the Arctic (Block *et al.*, 2009; Coulson *et al.*, 1993; Strathdee and Bale, 1998). The availability of liquid water also presents an important challenge. During the winter, water is locked up as snow and ice where it is inaccessible (Block *et al.*, 2009), but then inundates many habitats during the spring thaw. In summer, streams, lakes and rock pools, which form from melted ice and snow in spring, evaporate, resulting in drought (Convey *et al.*, 2003). In addition to the primary stressors of temperature and water availability, there are many other abiotic factors which impact upon the polar invertebrate fauna. These

include snow cover (Callaghan *et al.*, 1992), salinity (Elnitsky *et al.*, 2009), extremes of pH (Rinehart *et al.*, 2006), anoxia (Lopez-Martinez *et al.*, 2008), lack of photoperiodic cues (Strathdee *et al.*, 1993), UV radiation (Strathdee and Bale, 1998), and pollution (Avila-Jimenez *et al.*, 2010; Bindesbol *et al.*, 2009).

Plate 1. *Field site on Léonie Island, near to Rothera Research Station, Adelaide Island, western Antarctic Peninsula, maritime Antarctic (67°36'S, 68°21'W).*

1.2.3. Biota

As a consequence of the aforementioned stressors, the polar regions are unable to support a large diversity or abundance of terrestrial life (Block *et al.*, 2009). This paucity is well exemplified by the biological system most associated with the continental Antarctic, the "Chalikosystem", which can only support food chains of one

to three nematode species and rudimentary microorganisms (Block *et al.*, 2009). Likewise, the "Bryosystem" that is found over much of the ice-free areas of the maritime Antarctic (Block *et al.*, 2009) and High Arctic (Strathdee and Bale, 1998), is largely dominated by cryptogams, which are largely unproductive non-flowering plants, such as mosses and algae, and lichen (Plate 1). Arthropod species richness is also low in the Bryosystem, with only 553 arthropod species present from the Queen Elizabeth Islands (74^{o} 33'N) northwards in the Arctic, and just over 140 species found in the maritime and continental Antarctic (Callaghan *et al.*, 1992).

The soil faunas of the Antarctic and Arctic are largely dominated by Collembola (springtails) and Acari (mites), as well as tardigrades, rotifers, ticks, nematodes and enchytraeidae (Block *et al.*, 2009; Hodkinson and Coulson, 2004). The oribatid mite, *Alaskozetes antarcticus*, for example, ranges from the Falkland Islands (51^{o}S, 57^{o}W) to SE Alexander Island (71^{o}S, 70^{o}W) (Block and Convey, 1995) and can be found in aggregations numbering hundreds of thousands (Block and Convey, 1995), whereas Collembola, such as *Megaphorura arctica* (previously *Onychiurus arcticus*) have been found at densities as high as 268,000 individuals m^{-2} in the Arctic, and *Cryptopygus antarcticus*, as high as 1.5×10^{6} individuals m^{-2} in the Antarctic (Bengtson *et al.*, 1974; Burn, 1986; Convey and Smith, 1997; Tilbrook, 1967). There are a number of mite genera represented, including *Stereotydeus*, *Nanorchestes*, *Eupodes*, *Magellozetes* and *Globoppia*, that are comparatively common in the Antarctic, but have received far less attention than the larger "model" species. Together, these mites and Collembola, and other invertebrate groups like nematodes, play an important role in providing several ecosystem services, including decomposition, carbon mineralisation and nutrient cycling (Ávila-Jiménez *et al.*, 2010; Barrett *et al.*, 2008; Bokhorst *et al.*, 2007;

Freckman, 1988). The polar regions are also home to a number of higher invertebrate orders, including Coleoptera (Worland and Block, 1999), Hemiptera (Hulle *et al.*, 2008), Lepidoptera (Kukal and Kevan, 1987), Plecoptera (Hågvar, 2010), Mecoptera (Hågvar, 2010) and Diptera (Worland *et al.*, 2000). These higher orders are mainly found in the milder sub-Antarctic and Low and High Arctic regions (see Coulson, 2000 for a review of Svalbard), though there are some, such as the chironomids, *Belgica antarctica* and *Parochlus steinenii*, which are found in higher latitude Antarctic regions (Convey, 1996).

1.2.4. Alien species

Alien species are defined as those which occur outside of their natural range. The Antarctic is an isolated, remote landmass surrounded by the Southern Ocean, and, there has been little recent natural dispersal by species from other continents (Chwedorzewska, 2009). However, human presence in the Antarctic has risen over the last 200 years as a result of seal and whale hunting, scientific research and, more recently, tourism (Tin *et al.*, 2009; Chwedorzewska, 2009). Alien species, which are carried on cargo, vehicles and humans themselves, have consequently been able to bypass geographical barriers and colonize the Antarctic at an ever increasing rate (Hughes *et al.*, 2005, 2010; Frenot *et al.*, 2005). There have now been upwards of 200 species introductions, including mites, Collembola, aphids and spiders. Ninety-five percent of these introductions have occurred on the sub-Antarctic islands and there are now some islands, such as South Georgia, which possess more alien, than native, species in certain major groups (Frenot *et al.*, 2005). The result has not been entirely positive, with land disturbance (e.g. cattle trampling), competition, predation and

disease becoming ever more prevalent as more alien species establish (Bale, 2000; Chwedorzewska, 2009). As the Antarctic environment shows distinct regionalisation and evidence of local evolutionary intra-specific differentiation, intra-continental transfer is likewise of concern (Allegrucci *et al.*, 2006; Terauds *et al.*, 2012; Tin *et al.*, 2008).

Unlike the Antarctic, the Arctic is not isolated and its terrestrial habitats either lie on or are mostly close to surrounding landmasses. Alien species introductions are therefore more common, via natural or human means. There has even been evidence of invasion on the remote high Arctic islands of Svalbard by the diamondback moth, *Plutella xylostella*, the Hymenopteran parasitoids, *Atractrodes pusillus* and *Stenomacrus groenlandicus*, as well as other invertebrate species (Coulson *et al.*, 2002; Hughes *et al.*, 2010). Aside from accidentally transferring alien species, humans also pose a threat as an alien species themselves. Humans are impacting on the native biota through pollution, trampling, pedestrian approach, handling, construction and noise pollution (Tin *et al.*, 2009). For instance, recent evidence suggests that Collembola numbers are reduced by as much as 80% in areas disturbed by vehicles (Kevan *et al.*, 1995; Niwranski *et al.*, 2002).

1.3. Physiological adaptations of polar terrestrial invertebrates to stress

1.3.1. Thermal physiology

1.3.1.1. Trials of being an invertebrate

Invertebrates, more so than any other animal group, are at the whim of their environment. Unlike birds and mammals, which are able to regulate their internal body temperature, invertebrates are poikilothermic ectotherms and their body temperature is highly influenced by, and varies markedly with, the environmental temperature (Speight *et al.*, 2008). While cold-blooded vertebrates, such as fish, reptiles and amphibians, are also poikilothermic ectotherms, they are not generally as diminutive as invertebrates. Even the smallest vertebrate recorded, the Papua New Guinea frog *Paedophryne amanuensis* (7.7mm in length), dwarfs the vast majority of invertebrates (Rittmeyer *et al.*, 2012). Cold-blooded vertebrates accordingly have a smaller surface area to volume ratio than invertebrates and therefore have more time to respond to changes in temperature. This means that invertebrates are more susceptible to injuries following either rapid cooling (Czajka and Lee, 1990) or warming (Chidawanyika and Terblanche, 2011). A small body size also means invertebrates are generally more vulnerable to desiccation than their larger-bodied vertebrate relatives.

1.3.1.2. Responses to low temperature

Invertebrates that live in the polar regions can be at constant risk of their body fluids freezing and any associated injury (Mazur 1977). This risk is generally ameliorated by adoption one of two strategies - freeze-tolerance (= tolerance of internal ice formation)

or freeze-avoidance (= avoidance of internal ice formation) (Bale 2002; Block 1982; Cannon and Block 1988; Convey 1996; Sømme 1982; Storey and Storey 1988; Zachariassen 1985).

1.3.1.3. Freeze-tolerance

Various polar invertebrates have been shown to use this strategy, including Diptera (e.g. *Belgica antarctica* [Benoit *et al.*, 2009a], *Eretmoptera murphyi* [Worland, 2010] and *Heleomyza borealis* [Worland *et al.*, 2000]), Lepidoptera (e.g. *Gynaephora groenlandica* [Strathdee and Bale, 1998]), Coleoptera (e.g. *Hydromedion sparsutum* and *Perimylops antarcticus* [Worland and Block, 1999]) and nematoda (e.g. *Eudorylaimus coniceps* [Convey and Worland, 2000]). While the continental Antarctic nematode, *Panagrolaimus davidi* (Wharton and Ferns, 1995), has been shown to survive intracellular ice formation, perhaps indicative of a more general ability within polar nematodes, this form of injury is thought to be lethal to most other invertebrates (Block, 1990). The vast majority of freeze-tolerant invertebrates therefore restrict ice formation to extracellular compartments. Key to this process is the accumulation of ice nucleating agents (INAs), such as specialised proteins (Block *et al.*, 1990), food particles, crystalloid compounds (Lee *et al.*, 1996) and microorganisms (Klok and Chown, 1997; Worland and Block, 1999), which act as heterogeneous surfaces for the promotion of water molecule aggregation (Bale, 2002). By accumulating these agents in the haemolymph and gut, as well as in other tissues (Izumi *et al.*, 2009), ice formation (which occurs at the supercooling point - SCP) is encouraged to take place extracellularly at high sub-zero temperatures (-3 to -10^{o}C) (Duman and Horwath, 1983; Worland *et al.*, 1992, 1993; Worland and Block, 1999). At these temperatures, ice

crystal growth is slow, allowing water to flow from the cytoplasm of cells and join the newly formed ice crystals. Cells are subsequently dehydrated and less susceptible to cell lysis via intracellular freezing (Worland and Block, 1999).

However, freeze-tolerant invertebrates are still at risk from any one ice crystal in the extracellular space becoming too large and puncturing cells from the outside. They therefore also produce antifreeze proteins (AFPs) and/or antifreeze glycolipids (AFGLs). AFPs and AFGLs arrest the expansion of large crystals and instead promote the growth of many small crystals in a process called ice recrystallisation inhibition (Duman *et al.*, 2004). AFGLs may also stabilise membranes and prevent the propagation of ice into the cytosol, and slow the growth of extracellular ice, reducing the rate of water flux and solutes across the cellular membrane (Walters *et al.*, 2011). Even with the help of AFPs and AFGLs, ice formation is still able to distort proteins, membranes and other structures. Freeze-tolerant invertebrates thus accumulate polyhydric alcohols and sugars, such as glycerol, sorbitol and trehalose. These substances serve as cryo- and osmo-protectants and stabilise proteins and membranes. Polyols and sugars also provide other benefits and aid metabolism, and reduce cellular dehydration, maintaining water above the "critical minimum cell volume" (Calderon *et al.*, 2009; Holmstrup *et al.*, 1999; Montiel, 1998).

1.3.1.4. Freeze-avoidance

In contrast to freeze-tolerant species, invertebrates which are freeze-avoiding are unable to withstand any internal ice formation (Bale, 1996; Cannon and Block, 1988; Storey and Storey, 1988; Zachariassen, 1985). While seemingly disadvantageous in an environment which experiences temperatures close to an invertebrate's SCP, these

invertebrates avoid the dangers of extracellular ice formation and subsequent cellular dehydration that occur in freeze-tolerant species. Freeze-avoiding invertebrates range from Alaska (e.g. the red flat bark beetle, *Cucujus clavipes puniceus* [Sformo *et al.*, 2010]) and the high Arctic (e.g. the mite, *Diapterobates notatus* [Coulson *et al.*, 1995a]) to the Antarctic continent (e.g. *Cryptopygus antarcticus* [Block and Worland, 2001; Cannon and Block, 1988]), and outnumber freeze-tolerant species in almost all cases. Freeze avoiding invertebrates can be separated into several different categories to better define them ecologically and physiologically. These include, for instance, true freeze-avoiding (lower lethal temperature [LLT] = SCP), chill tolerant (show minimal pre-freeze mortality), chill susceptible (die well above their SCPs) and opportunistic survival (unable to survive below their developmental threshold) (see Bale, 1993). The SCP can also vary greatly between and within species, and such classifications can thus become misleading, unless accompanied by details of the species SCP. For example, the summer acclimatised collembolan, *Megaphorura arctica*, is classified as true freeze-avoiding or chill tolerant, while the aphid *Myzus persicae* is classified as chill susceptible. The reader may therefore infer that summer acclimatised *M. arctica* is more cold tolerant. However, *M. arctica* in summer has a high SCP of only -6^{o}C (Worland, 1996), which is higher than the LLT of *M. persicae* (Clough *et al.*, 1990).

Mechanistically, freeze-avoidance revolves around a process termed supercooling - the prevention of internal ice formation below the environmental freezing point. Supercooling is principally achieved via three processes (Bale, 2002). The first is the removal of INAs and has been shown to lower the SCP by up to 20^{o}C in some invertebrates (Zachariassen *et al.*, 1980; Burns *et al.*, 2010). INAs are often removed by moulting or ecdysis, the removal of the outer layer of the body and gut contents, which

is a necessary stage in the somatic development of arthropods (Hawes *et al.*, 2007). Recent studies by Worland and Convey (2008) and Burns *et al.* (2010) have shown that moulting is highly dependent on temperature. Both the proportion of *C. antarcticus* moulting at any one time, and the expression of moult-associated genes, increase as temperatures fall, suggesting that the timing of moulting is both an adaptive trait and a developmental one. Starvation (Cannon and Block, 1988; Sømme and Block, 1982) and food selection (Bokhorst *et al.*, 2007; Worland and Lukešová, 2000; Owen *et al.*, 2013) may also be adaptive processes which aid INA removal. While these processes help to rid INAs from the gut, those in the rest of the body remain largely unaffected. To arrest ice nucleation here, as well as any remaining INAs in the gut, freeze-avoiding invertebrates initiate a second supercooling process - the accumulation of AFPs. Through a non-colligative mechanism (thermal hysteresis) of adsorption onto, and consequently inhibition of, embryonic ice crystals or INAs (Clark and Worland, 2008; Davies and Sykes, 1997), AFPs reduce an organism's SCP relative to its melting point (MP) (Bale, 2002). Thermal hysteresis has been recorded in a number of polar terrestrial invertebrates, including Antarctic and Arctic mites (*A. antarcticus* [Block *et al.*, 2009] and *Phauloppia* sp. [Sjursen and Sømme, 2000]), Antarctic Collembola (e.g. *C. antarcticus* [Block *et al.*, 2009] and *Gressittacantha terranova* [Hawes *et al.*, 2011]), Alaskan beetles (e.g. *Cucujus clavipes* [Sformo *et al.*, 2010]) and Alaskan lacewings (e.g. *Hemerobius simulans* [Duman *et al.*, 2004]). AFPs provide further protection by stabilising the supercooled state and preventing inoculative freezing (Bale, 1993), and preserving membranes during phase transitions (Duman *et al.*, 2004). In a similar manner to freeze-tolerant species, freeze-avoiding invertebrates also utilise polyols and sugars for cryoprotection and the enhancement of metabolism at lower temperatures

(third supercooling process) (Block *et al.*, 2009; Clark and Worland, 2008; Muise and Storey, 2001). Polyols and sugars also help to lower the SCP in a non-colligative manner like AFPs (Lee *et al.*, 1996).

1.3.1.5. Responses to chilling injury

Freeze-tolerance and freeze-avoidance are mechanistically distinct from each other. However, there is also commonality between the two strategies, as they are both susceptible to, and therefore must also guard against, chilling injury. Chilling is defined as cooling sufficient to induce damaging effects or even death in the absence of freezing (Hayward *et al*. 2014). Extreme chilling injury can result from rapid cooling (cold shock or acute stress), as well as long-term exposure to low temperatures (chronic stress) and/or experience of temperature extremes (Czajka and Lee, 1990). In truth, chilling and cold stress are relative terms, and the temperatures at which they occur will depend on multiple factors, ranging from the species' evolutionary history and geographic origin, to an individual's physiological status and recent thermal history. Chilling-induced damage includes the loss of integrity, fluidity, and thus function, of the membrane (Izumi *et al.* 2009), the deterioration of intracellular organelles (Strange and Dark 1962), the disruption of enzymes and electrochemical ion potentials (Denlinger and Lee 2010), and the destruction of whole cells through apoptosis (Yi *et al.* 2007).

The membranes which surround cells and organelles of all life forms allow for the selective transfer of solutes across the cell, intra and inter cell communication, the application of energy harnessed through transmembrane ion gradients, and function as a barrier to pathogens (Hazel, 1995). It is therefore necessary for plants, microbes and animals, including invertebrates, to maintain membrane fluidity as temperature falls.

This is achieved through homeoviscous adaptation, which permits the maintenance of membrane fluidity through alterations in the composition of membrane phospholipid fatty acid chains (Hazel, 1995). Under low temperatures and subsequent packing of phospholipids, the fluidity is maintained by raising the number of unsaturated fatty acids. These introduce double bonds (or kinks) into the phospholipid matrix and reduce phospholipid aggregation. The fluidity of the membrane and the transition phase (Tm = fluid to gel) are also influenced by the position of double bonds and the length of fatty acid chains (Baenzinger *et al.*, 1992), and some invertebrates, including *M. arctica*, respond by augmenting these attributes (Bahrndorff *et al.*, 2007).

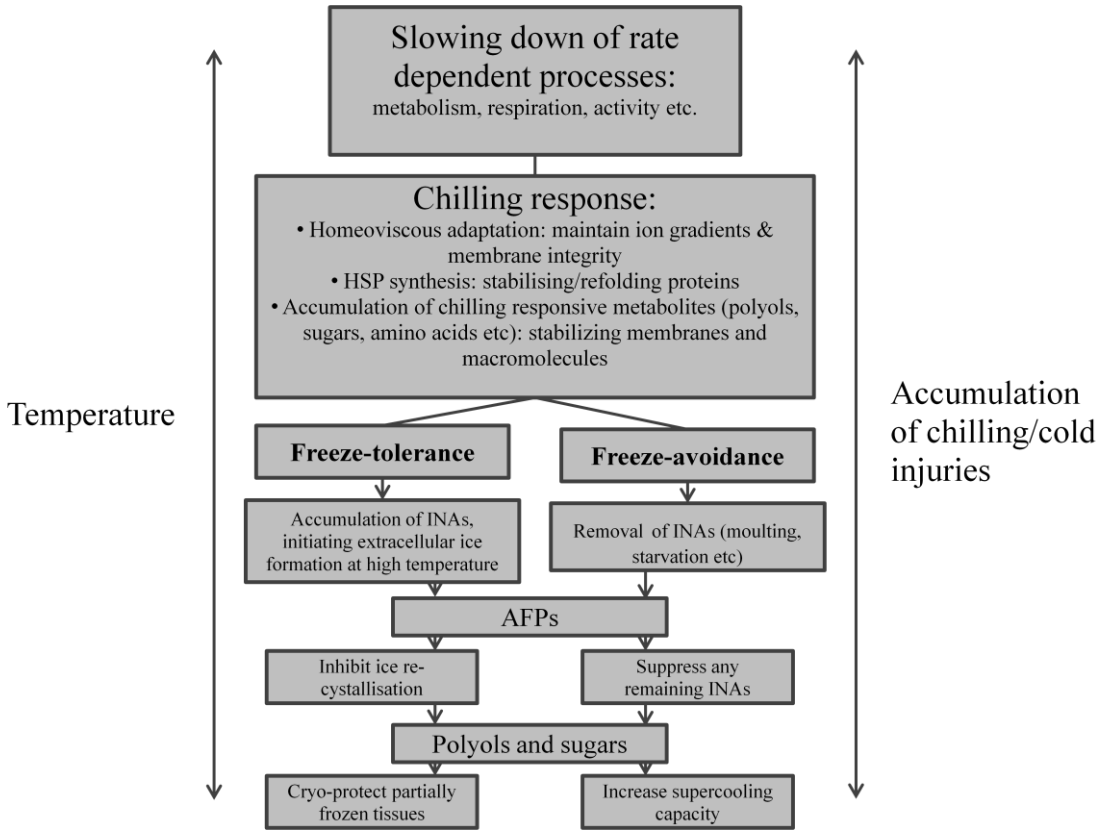

Fig. 1.1. *Schematic representation of the physiological and molecular responses of terrestrial invertebrates to temperature, adapted from Bale (2002).*

HSPs also play an important role in response to chilling injury, as they are involved in refolding and stabilising denatured proteins, recovering microfilament dynamics and regulating apoptosis at low temperatures (Benoit and Lopez-Martinez, 2012; Clark and Worland, 2008; Tammariello *et al.*, 1999; Yi *et al.*, 2007). Hsps have been shown to be constitutively expressed in larva of the Antarctic midge, *B. antarctica*, which experience chronic cold, whereas adults, found in more variable environments, only expressed Hsps as a direct response to stress (Rinehart *et al.*, 2006). The latter scenario represents the 'classic' Hsp response, as seen in non-polar species like the potato beetle, *Leptinotarsa decemlineata* (Yocum, 2001) and the onion fly, *Delia antiqua* (Chen *et al.*, 2006), amongst others. Direct evidence of their contribution to stress tolerance has also been shown in the firebug, *Pyrrhocoris apterus* (Kostal and Tollarova-Borowanska, 2009), and the flesh fly, *S. crassipalpis* (Rinehart *et al.*, 2007). In both species, injection with *hsp70* double-stranded RNA (RNAi) was used to suppress *hsp70* mRNA and protein levels, which resulted in reduced survival (Rinehart *et al.*, 2007), or restricted ability to repair chilling injury and mate successfully (Kostal and Tollarova-Borowanska, 2009).

Further mechanisms suggested to play roles in chilling injury include mitochondrial degradation, which reduces metabolism and energy use (Levin *et al.*, 2003), the accumulation of polyols and sugars, which act as cryoprotectants (Montiel *et al.*, 1998), and the build-up of amino acids, which may serve as a reserve for HSPs or stabilise supercooling (Kostal *et al.*, 2011). A modification of the schematic representation of freeze-avoidance and freeze-tolerance strategies of Bale (2002), which now includes the physiological response to chilling injury (common to both strategies), is presented in Fig. 1.1.

1.3.1.6. Cryoprotective dehydration

Cryoprotective dehydration was first discovered by Holmstrup (1992) in cocoons of the earthworm, *Dendrobaena octaedra*, and has since been described in nematodes (e.g. *Panagrolaimus davidi* [Smith *et al.*, 2008]), enchytraeid worms (e.g. *Fridericia ratzeli* [Pedersen and Holmstrup, 2003]), Collembola (Elnitsky *et al.*, 2008a; Sorensen and Holmstrup, 2011; Worland *et al.*, 1998) and even mammalian cells (Pegg, 2001). This process occurs in an environment in equilibrium with the vapour pressure of ice. Under these conditions, invertebrates continue to lose water along a diffusion gradient between their supercooled body fluids and the surrounding ice until the vapour pressure of their body fluids is equal to that of the environment (Wharton *et al.*, 2003). The subsequent concentration and *de novo* synthesis of solutes (Elnitsky *et al.*, 2008a) causes the SCPs of invertebrates to be reduced and their melting points (MPs) to become equilibrated with the ambient temperature (Elnitsky *et al.*, 2008a; Holmstrup *et al.*, 2002; Pedersen and Holmstrup, 2003). In this state, the risk of freezing is eliminated (Elnitsky *et al.*, 2008a).

Cryoprotective dehydration is perhaps best exemplified in the Arctic collembolan, *M. arctica*. The response was first described in this collembolan by Worland *et al.* (1998) and Holmstrup and Sømme (1998), who showed the SCP of *M. arctica* to fall as low as -30^{o}C when the temperature was reduced to -12.4^{o}C. The MP was also shown to decrease with temperature (Holmstrup and Sømme, 1998), and was later shown by Holmstrup *et al.* (2002) to decline in parallel with the environmental temperature, before equilibrating with this temperature after a 1-6 day lag period. Cryoprotective dehydration is not restricted to freeze-avoiding invertebrates such as *M. arctica*, but also

extends to freeze-tolerant species. For instance, larvae of *B. antarctica* have been demonstrated to lose water in the presence of ice when cooled to -3^{o}C, and have subsequently shown to have a three-fold depression of their MP (Elnitsky *et al.*, 2008a).

The solutes accumulated during cryoprotective dehydration are similar in *B. antarctica* and *M. arctica* and include glucose and trehalose (Elnitsky *et al.*, 2008b; Holmstrup *et al.*, 2002). Glucose is likewise accumulated during cryoprotective dehydration in the earthworm, *F. ratzeli* (Pedersen and Holmstrup, 2003). Other polyols, sugars and/or amino acids may also be involved in the process (Elnitsky *et al.*, 2008b). The accumulation of these solutes has already been shown to lower the SCP and MP, and they may also lead to a vitrified state, as has been shown in at least one invertebrate, the red flat bark beetle, *Cucujus clavipes puniceus* (Sformo *et al.*, 2010).

For cryoprotective dehydration to be a viable strategy, invertebrates must be in possession of two features; cuticular permeability and desiccation tolerance (Bahrndorff *et al.*, 2007). The former is required for the transport of water from the supercooled body fluids to the external environment at a rate equivalent to that of the lowering of ice vapour pressure with temperature (Holmstrup *et al.*, 2002), while desiccation tolerance is imperative if the organism is to survive considerable water loss. Briefly, desiccation tolerance is attained via the accumulation of trehalose and other low molecular weight carbohydrates (Worland *et al.*, 1998), mobilisation of HSPs (Sorensen *et al.*, 2010), reduction of reactive oxygen species (ROS) and repair of oxidative damage (Clark *et al.*, 2009b), stabilisation of the cytoskeleton (Clark *et al.*, 2009b), lowering of metabolism (Sorensen *et al.*, 2010) and desaturation of plasma membranes (Bahrndorff *et al.*, 2007).

1.3.1.7. Vitrification

Under crystalline conditions, cells eventually degrade and lose their viability when exposed to chronic chilling or cold shock (Katkov, 2006). Freezing can also irreversibly damage cells. Vitrification (or the attainment of a glass-like state) in contrast does not lead to such injuries. Vitrified fluids behave more like a solid and yet also show little change in their molecular state. Under these conditions, an organism's fluids are stable and immune from molecular degradation. It is speculated that vitrification could further aid freeze-avoidance and freeze-tolerance strategies. Clarke *et al.* (2013) have shown vitrification in a number of unicellular organisms, including bacteria, and photosynthetic and heterotrophic eukaryotes, when cooled slowly in the presence of extracellular ice. Cellular dehydration resulting from the movement of water out of the cell to join newly formed ice crystals and the subsequent increase in fluid viscosity inside the cell, under slow rates of cooling, allows these unicellular organisms to vitrify prior to intracellular freezing.

Clarke *et al.* (2013) hypothesised that vitrification would also occur in the cells of freeze-tolerant multicellular organisms, where ice formation in the extracellular space, rather than in the environment would encourage intracellular dehydration and the consequential formation of a glass state. As an example, Clarke *et al.* (2013) postulated that the Alaskan tenebrionid beetle, *Upis ceramboides*, would vitrify. *Upis ceramboides* freezes at high sub-zero temperatures and is able to survive in this frozen state to -60°C (Miller, 1978). It is likely that the dehydration induced by extracellular ice formation would eventually also lead to glass formation, and would only be limited by the rate at which the beetle is cooled (Miller, 1978). Consequently, it has been speculated that

organisms which employ cryoprotective dehydration as a cold tolerance strategy may also be capable of vitrification. A recent study by Sformo *et al.* (2010) indicates that this is the case, having shown the freeze-avoiding red flat bark beetle, *Cucujus clavipes puniceus*, to vitrify at a very low temperature (-58°C). As a result, 43% of the beetles were able to survive between -70 and -73°C, and a further 7% were able to survive -100°C (Sformo *et al.*, 2010). Although the ecological relevance of vitrification in this beetle is unclear, with temperatures in Alaska rarely falling to -58°C, confirmation of the presence of this trait is significant.

1.3.1.8. Rapid cold hardening (RCH)

RCH is defined as the rapid induction of cold tolerance (within minutes to hours) to otherwise lethal temperatures (Lee *et al.*, 2006b; Yi *et al.*, 2007). RCH was first described in the flesh fly, *S. crassipalpis* (Lee *et al.*, 1987), and has since been observed in a wide range of other organisms, including polar species such as *B. antarctica* (Lee *et al.*, 2006b), *H. belgicae* (Hawes *et al.*, 2007; Worland and Convey, 2001), *C. antarcticus* and the mite, *Alaskozetes antarcticus* (Worland and Convey, 2001). The response generally provides only moderate survivorship benefits, with survival being extended for, at most, 10 hours at the discriminating temperature (temperature at which there is between 10 and 20% survival upon direct transfer) and for just 2-3^{o}C below it (Bale, 2002).

RCH also impacts on sub-lethal characteristics, including at temperatures above 0°C. In the fruit fly, *Drosophila melanogaster*, courting and reproduction were 35 and 55% greater at 16^{o}C, respectively, following RCH (Shreve *et al.*, 2004). Further sub-lethal improvements have included the maintenance of the proboscis extension reflex and

grooming behaviour in flesh flies (Kelty *et al.*, 1996), the preservation of learning and spatial conditioning (Kim *et al.*, 2005), and the sustenance of flight (Larsen and Lee, 1994). Similar improvements are likely to be found in polar invertebrates though, as yet, they have not been explored.

The survival and behavioural improvements of RCH are likely to be highly advantageous and may allow invertebrates to adjust quickly to, and track, environmental temperatures on both a temporal (daily) and spatial (microhabitat) scale (Kelty and Lee, 1999; Powell and Bale, 2004, 2005, 2006; Shreve *et al.*, 2004; Worland and Convey, 2001). Analogous to acclimation over weeks and months, a gradual rate of cooling that is more in line with nature tends to elicit greater protection (Chidawanyika and Terblanche, 2011; Kelty and Lee, 1999, 2001; McDonald *et al.*, 1997; Wang and Kang, 2003). As suggested by Wang and Kang (2003) and others, this enhanced protection is presumably because of the greater time these individuals spend at protection-inducing temperatures.

Although the ecological role of RCH is well established, relatively little is known about the physiological mechanisms underlying the response (Teets and Denlinger, 2013). Recent studies suggest that RCH is driven by a calcium signaling cascade involving calmodulin, which allow cells to sense changes in temperature and trigger downstream physiological responses (Teets *et al.*, 2008). Protection against cold-induced apoptosis is likely to be one such physiological response. RCH is able to impair apoptosis by down-regulating promoters of the response and up-regulating apoptosis inhibitors. In *D. melanogaster* and *S. crassipalpis*, apoptosis was reduced by >34% following RCH (Yi *et al.*, 2007; Yi and Lee, 2011). RCH also involves a homeoviscous adaptation response. With the use of solid state NMR spectroscopy, Lee *et al.* (2006a) were able to

demonstrate enhanced membrane unsaturation and a subsequent rise in membrane fluidity in *B. antarctica* following RCH. Metabolic adjustments, including the accumulation of polyols and sugars during RCH and the minimisation of metabolic perturbations during cold shock recovery, may likewise play a key role (Michaud and Denlinger, 2007; Overgaard *et al.*, 2007; Teets *et al.*, 2012). However, the universality of homeoviscous adaptation and metabolic adjustment is in question, as some invertebrates show an RCH response in the absence of either the elevation of polyols and sugars or alterations in membrane composition (MacMillan *et al.*, 2009). Because apoptosis inhibition and homeoviscous adaptation, as well as metabolic adjustments to a large degree, concern responses to chilling injury, this suggests that chilling injury, rather than freezing damage, is the primary target of RCH in the chill-susceptible and freeze-tolerant invertebrates studied. The same cannot be said for freeze-avoiding invertebrates, however, in which the SCP is lowered during RCH (Worland and Convey, 2001). Worland and Convey, (2001) also confirmed that the body water content and solute concentration of freeze-avoiding invertebrates were unaffected by RCH, and hypothesised that RCH in these organisms could be understood by the inhibition of INAs, though this remains unconfirmed.

1.3.1.9. Thermal thresholds of activity

Analogous to survival, sub-lethal characteristics, such as foraging, courtship and reproduction, are also affected by temperature. Activity, which underlies all of the aforementioned characteristics, is particularly susceptible. As temperature decreases, neuromuscular function slows and invertebrates begin to lose their coordination (Critical Thermal minimum or CTmin) and eventually their ability to maintain

electrophysiological activity and movement (chill coma) (Hazell and Bale, 2011). In temperate insects, chill coma is attained at positive temperatures, sometimes well above 0^{o}C. For *D. melanogaster*, chill coma is reached at temperatures above 5^{o}C (Kelty and Lee, 1999), whereas for the bumble bee, *Bombus terrestris*, the chill coma temperature is even higher (Emily Owen Pers. Comm.). Polar terrestrial invertebrates, in contrast, remain active at or below 0^{o}C (Coulson *et al.*, 1995a). The dipteran, *Scoliocentra nigrinervis*, for example, has been observed performing activity at -12.7^{o}C, and *Trichocera regelationus* has even been found attempting to fly at -4^{o}C (Hågvar, 2010). Block (1990) and Sinclair *et al.* (2006) have also shown sub-zero activity in the Antarctic mites, *A. antarcticus* and *Nanorchestes antarcticus*, and Collembola, *Isotoma klovstadi*, *Cryptopygus cisantarcticus* and *Friesea grisea*. By remaining active at these temperatures, polar terrestrial invertebrates are able to take advantage of relatively warmer spells in spring, summer and autumn, and continue to develop and reproduce (Hågvar, 2010).

The physiological mechanisms underlying CTmin and chill coma are beginning to be understood. Recent studies suggest it is reduced muscle action, and resting, potential frequency that is responsible and that this is likely caused by disrupted ion regulation (Macmillan and Sinclair, 2010). Three main routes of disruption have been identified, namely ion-motive pumps, ion channels and the membrane (Macmillan and Sinclair, 2010). Ion-motive pumps, such as Na^{+}/K^{+} and $Ca2^{+}$ ATPases, which are involved in ion regulation and neurotransmitter release, are directly influenced by the thermal sensitivity of enzymes. Lowered temperatures reduce the capability of pumps to transport ions and in turn reduce the capacity of the cell to maintain cell potential. Ion channels are similarly influenced by temperature. In *D. melanogaster*, the current

amplitude of K^+ channels was lowered by 25% and the time to current peak was raised by 1.2 ms when the temperature was decreased by just 4°C (25 to 21°C). Lastly, ATPases, ion channels and the release of neurotransmitters are heavily impacted by the phospholipid composition of the pre-synaptic membrane, which alters drastically under low temperatures (Hazel, 1995; Macmillan and Sinclair, 2010).

1.3.2. Hygric physiology

Water is a requirement for all life on Earth (Hodkinson *et al.*, 1999). Without it, living organisms are exposed to desiccation and its associated injuries, which include protein denaturation and unwanted macromolecular interactions (Sano *et al.*, 1999; Tang and Pikal, 2005), crystalline to gel membrane phase transitions (Hazel, 1995), oxidative damage (Lopez-Martinez *et al.*, 2008), mechanical stress and the rapid influx of water following rehydration (Bayley and Holmstrup, 1999). The possibility of such injuries is particularly high in the Antarctic and Arctic, where water is unavailable for extended periods of the year (Block *et al.*, 2009). Polar terrestrial invertebrates protect against this threat physiologically through the adoption of one of two strategies, desiccation resistance or desiccation tolerance (Fig. 1.2, Danks, 2000).

1.3.2.1. Desiccation resistance

Desiccation resistance is defined as the capacity to prevent water loss from the body. The extent to which this occurs varies greatly amongst invertebrates, leading to the recognition of three groups - hygric, which have little or no control over their water loss, and transitional and mesic, which are increasingly able to regulate the loss of their body water (Eisenbeis, 1983). The mesic status of some invertebrates is partly due to their lowered cuticular permeability. Reduced permeability is largely achieved through

the modulation of the wax layer, which coats the cuticle and consists of bipolar molecules with hydrophobic and hydrophilic ends (Speight *et al.*, 2008). In the majority of invertebrates, the hydrophobic ends face outward and limit the rate of water loss. However, mesic species go a little further and tend to either accumulate or increase the length of hydrocarbons or hydrophobic molecules, resulting in tighter packing and a greater reduction of water loss (Benoit *et al.*, 2007b). For instance, the mesic mite, *A. antarcticus*, experienced a lower rate of water loss than the mites, *Hydrogamasellus antarcticus* and *Rhagidia gerlachei*, which had two to three times less hydrocarbons. *Alaskozetes antarcticus* was also shown to have a high critical transition temperature of 25^{o}C, below which hydrocarbons remained stable and cells remained relatively watertight (Benoit *et al.*, 2007b).

The accumulation of polyols and sugars, and subsequent absorption of water has also proven a beneficial strategy in a number of species, such as the collembolan, *Folsomia candida*. Having lost almost half of its osmotically active water under 98.2% RH, the collembolan was able to recover nearly all of the loss within 5-7 d, via the accumulation and synthesis of myo-inositol, glucose and trehalose (Bayley and Holmstrup, 1999; Timmermans *et al.*, 2009). The Antarctic species, *C. antarcticus* (Elnitsky *et al.*, 2008a) and *B. antarctica* (Benoit *et al.*, 2009a), are similarly able to depress the rate of water loss through the accumulation of osmolytes. There are also some species, including astigmata mites, that are able to maintain an equilibrium with the environment at between 70 and 98% RH from the outset (Benoit *et al.*, 2007b, 2009a). Further means of resisting desiccation are freezing (Convey, 1992), membrane alterations, metabolic suppression (Michaud *et al.*, 2008) and specialised respiration (Convey *et al.*, 2003; Danks, 2000; Slama, 1988).

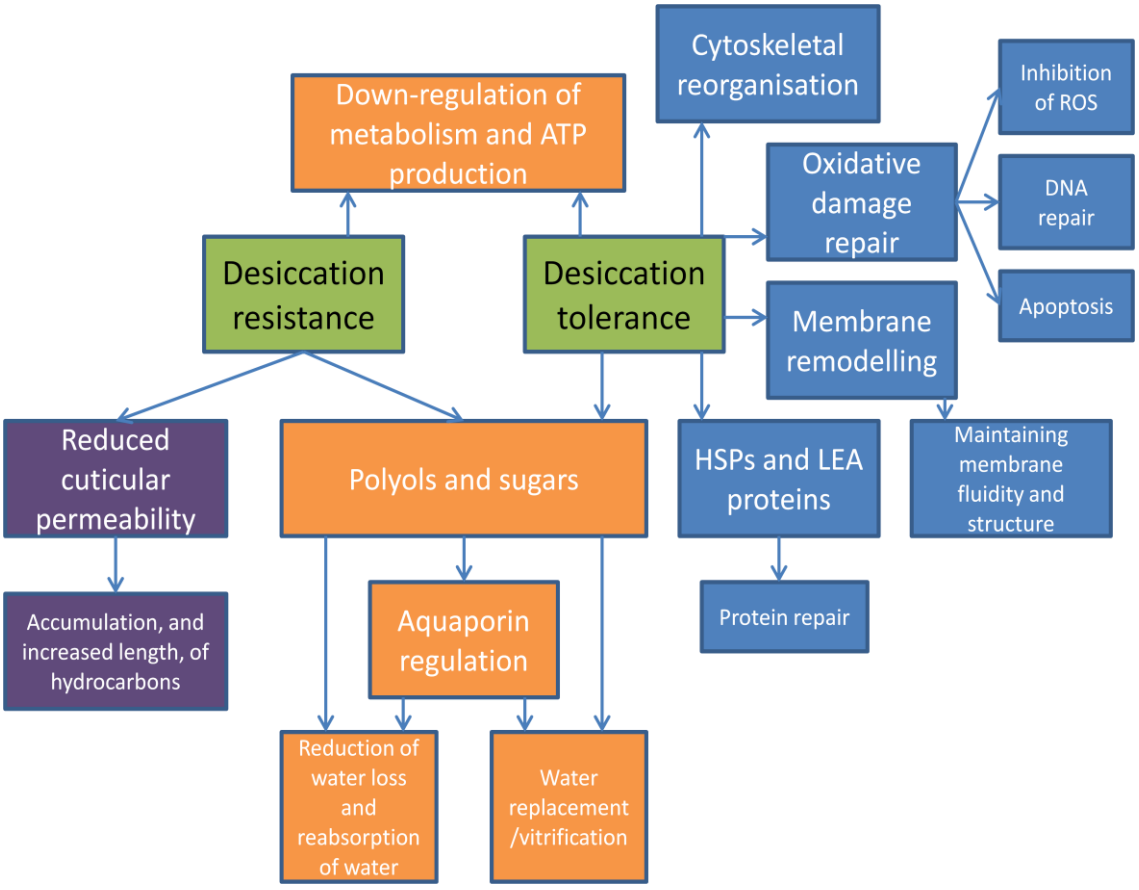

Fig. 1.2. *Schematic representation of the physiological responses of desiccation resistance and desiccation tolerance.*

1.3.2.2. Desiccation tolerance

For invertebrates that are less desiccation resistant, an ability to tolerate water loss is crucial for survival (Danks, 2000). Some invertebrates are particularly tolerant - *Dendrobaena octaedra* cocoons (Holmstrup and Zachariassen, 1996) and larvae of *B. antarctica* (Hayward *et al.*, 2007) are able to endure >75% loss of their water content, and nematodes and tardigrades are able to survive the loss of virtually all of their osmotically active water and most of their osmotically inactive water in a process called anhydrobiosis (Crowe and Madin, 1975; Hengherr *et al.*, 2010; Watanabe *et al.*, 2002; Wharton, 1993; 2003, 2011; Wharton and Worland, 2001). The mechanisms which

these organisms utilise to confer tolerance are many and include the accumulation of polyols and sugars, the activation of HSPs and Late Embryogenesis Abundant (LEA) proteins, membrane remodelling and oxidative damage repair (Gusev *et al.*, 2010; Watanabe, 2006).

1.3.2.2.1. Polyols and sugars

Polyols and sugars are used in response to desiccation in a number of polar invertebrate groups, including nematodes (e.g. *Plectus murrayi* [Adhikari *et al.*, 2010]), midges (e.g. *B. antarctica* [Benoit *et al.*, 2009a]), beetles (e.g. *H. sparsutum* [Bale *et al.*, 2000]) and Collembola (e.g. *C. antarcticus* [Elnitsky *et al.*, 2008a]). Of these, *B. antarctica* has been especially well studied. As a result of water loss and *de novo* synthesis, larvae of this species raise the level of glycerol and trehalose two to three fold (Benoit *et al.*, 2007a). Two hypotheses have been put forward for the function of polyols and sugars during desiccation. The first hypothesis is that polyols and sugars, particularly trehalose, are used for the replacement of water. Subsequently, cellular damage and deleterious protein interactions, which would otherwise occur in the absence of water, are reduced (Benoit *et al.*, 2009a). The second hypothesis is that the low molecular weight compounds aid the production of amorphous sugar glasses (e.g. through the formation of hydrogen bonds with membrane phospholipids [Sakurai *et al.*, 2008]). These glasses stabilise proteins and membranes by minimising physical and chemical reactions involving molecular diffusion, such as solute crystal nucleation (Bahrndorff *et al.*, 2009; Danks, 2000; Hengherr *et al.*, 2009).

1.3.2.2.2 HSPs and LEA proteins

Protein denaturation is a common injury following desiccation. HSPs are induced in direct response to protein damage, and are well recognised as being involved in the reformation or degradation of affected proteins (Benoit and Lopez-Martinez, 2012; Feder and Hofmann,1999). Thus, it is unsurprising that HSPs are up-regulated in response to desiccation in several invertebrates, including tardigrades (Hengherr *et al.*, 2008), Collembola (Timmermans *et al.*, 2009) and Antarctic midges (Lopez-Martinez *et al.*, 2009). The group of proteins known as LEA proteins has also been shown to play a role in desiccation tolerance in a number of invertebrates, including polar species, such as *M. arctica* (Bahrndorff *et al.*, 2009; Browne *et al.*, 2002, 2004; Gal *et al.*, 2004; Goyal *et al.*, 2005; Watanabe *et al.*, 2003). LEA proteins possess many of the same attributes as HSPs, being able to prevent protein aggregation and preserve enzymatic activity. These proteins may also be able to suppress unwanted macromolecular interactions and maintain membrane fluidity *in vitro* (Bahrndorff *et al.*, 2009), reduce water loss, prevent ice crystal formation (Bokor *et al.*, 2005) and stabilise sugar glasses (Wolkers *et al.*, 2001). There is even evidence that LEA proteins are fragmented into smaller, but still functional, components in response to increased desiccation and are thereby better able to counteract damage (Kikiwada *et al.*, 2006).

1.3.2.2.3 Membrane remodelling

As with low temperature, the loss of water from cells and membranes leads to the transition of the plasma membrane from a crystalline to a gel phase (Hazel, 1995). Some invertebrates ameliorate this stress via homeoviscous adaptation. In *B. antarctica*, enzymes such as Δ9 FAD and fatty acyl CoA Δ9 desaturases are used to increase

unsaturation, and thus also fluidity, of the membrane (Lopez-Martinez *et al.*, 2009). Membrane remodelling in this midge may also involve the replacement of unsaturated membrane fatty acids with saturated forms. Although seemingly counterintuitive, this substitution minimises the impact of singlet oxygen, a product of desiccation, which reacts directly with polyunsaturated fatty acid side chains and subsequently causes lipid peroxidation (Lopez-Martinez *et al.*, 2009).

1.3.2.2.4. Oxidative damage repair

Desiccation of invertebrates results in the production of ROS, such as hydrogen peroxide and superoxide radicals. Reactive oxygen species cause numerous injuries, including the disruption of membrane fluidity, apoptosis of mitochondria, denaturation of proteins and fragmentation of DNA (Lopez-Martinez *et al.*, 2008). Antioxidants, primarily superoxide dismutase (SOD), catalase and glutathione peroxidase, are accumulated in organisms in response to these injuries and inhibit ROS. Such accumulation has been observed in a number of organisms, including plants (Jenks and Wood, 2007), nematodes (Reardon *et al.*, 2010) and the Antarctic midge, *B. antarctica* (Lopez-Martinez *et al.*, 2008). However, antioxidants are unable to completely arrest the effects of oxidation in some species. There is therefore a need for other defences, such as apoptosis of damaged cells or a DNA repair system. The latter is used in the anhydrobiotic midge, *Polypedilum vanderplanki*, and is achieved through the up-regulation of Rad23 and Rad51, which are genes associated with the repair of DNA breaks (Gusev *et al.*, 2010).

1.3.2.2.5. Additional mechanisms

There are several other processes known to be involved in desiccation tolerance which may be utilised in polar terrestrial invertebrates. These include cytoskeletal reorganization, such as the synthesis of actin, tropomyosin and myosin for the maintenance of the cytoskeleton (Lopez-Martinez *et al.*, 2009; Li *et al.*, 2009), the accumulation of aquaporins for the efficient transport of water and solutes from and into the body (Li *et al.*, 2009; Philip *et al.*, 2008, 2010), the removal and redistribution of osmolytes during rehydration (Bayley and Holmstrup, 1999; Hayward *et al.*, 2007), the regulation of autophagy (Teets *et al.*, 2012), the down-regulation of metabolism and ATP production (Teets *et al.*, 2012), and the possession of a high initial water content (Hayward *et al.*, 2007).

1.3.3. Supplementary stresses

Aside from desiccation and low temperatures, there are a number of other stresses, including salinity (Elnitsky *et al.*, 2009), pollution (Avila-Jimenez *et al.*, 2010), pH (Rinehart *et al.*, 2006), biotic interactions (Hodkinson and Coulson, 2004) and anoxia (Hodkinson and Bird, 2004), which may have at least a small, if not substantial, influence over the invertebrate fauna in the polar regions. Anoxia is especially notable, as flooding during the summer and ice entrapment during the winter may leave invertebrates without oxygen for days, weeks and even months (Hodkinson and Bird, 2004; Sømme and Block, 1982).

1.3.4. Acclimation and cross-tolerance

The variation in temperature between summer and winter in the Antarctic and Arctic is great, and temperatures annually can fall by tens of degrees in buffered microhabitats, and by as much as 100^{o}C on the soil and rock surface (Convey, 1996). There are some invertebrates, such as the nunatak inhabiting springtail, *Cryptopygus sverdrupi* (Sømme, 1986), which manage this scenario by remaining in a cold hardy state all year round, and larvae of *B. antarctica*, which have constitutive Hsp expression (Rinehart *et al.*, 2006). However, most polar invertebrates are not in a constant state of readiness for the winter months and instead prepare themselves physiologically (Deere *et al.*, 2006). This transition from a stress susceptible to stress tolerant state, which is induced by changing environmental conditions, is referred to as either "acclimation" – in response to one abiotic factor (e.g. in the lab); or acclimatisation, when in response to multiple abiotic variables (e.g. under field conditions). Examples of acclimation have been shown in the chironomid *E. murphyi* (Worland, 2010), the beetle, *Hydromedion sparsutum* (Bale *et al.*, 2000), the aphid, *Myzus polaris* (Hazell *et al.*, 2010), the mite *H. belgicae* (Hawes *et al.*, 2007), the collembolan, *Cryptopygus antarcticus* (Worland *et al.*, 2007), the nematode, *Plectus murrayi* (Adhikari *et al.*, 2010), and a number of other polar invertebrates. The greatest phenotypic changes in stress tolerance are typically associated with gradual changes in environmental conditions, as these mirror the slow cooling rates seen under seasonal change. Certainly, it is well known that faster cooling rates reduce the survival of freeze-tolerant species, raise the SCP of freeze-avoiding species, and reduce the capacity of these animals to respond to chilling injury (Sinclair *et al.*, 2003), but see detail of RCH mentioned previously.

Interestingly, acclimation to low temperatures does not just confer enhanced cold tolerance, but also confers enhanced desiccation tolerance. This phenomenon is known as cross-tolerance and is understood through the interrelationship that exists between low temperature and low water availability. Both stressors result in similar injuries and physiological challenges to an invertebrate, including reduction of membrane fluidity, stability and function (Bayley *et al.*, 2001); impairment of protein folding (Ring and Danks, 1994); and, in the case of freezing, increased pH and osmolality of cellular fluid (Ring and Danks, 1994). It therefore follows that the physiological mechanisms induced by an invertebrate in response to these stresses are also similar, or at least complementary. Several studies have suggested that the mechanisms used in response to low temperature evolved from those used in response to low water availability either as aquatic organisms colonised the land, or as they moved from generally less stressful tropical and temperate latitudes towards the poles (Block, 1996; Pullin, 1996; Ring and Danks, 1994, 1998).

In addition to cross-tolerance between low temperature and desiccation, cross-tolerance has also been observed between other combinations of stresses. In particular, cross-tolerance has been observed between desiccation and low-linear energy transfer (LET) and high-LET radiation (Gladyshev and Meselson, 2008; Gusev *et al.*, 2010; Jonsson *et al.*, 2008), desiccation and CO2 and N2 exposure (Ricci *et al.*, 2005), desiccation and high hydrostatic pressure (Seki and Toyoshima, 1988), low temperature and salinity (Elnitsky *et al.*, 2009), low temperature and anoxia (Yoder *et al.*, 2006) and low and high temperature (Yoder *et al.*, 2006).

1.4. Climate warming

Over the last two to three decades, climate warming has received considerable public attention and has become the focus of the largest scientific collaboration in human history. There is now an almost universal consensus that atmospheric CO_2 levels are rising as a result of human activity and are leading to warming on a global scale (IPCC, 2013). Temperatures have so far risen by 0.85^{o}C across the Earth's surface over the last century (IPCC, 2013). The rate of increase in temperature has been particularly high in parts of the polar regions ('polar amplification'), averaging 2^{o}C over the past 50 years (Arctic Council, 2005; Convey *et al.*, 2009; Turner *et al.*, 2009). General circulation models suggest these temperature trends will continue (Convey *et al.*, 2009; Turner *et al.*, 2009). Water availability is also likely to change as a result of climate warming. Precipitation is predicted to increase by 0.5-1% per decade at higher latitudes (Walther *et al.*, 2002; Turner *et al.*, 2009). However, as temperatures rise, snow cover is expected to decrease and melt is expected to occur earlier in the season. In turn, the thawing of glaciers and evaporation of meltwater are also expected to take place earlier in the summer season (Avila-Jimenez *et al.*, 2010; Walther *et al.*, 2002).

1.4.1. Temperature and Heat tolerance

As mentioned previously (sub-section 1.3.1.1), invertebrates are poikilothermic ectotherms and are unable to regulate their body temperature. These organisms must therefore remain within their physiological thresholds of temperature tolerance, below or above which they will not be able to survive (Walther *et al.*, 2002). Invertebrates can remain within their physiological thresholds through alterations in their behaviour, phenology, physiology and genetic make-up, with these responses acting within or

between generations (Lachenicht *et al.*, 2010). However, it has been hypothesised that with increasing latitude, the sensitivity of invertebrates to a temperature rise decreases (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008). It has even been suggested that polar terrestrial invertebrates may cope well, and benefit, from a climate warming scenario (see Bale and Hayward, 2010 for review). Studies by Deere *et al.* (2006), Sinclair *et al.* (2006) and Slabber *et al.* (2007), have shown Antarctic arthropods to survive above 30^{o}C, indicating that at an organismal level they have the capacity to fare well under climate warming. Several studies have also investigated the impact of global warming at the community level, but these have given mixed results. As a result of an enhanced temperature manipulation on Alexander Island, the number of nematodes was raised several fold in the first year (Convey and Wynn-Williams, 2002). Similar findings have also been found in other life groups, including in plants for example, Day *et al.* (2009) showed the aboveground biomass of vegetation to increase by 36% overall following a temperature rise. However, in studies by Convey *et al.* (2002) and Day *et al.* (2009), the abundance of arthropods was shown to decrease under warming conditions.

1.4.2. Water availability

As temperature rises, snow melt will likely come sooner. In turn, the thawing of glaciers and evaporation of meltwater will also occur earlier, increasing the threat of desiccation for invertebrates. Evidence for this is supported by an 11 year study on *C. antarcticus*, which exhibited a fall in water content during the summer period (Convey *et al.*, 2003). However, there is also evidence that water availability may increase as a result of global warming. Under these conditions, invertebrates may fare better. In a study by Convey *et al.* (2002), water amendments showed an increase in the collembolan, *Isotoma*

octooculata, and mite populations. Likewise, in a study by Day *et al.* (2009), raised water availability increased the abundance of *C. antarcticus* by as much as 81% in the first season. Schulte *et al.* (2008) has also provided records of the largest ever aggregations of the Collembola, *C. antarcticus* and *Friesea grisea*, on Humble Island, Antarctica, with the biggest being up to 2,000,000 eggs strong. These aggregations took place during a particularly warm period that may have resulted in greater moister, and a greater abundance of *Prasiola crispa*. This algae is a preferred food source of many Antarctic invertebrates, including *C. antarcticus* and *A. antarcticus* (Worland and Lukesova, 2000). A possible risk of increased moisture availability is habitat inundation for extended periods. This could have devastating effects on species unable to survive prolonged submergence, especially if they cannot respire under water and/or have limited anoxia tolerance.

1.4.3. Pollution

CO_2 levels have increased greatly over the last century and are predicted to rise further (Convey *et al.*, 2009; Turner *et al.*, 2009). As a consequence, the carbon: nitrogen ratio in plants may be enhanced. Invertebrates will therefore need to eat more to gain their fill of nitrogen. Further, the carbon: nitrogen ratio of plant litter will be raised, resulting in a reduced rate of decomposition and subsequent decrease in available soil nutrients for plants. The reduction in nutrients will lead to the reduced availability of food for herbivorous invertebrates (Callaghan *et al.*, 1992). CFCs and other ozone-depleting substances, which are degrading the ozone layer, have also been rising. The rise has lead to increases in the flux of ultraviolet-B radiation. UV-B can cause deleterious effects to polar terrestrial invertebrates. In a multi-factorial climate manipulation study

by Convey *et al.* (2002), both UV-A and UV-B were shown to invoke a negative response in the Antarctic arthropod fauna. The negative response may be attributed to the reduced quality of vegetation, with the two flowering plants, *C. quitensis* and *Deschampsia antarctica*, both showing lower biomass under high UV-B conditions (Convey *et al.*, 2002).

1.4.4. Alien species

As outlined earlier, introductions of non-native species are an increasing concern at polar latitudes. Climate warming trends are also aiding these introductions. By raising the average temperature of parts of the Antarctic and Arctic, warming has opened up areas which were previously too stressful for the organisms being transferred (Chwedorzewska, 2009; Convey *et al.*, 2009; Frenot *et al.*, 2005). However, in the maritime and continental Antarctic, instances of establishment of alien (or introduced) species remain small, with only eight known establishment events to date (Hughes and Convey, 2012; Tin *et al.*, 2009). This minimal transfer is best explained by the severity and isolation of the maritime and continental Antarctic eclipsing the alleviation of recent warming, though the length of time these environments will remain this way in an era of climate warming is likely finite. For invertebrates that are specifically adapted to the extreme maritime and continental Antarctic, which do not have a wealth of biotic diversity, competition, predation and disease from alien species poses a serious threat.

1.5. Polar invertebrate study species

The invertebrates studied in this thesis include the Collembola, *C. antarcticus* and *M. arctica*, the mite, *A. antarcticus*, and midges, *Eretmoptera murphyi* and *H. borealis*. All of these invertebrates are considered models in their respective habitats. Primarily, this

is due to their ease of collection, for they are found in large numbers, are easily accessible, and are easily identifiable, which has meant that the vast majority of polar terrestrial invertebrate work has been carried out on these species to date. Further, because these species are abundant within their habitats, it is assumed they play a larger than average role in ecosystem services, such as nutrient cycling and carbon mineralisation.

Eretmoptera murphyi is also studied because it is a rare example of a successful alien species in the maritime Antarctic, with only 7 other alien species known to have established in the region. Identifying the physiological traits that have allowed this midge to succeed may offer a template onto which other potential alien species can be compared against.

1.5.1. Antarctic invertebrates

1.5.1.1. Cryptopygus antarcticus

Cryptopygus antarcticus is found across the sub- and maritime Antarctic in large aggregations, numbering up to 1.5 x 10^6 individuals m^{-2} at some sites (Burn, 1986; Convey and Smith, 1997; Tilbrook, 1967). It is found in lichen and moss, though it prefers algae, such as *Prasiola crispa* (Worland and Lukešová, 2000). *Cryptopygus antarcticus* is a characteristic black colour, is 1-2 mm long, and weighs just a few micrograms. Like most other polar invertebrates, this collembolan has a multi-year life cycle spanning 3-5 years (Block *et al.*, 2009).

1.5.1.2. Alaskozetes antarcticus

Alaskozetes antarcticus, like *C. antarcticus*, is widespread, ranging from the Falkland Islands (51°S, 57°W) to SE Alexander Island (71°S, 70°W) (Block and Convey, 1995). The mite is found in moss, lichen and algae in large aggregations of hundreds of thousands, though it is more usually observed in small clusters of tens or hundreds on the underside of rocks. The morphology of *A. antarcticus* is equivalent to the Antarctic collembolan, as it is black and 1-2 mm in length. Its lifecycle is also approximately 5 years in duration (Block *et al.*, 2009).

1.5.1.3. Eretmoptera murphyi

Eretmoptera murphyi is a non-biting, parthenogenetic midge. It is native to the sub-Antarctic island of South Georgia and was recently introduced to maritime Antarctic Signy Island in the 1970s (Block *et al.*, 1984; Convey and Block, 1996). The midge is flightless and is assumed to have been introduced by humans during a plant trial. It inhabits the upper soil layer and is found over an area greater than 2000 m^2, with upper estimates of abundance at 400000 m^{-2} (Worland and Hughes, 2010). *Eretmoptera murphyi* has a biennial life cycle, which is mostly spent as a larva (Convey and Block, 1996). There are four larval stages, with each proceeding stage increasing in size and becoming more opaque and yellow. Adults are only active for a few weeks in the second summer.

1.5.2. Arctic invertebrates

1.5.2.1. Megaphorura arctica

Megaphorura arctica was recently known as *Onychiurus arcticus* prior to its reclassification. It is found across the Palaearctic and Canada in densities as high as 286000 individuals m^{-2} (Bengtson *et al.*, 1974). Like *A. antarcticus* though, it is mostly found in small groups of tens of individuals on the underside of rocks and upper soil and moss layers. It has a distinctive yellow colour and is larger than most other polar collembola, measuring up to 5 mm in length. *Megaphorura arctica* has a multi-year life cycle.

1.5.2.2. Heleomyza borealis

Heleomyza borealis is a non-biting midge found on the island of Spitsbergen, Svalbard. Largely, it is found underneath bird nesting sites and is collected from plant litter, bird excrement and fungi (Worland *et al.*, 2000). Larvae of the midge are off white in colour and tend to be found in small aggregations, but can be found numbering hundreds of individuals in certain habitats.

1.6. Thesis outline

Against the background introduced in this chapter, this thesis further explores the stress ecophysiology of the aforementioned polar terrestrial invertebrates by addressing the four objectives outlined at the beginning of this chapter – i) identifying stresses experienced by the invertebrates, ii) quantifying their capacity to tolerate these stresses, iii) investigating the physiological strategies they show in response to stress, and iv) determining the impact of climate warming.

1.6.1. Temperature stress: Chapters 2 and 3

Invertebrates are small, poikilothermic ectotherms and therefore their spatial and temporal distribution is constrained by the temperatures that they can tolerate and by those at which they can perform optimally (Speight *et al.*, 2008). Significantly, these temperatures will vary depending on their ability to acclimatise. Many studies have recognised this and have investigated the acclimatory capacity of invertebrates with respect to their survival (e.g. Worland, 2010). However, relatively few studies have explored the capacity of invertebrates, particularly polar invertebrates, to acclimatise their sub-lethal characteristics (e.g. development, reproduction etc.). In Chapter 2, the impact of long-term temperature acclimation on thermal activity thresholds is explored for three polar species; *C. antarcticus, M. arctica* and *A. antarcticus*.

Unlike the aforementioned Antarctic invertebrates, *C. antarcticus* and *A. antarcticus*, *E. murphyi* is not native to its maritime Antarctic habitat. It was introduced by humans (Block *et al.*, 1984; Convey and Block, 1996). Establishments of this kind have been frequent on the sub-Antarctic islands, as a result of increasing human activity and climate warming, and the impact they are having is considerable (Chwedorzewska, 2009; Frenot *et al.*, 2005). While less frequent, establishment events in the maritime Antarctic are also having a substantial impact (Hughes *et al.*, 2013). There is therefore an imperative to understand the traits that underlie a successful alien (or introduced) species, so that they can be prevented or better managed in future. The traits that underlie *E. murphyi*'s success are further explored in Chapter 3, with respect to its short term acclimation, or rapid cold hardening, response.

1.6.2. Water stress: Chapters 4, 5 and 6

While temperature is often recognised as the principal stressor in the polar regions, low water availability may be of equal, or of greater, importance to resident invertebrates (Block *et al.*, 2009). *Cryptopygus antarcticus* is often trapped on the surface of sea water or supra littoral pools and under these conditions, the collembolan is vulnerable to desiccation (Hopkin, 1997; Hawes, 2011; McGaughran *et al*., 2011). Previous studies have demonstrated extensive tolerance of *C. antarcticus* on sea water, but have not looked at its tolerance of higher salinities (e.g. Hawes *et al.*, 2008). In Chapter 4, the tolerance of *C. antarcticus* to these higher salinities is explored.

Polar invertebrates are also exposed to low water availability when meltwater evaporates in summer, and when water is frozen as ice during winter (Block *et al.*, 2009). Because invertebrates are small and have a large surface area to volume ratio, they are more vulnerable to these environmental circumstances than larger animals. Their success will therefore be in part determined by their capacity to either resist or tolerate any resulting desiccation. In Chapter 5, the physiological strategies employed in response to low water availability by the midges, *E. murphyi* and *H. borealis*, are compared. It should also be noted that low water availability does not often occur in isolation and is usually encountered together with other stressors such as low or high temperature (Convey, 1996). The cross-tolerance capacity of the two midges, as well as *C. antarcticus*, is therefore also investigated in Chapters 4 and 5.

Chapter 6 looks at the opposite extreme to desiccation: inundation. When snow and ice melt in spring, polar habitats can flood with water (Convey, 1996). On South Georgia and Signy Island, where the midge, *E. murphyi*, resides this is also the case. This

presents a major challenge for an invertebrate that predominantly respires and develops on land. In Chapter 6, the ability of the midge to respire in water is assessed and its capacity to tolerate anoxia, which may occur in water or when trapped in ice, is explored.

1.6.3. Climate warming: Chapters 7 and 8

Through Chapters 2 to 6 the capacity of polar terrestrial invertebrates to tolerate current climatic conditions is investigated. However, it is also important to anticipate the future, and this is especially true when considering climate warming. Global temperatures have risen by 0.85°C over the last 100 years, and this trend is expected to continue (IPCC, 2013). Significantly, climate warming is occurring most rapidly in the polar regions, and, as invertebrates are small, poikilothermic ectotherms, they are likely to be affected most. Polar terrestrial invertebrates therefore provide an important study system. Over Chapters 7 and 8, the tolerance and physiological plasticity of *C. antarcticus*, *A. antarcticus*, *M. arctica* and *E. murphyi* to high temperature are explored and are discussed in the context of climate change.

CHAPTER 2: THE EFFECT OF ACCLIMATION TEMPERATURE ON THERMAL ACTIVITY THRESHOLDS IN POLAR TERRESTRIAL INVERTEBRATES

The work presented in this chapter has been published in the Journal of Insect Physiology (Everatt, M. J., Bale, J. S., Convey, P., Worland, M. R. and Hayward, S. A. L. (2013) The effect of acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates. Journal of Insect Physiology. 59, 1057-1064.)

2.1. Abstract

In the Maritime Antarctic and High Arctic, soil microhabitat temperatures throughout the year typically range between −10 and +5 °C. However, on occasion, they can exceed 20 °C, and these instances are likely to increase and intensify as a result of climate warming. Remaining active under both cool and warm conditions is therefore important for polar terrestrial invertebrates if they are to forage, reproduce and maximise their fitness. In the current study, lower and upper thermal activity thresholds were investigated in the polar Collembola, *Megaphorura arctica* and *Cryptopygus antarcticus*, and the mite, *Alaskozetes antarcticus*. Specifically, the effect of acclimation on these traits was explored. Sub-zero activity was exhibited in all three species, at temperatures as low as −4.6 °C in *A. antarcticus*. At high temperatures, all three species had capacity for activity above 30 °C and were most active at 25 °C. This indicates a comparable spread of temperatures across which activity can occur to that seen in temperate and tropical species, but with the activity window shifted towards lower

temperatures. In all three species following one month acclimation at −2 °C, chill coma (=the temperature at which movement and activity cease) and the critical thermal minimum (=low temperature at which coordination is no longer shown) occurred at lower temperatures than for individuals maintained at +4 °C (except for the CTmin of *M. arctica*). Individuals acclimated at +9 °C conversely showed little change in their chill coma or CTmin. A similar trend was demonstrated for the heat coma and critical thermal maximum (CTmax) of all species. Following one month at −2 °C, the heat coma and CTmax were reduced as compared with +4 °C reared individuals, whereas the heat coma and CTmax of individuals acclimated at +9 °C showed little adjustment. The data obtained suggest these invertebrates are able to take maximum advantage of the short growing season and have some capacity, in spite of limited plasticity at high temperatures, to cope with climate change.

2.2. Introduction

As poikilothermic ectotherms, invertebrates have limited means of regulating their own body temperature and are instead dependent on the thermal conditions of their environment (Speight *et al.*, 2008). It is widely acknowledged therefore that the spatial and temporal distribution and abundance of invertebrates are partly determined by the range of temperatures they can tolerate and by the range of temperatures at which they perform optimally (Gaston, 2009; Terblanche *et al.*, 2011). Investigations into the thermal tolerance limits of invertebrates are accordingly necessary to fully understand the ecology of a species or population and to infer the impact of climate change (e.g. Deutsch *et al.*, 2008; Everatt *et al.*, 2013a; Somero, 2005). A common limitation of many current thermal biology studies, however, is their emphasis on organismal

survival. While survival clearly underpins the fitness of a species, there are also a number of other attributes which are greatly affected by temperature (Bale, 2002). These attributes, termed sub-lethal characteristics, include courtship, reproduction, foraging/feeding and predator avoidance (Kelty and Lee, 1999; Korenko *et al.*, 2010). When these attributes can occur is governed by the upper and lower activity thresholds of the organism, and this thermal activity 'window' demonstrates phenotypic plasticity depending on the geographic location and the thermal/physiological history of the organism being studied (Addo-Bediako *et al.*, 2000; Bale and Hayward, 2010). Because thermal activity thresholds are affected by less extreme temperatures, more regularly encountered than those which cause mortality, the extent to which sub-lethal characteristics are affected could be of more importance than the ability to survive temperature extremes *per se*.

The limits of movement under low temperatures have been a source of fascination since the late 19th Century. Rossbach (1872) observed the frequency of contractions of the contractile vesicle of three protist species and noticed that, at some low temperature, contractions ceased. He termed the absence of movement 'chill coma'. By 1939, the terminology relating to chill coma encompassed four potential states; chill coma^{1} – absence of activity and movement, chill coma^{2} – final peak of activity and movement, chill coma^{3} – loss of coordination, and chill coma^{4} – absence of spontaneous movement, and these terms have remained in use to this day (Hazell and Bale, 2011). Within this paper, the first definition will be used, i.e. the absence of activity and movement. Cowles and Bogert (1944) applied a new term to describe chill coma^{3} or the loss of coordination. This term was the 'Critical Thermal minimum' (CTmin) and will be used here to define the complete loss of coordination (inability to walk or move forward).

The upper thermal thresholds of activity are analogous to those of low temperature and include heat coma and the Critical Thermal maximum (CTmax) (Hazell *et al.*, 2008).

The Antarctic and Arctic are characterised by long, cold winters and brief, cool summers (Ávila-Jiménez *et al.*, 2010; Block *et al.*, 2009). During the winter, air temperatures regularly fall below −10 °C, and to lower than −40 °C, in regions of the High Arctic and maritime and continental Antarctic (Block *et al.*, 2009; Coulson *et al.*, 1993; Strathdee and Bale, 1998; Walton, 1984). Buffered microhabitat temperatures in the soil or underneath the snow are likewise sub-zero during winter, though generally these temperatures do not fall much lower than −10 °C (Coulson *et al.*, 1993; Davey *et al.*, 1992; Rinehart *et al.*, 2006; Strathdee and Bale, 1998). Water is also transformed into ice in winter and is inaccessible to living organisms (Block *et al.*, 2009). Activity is virtually impossible under these conditions. Accordingly, polar terrestrial invertebrates are dormant during this period and wait until the short, four to six month, summer period to resume activity (Convey, 1996). Summer air temperatures are still very cool, however, rarely rising above 0 °C in the continental Antarctic, 5 °C in the maritime Antarctic, and slightly higher in the Arctic (Davey *et al.*, 1992; Block *et al.*, 2009; Coulson *et al.*, 1993; Strathdee and Bale, 1998). To benefit from these relatively favourable conditions, these invertebrates are capable of activity at low and even sub-zero temperatures. Hågvar (2010) has identified several invertebrate groups, including Collembola, Mecoptera, Diptera, Plecoptera and Araneae, which are active at or below 0 °C on the snow of Fennoscandinavia. Block (1990) and Sinclair *et al.* (2006) have also shown sub-zero activity in the Antarctic mites *Alaskozetes antarcticus* and *Nanorchestes antarcticus*, and the Collembola *Isotoma klovstadi*, *Cryptopygus cisantarcticus* and *Friesea grisea*, respectively.

Activity at high temperatures may also be important in the polar regions. Currently, buffered microhabitat temperatures range up to c. 20 °C in the maritime Antarctic (Convey *et al.*, 2009; Davey *et al.*, 1992; Everatt *et al.*, 2013a), and to slightly higher temperatures in the Arctic (Coulson *et al.*, 1993). Climate warming is also rapidly affecting the polar regions. Over the last 50 years, polar amplification of global climate trends has led to an average 2 °C rise in air temperatures in parts of the Arctic and Antarctic, with even greater increases experienced in regions such as the northern and western Antarctic Peninsula, or when looked at on a seasonal basis (Arctic Council, 2005; Convey *et al.*, 2009; Turner *et al.*, 2009). This trend is set to continue, with general circulation models predicting particularly rapid warming at polar latitudes (Convey *et al.*, 2009; Kattenberg *et al.*, 1996). In addition, specific microhabitats, such as the surfaces of rocks and bryophyte clumps, can experience maximum temperatures approaching or exceeding 30 °C (Convey, 1996; Everatt *et al.*, 2013a; Smith, 1988). Climate warming may increase the prevalence and duration of these exposures (Bokhorst *et al.*, 2011; Nielsen and Wall, 2013). The ability of polar terrestrial invertebrates to remain active at high temperatures has only as yet been explored in three continental Antarctic Collembola, and all show a remarkable capacity to remain active above 30 °C (Sinclair *et al.*, 2006).

The vast majority of polar terrestrial invertebrates express seasonal and shorter term thermal tolerance strategies to enable survival of shifts in temperature (Cannon *et al.*, 1988; Worland, 2001; Denlinger and Lee, 2010). However, the ability of polar terrestrial invertebrates to acclimate or acclimatise their thermal activity thresholds is less well known. Only two polar species, the aphid, *Myzus polaris*, and the collembolan, *Isotoma klovstadi*, have been demonstrated to have this ability, with a depression in the

CTmin of individuals reared at, or taken from, lower temperatures (Hazell *et al.*, 2010; Sinclair *et al.*, 2006). In the current study, the lower and upper thermal activity thresholds are characterised in three common polar invertebrates widely regarded as 'model' species in their respective ecosystems: *Cryptopygus antarcticus* (Block *et al.*, 2009; Tilbrook, 1967) and *Alaskozetes antarcticus* (Block and Convey, 1995; Burn, 1986) from the maritime Antarctic, and *Megaphorura arctica* (Fjellberg, 1994) from the High Arctic. In particular, how the thermal activity thresholds of these species respond to acclimation is explored.

2.3. Materials and methods

2.3.1. Invertebrate collection and storage conditions

Summer acclimatised individuals of *M. arctica* were collected from moss-covered slopes at Krykkefjellet and Stuphallet, near Ny-Ålesund, Spitsbergen, Svalbard (78°55′N, 11°56′E) in August 2011. Summer acclimatised individuals of *C. antarcticus* and *A. antarcticus* were collected from moss and algae, and the underside of rocks, on Lagoon Island (67°35′S, 68°16′W) and Léonie Island (67°36′S, 68°21′W), near to Rothera Research Station, Adelaide Island (western Antarctic Peninsula, maritime Antarctic), between January and March 2012.

Samples of *C. antarcticus* and *A. antarcticus* were held at +4 °C (24:0 L:D) in plastic bags or boxes containing substratum from the sites at which they were found whilst at Rothera Research Station and were used shortly after collection in experiments 2.3, 2.4 and 2.6. These individuals were designated as the "summer acclimatised" group. Following each respective field season, samples of *M. arctica*, and *C. antarcticus* and *A. antarcticus*, were transported to the University of Birmingham under refrigerated

conditions and then held in plastic boxes containing substratum from the site of collection at +4 °C (0:24 L:D). The duration of travel was ~2 d from the Arctic and ~2 months from the Antarctic. Each species was split into two additional acclimatory groups (−2 and +9 °C, 0: 24 L:D), representing early spring/late autumn microhabitat temperature and upper summer microhabitat temperature, respectively. Samples were held for at least two weeks at +9 °C, and for at least one month at −2 °C prior to experimentation. The age of individuals used for experimentation was not uniform, as it was not possible to breed same age populations of the polar invertebrates in a laboratory setting. Difficulties in obtaining active individuals of *M. arctica* from acclimation at −2 °C meant that individuals used in observations of locomotion (Section 2.5) were instead taken from a one month acclimation at 0 °C.

2.3.2. Experimental conditions

Activity thresholds were assessed within an aluminium block arena. The temperature within the arena was regulated using an alcohol bath (Haake Phoenix II C50P, Thermo Electron Corporation), and activity monitored using a digital video camera with a macro lens (see Hazell *et al.*, 2008). Thirty individuals were transferred into the arena in groups of 10 (initially set to +4 °C), and were allowed to settle before video recording (Studio Capture DT, Studio86Designs, Lutterworth, UK) and the alcohol bath programme began. This procedure was performed for each species and for each acclimation treatment.

2.3.3. CTmin and chill coma

The temperature of the arena was reduced from +4 to −10 °C at 0.2 °C min^{-1}. Although a rate of change more closely in line with that experienced by the study species would

have been preferable, a rate of 0.2 °C min^{-1} was chosen due to time constraints. The temperatures at which each individual last walked or moved forward (CTmin) and last moved its body, legs and/or antennae (chill coma) were subsequently recorded.

2.3.4. CTmax and heat coma

The temperature of the arena was raised from +4 to +40 °C at 0.2 °C min^{-1}. The temperatures at which each individual last walked or moved forward (CTmax) and last moved its body, legs and/or antennae (heat coma) were recorded.

2.3.5. Locomotion analysis

The arena and video equipment, as described in Section 2.2, was used to record the total distance travelled by individuals within a 5 min observation period at temperatures representative of either current spring/winter conditions, or current and future (predicted) summer microhabitat conditions. Spring/winter conditions: +4, 0, −4 and −8 °C; summer conditions: 10, 15, 20, 25, 30 and 35 °C. Groups of 5 individuals were held in the arena for each recording, and cooled or warmed from +4 °C at a rate of 0.2 °C min^{-1}. For each acclimation group, the same 10 individuals were used for the +4, 0, −4 and −8 °C exposures, and a second set of 10 individuals were used for 10, 15, 20, 25, 30 and 35 °C. Thus, in the spring/winter temperature exposures, individuals were observed at +4 °C for 5 min, then ramped to 0 °C and observed for 5 min, then ramped to −4 °C and so on. This technique more accurately reflects the gradual change in microhabitat conditions within terrestrial habitats than would be represented by direct transfer to each temperature. The distance travelled within each 5 min holding period was measured using Studio Measure (Studio86Designs, Lutterworth, UK). Inactive

periods were not screened out so as to take account of both the propensity and ability of each species to move at each temperature.

2.3.6. Supercooling points (SCPs)

The supercooling points (SCP = freezing point of body fluids) of each acclimation group were determined by cooling 32 (24 in summer acclimatised group) individuals of each species from +4 to −30 °C at 0.5 °C min^{-1}. Each individual was placed in contact with a thermocouple (one individual per thermocouple, except in the "summer acclimatised" groups in which there were three individuals per thermocouple). This was housed within an Eppendorf tube, itself in a glass test tube plugged with sponge, inside an alcohol bath. The SCP was defined as the temperature at the onset of the freezing exotherm and was recorded using Picolog Recorder Software (Pico Technology Limited, UK) (cf. Hawes *et al.*, 2006). The SCP is known to be the lower limit of survival, and equivalent to the lower lethal temperature, in the three species studied (Cannon *et al.*, 1988; Worland *et al.*, 1998).

2.3.7. Statistical analysis

The Kolmogorov–Smirnov test was used to determine whether activity threshold and SCP data were normally distributed. Normally distributed data were analysed using analysis of variance (ANOVA) and Tukey's multiple range test, and non-normally distributed data were analysed using the Kruskal–Wallis test.

2.4. Results

2.4.1. CTmin and chill coma

2.4.1.1. Interspecific comparisons

The point at which each species (+4 °C acclimation) no longer showed coordination (CTmin) and lost mobility entirely (chill coma) both typically occurred at temperatures below 0 °C (Fig. 2.1). The chill coma temperature was lower than −3.8 °C in all species, and was lowest in *A. antarcticus* (−4.6 °C). The CTmin occurred at similarly low temperatures in the two collembolan species (*C. antarcticus*: −3.5 °C, *M. arctica*: −4 °C), but was significantly higher in the mite (−0.6 °C, $P < 0.05$ Kruskal–Wallis test).

2.4.1.2. Effect of acclimation

Following 1 month at −2 °C, all species showed significantly lower chill coma values ($P < 0.05$ Kruskal–Wallis test [*C. antarcticus* and *M. arctica*], $P < 0.05$ Tukey's multiple range test [*A. antarcticus*]), and generally lower or equivalent CTmin values, than individuals maintained at +4 °C (Fig. 2.1). Individuals of *A. antarcticus* (−2 °C acclimation) also exhibited significantly lower CTmin and chill coma values in comparison with summer acclimatised individuals ($P < 0.05$ Tukey's multiple range test). There were no significant differences in the CTmin and chill coma values between species acclimated at +9 °C and those at +4 °C, except for *M. arctica* in which the CTmin was significantly higher in the +9 °C acclimated group ($P < 0.05$ Kruskal–Wallis test).

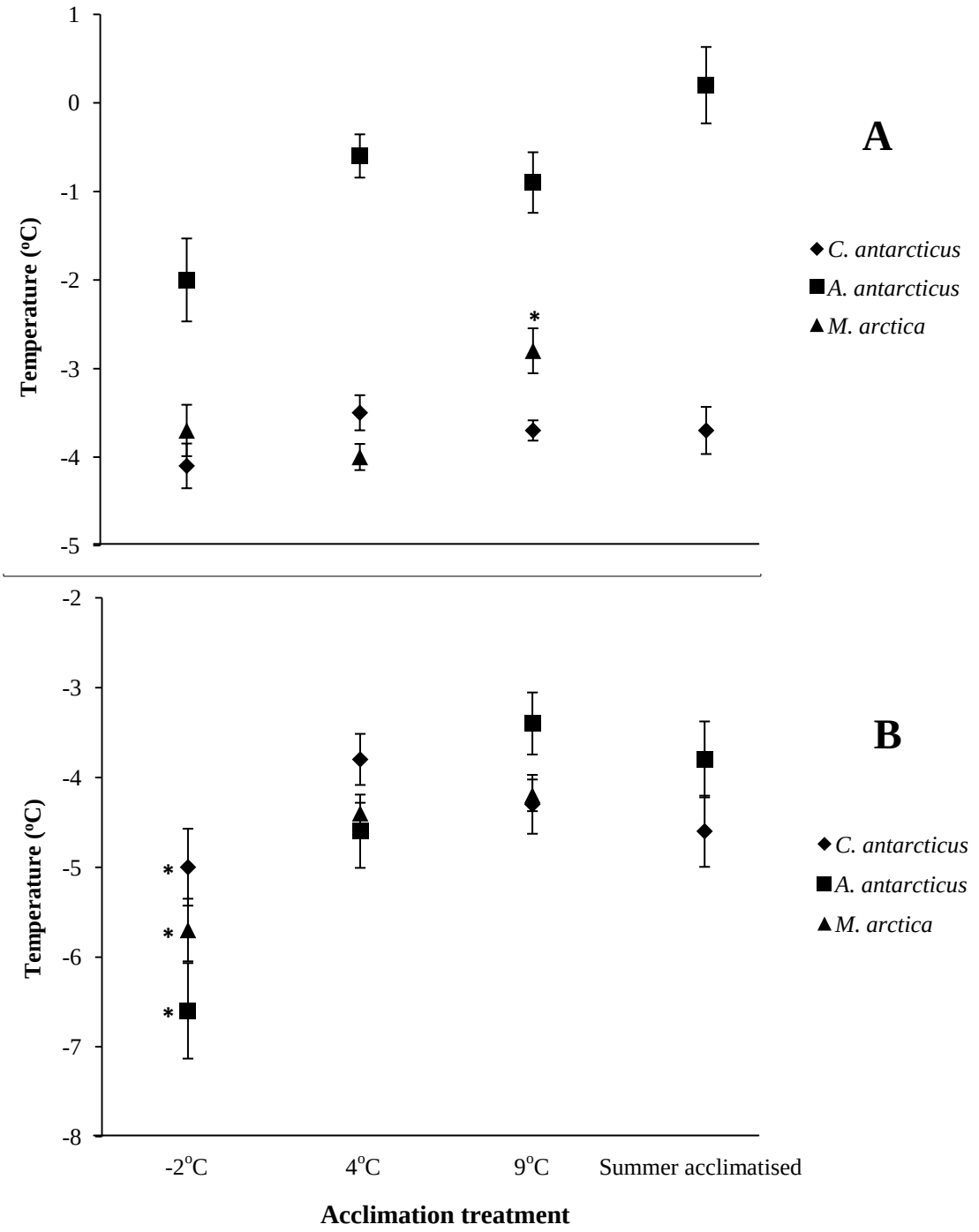

Fig. 2.1. *CTmin (A) and chill coma (B) of C. antarcticus, A. antarcticus and M. arctica, following acclimation at 4, 9 and -2°C, and individuals acclimatised to the Antarctic summer (C. antarcticus and A. antarcticus only). Means ± S.E.M. are presented for approximately 30 individuals. Asterisks indicate a treatment significantly different from 4°C acclimated individuals for each species at P < 0.05 (Kruskal-Wallis test; Tukey's multiple range test).*

2.4.2. CTmax and heat coma

2.4.2.1. Interspecific comparisons

In all species maintained at +4 °C, both CTmax and heat coma temperatures were typically above 30 °C (Fig. 2.2). Both CTmax and heat coma values were significantly different between species and were progressively greater from *C. antarcticus* (30.1 and 31.8 °C), through *M. arctica* (31.7 and 34.6 °C), to *A. antarcticus* (34.1 and 36.9 °C) ($P < 0.05$ Tukey's multiple range test, variances not equal).

2.4.2.2. Effect of acclimation

A one month acclimation at −2 °C significantly reduced CTmax and heat coma temperatures compared to individuals maintained at +4 °C in all species (Fig. 2.2, $P < 0.05$ Kruskal–Wallis test). A two week acclimation at +9 °C also led to lower (or unchanged – *C. antarcticus*) CTmax and heat coma temperatures, though this was only significant for the heat coma temperature of *A. antarcticus* ($P < 0.05$ Kruskal–Wallis test). Summer acclimatised individuals of *C. antarcticus* exhibited significantly lower CTmax and heat coma temperatures than individuals acclimated at either −2 °C or +4 °C, while summer acclimatised individuals of *A. antarcticus* only showed significantly lower CTmax and heat coma temperatures than individuals maintained at +4 °C.

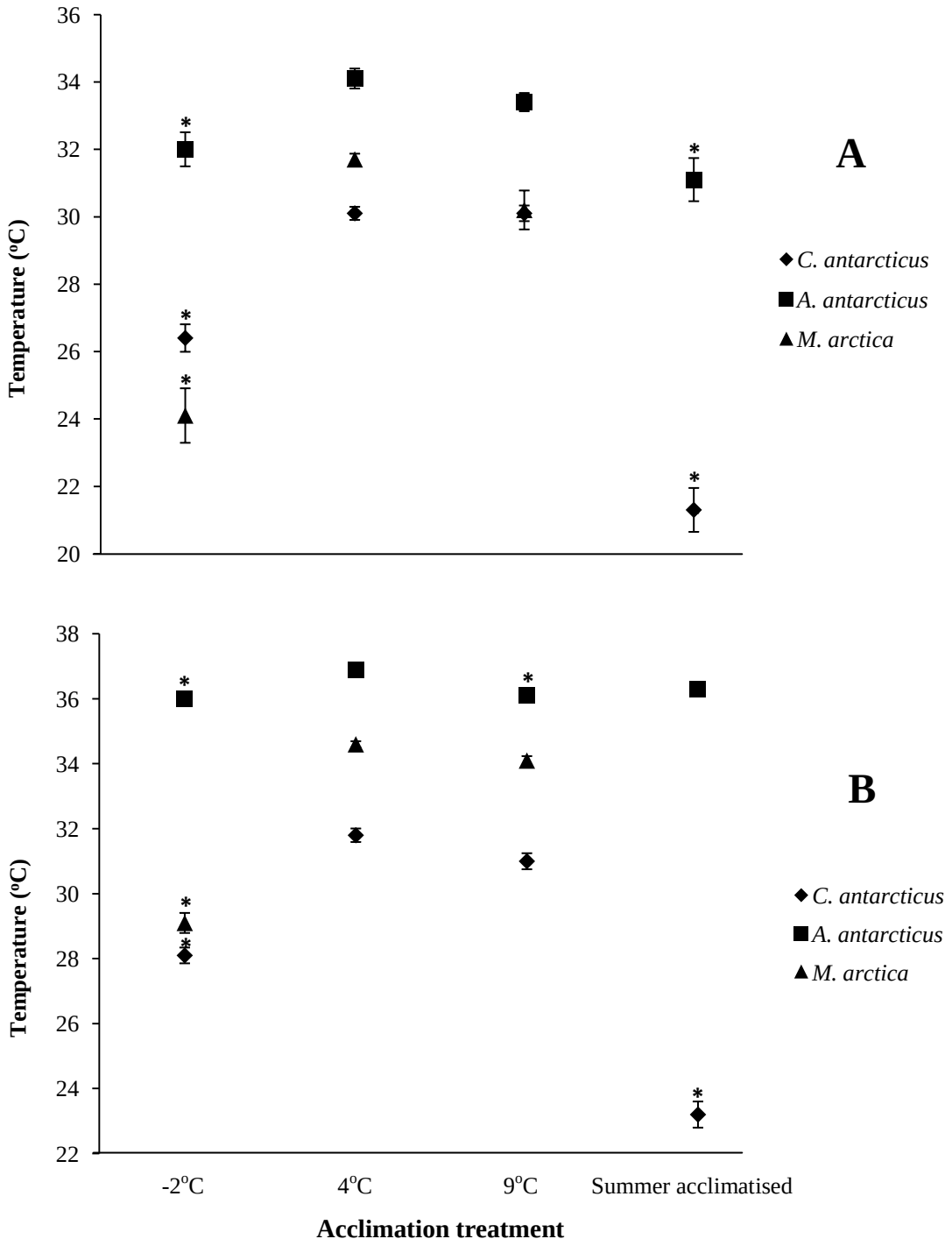

Fig. 2.2. *CTmax (A) and heat coma (B) of C. antarcticus, A. antarcticus and M. arctica, following acclimation at 4, 9 and -2°C, and individuals acclimatised to the Antarctic summer (C. antarcticus and A. antarcticus only). Means ± S.E.M. are presented for approximately 30 individuals. Asterisks indicate a treatment significantly different from 4°C acclimated individuals for each species at P < 0.05 (Kruskal-Wallis test).*

2.4.3. Locomotion analysis

2.4.3.1. Interspecific comparisons

Across all temperatures between −4 and 20 °C, both collembolan species were significantly more active and travelled a greater distance than the mite ($P < 0.05$ Kruskal–Wallis test, 4 °C acclimation, Fig. 2.3). In all species previously acclimated at +4 °C, movement increased with temperature up to 25 °C (except at 9 °C in *M. arctica*), before decreasing again at temperatures < 30 °C.

2.4.3.2. Effect of acclimation

Following an acclimation period at −2 °C (0 °C for *M. arctica*), there was no significant difference in locomotion at temperatures < 0 °C, except for *M. arctica*, in which movement was significantly greater at −4 °C ($P < 0.05$ Tukey's multiple range test, variances not equal) (Fig. 2.3). At 15 and 20 °C, movement was most rapid in *C. antarcticus* acclimated at −2 °C, as compared with the two other acclimation groups. The movement of *M. arctica*, acclimated at 0 °C, was also more rapid at 20 °C. Individuals of both collembolan species given an acclimation period at +9 °C exhibited considerably slower movement at temperatures above +4 °C than individuals maintained at +4 °C. In contrast, movement was greater across all temperatures between 0 and 25 °C in +9 °C acclimated individuals of *A. antarcticus*.

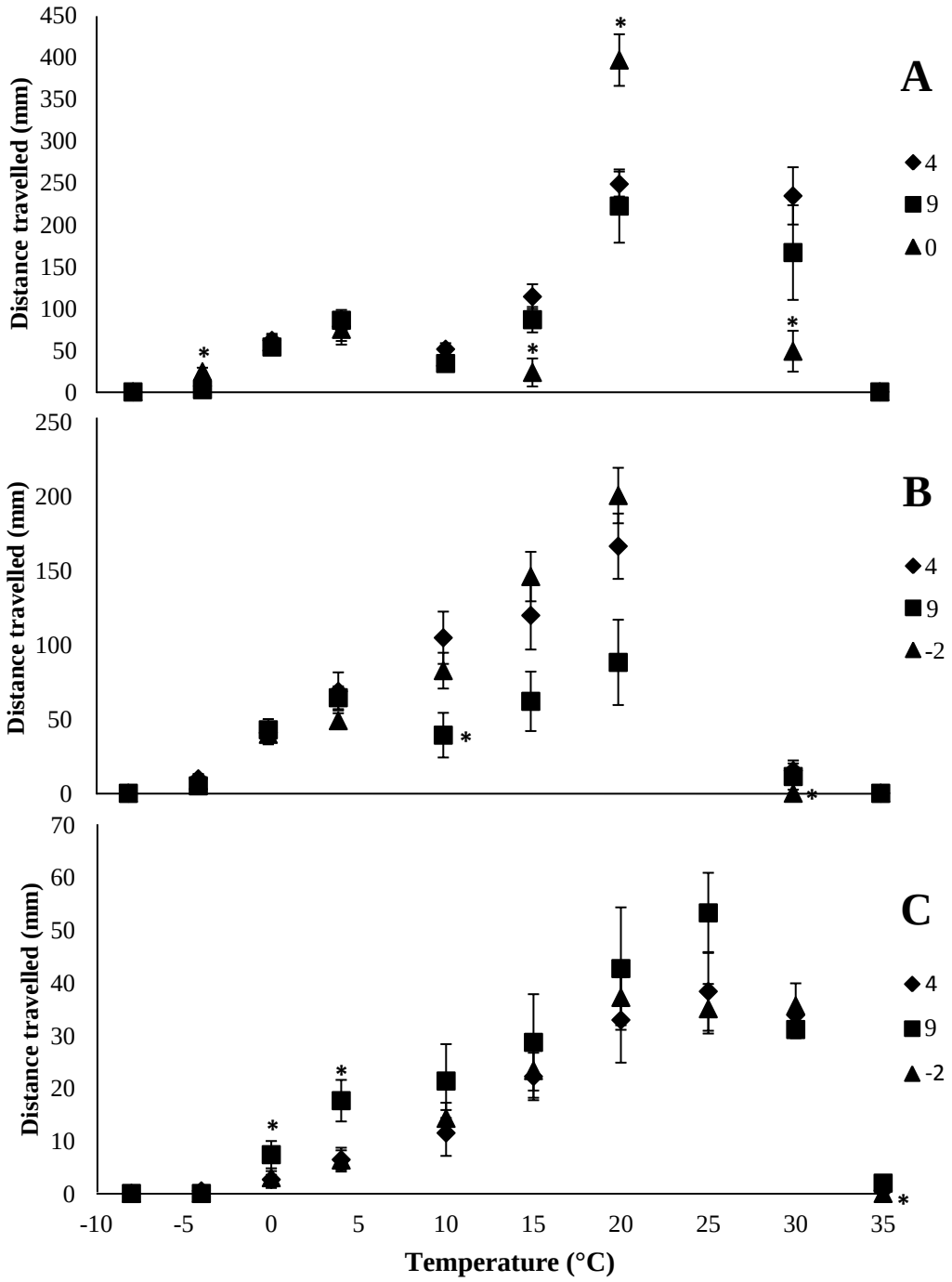

Fig. 2.3. *Locomotion analysis (distance travelled in 5 min) of M. arctica (A), C. antarcticus (B) and A. antarcticus (C), following acclimation at 4, 9, and -2°C (0°C for M. arctica). Means ± S.E.M. are presented for approximately 10 individuals. Asterisks indicate a treatment significantly different from 4°C acclimated individuals for each species at P < 0.05 (Kruskal-Wallis test; Tukey's multiple range test). Movement speeds at 25°C were not analysed for M. arctica and C. antarcticus.*

2.4.4. SCPs

2.4.4.1. Interspecific comparisons

There were no significant differences in the SCPs of the three species when maintained at +4 °C (Table 2.1, $P < 0.05$ Kruskal–Wallis test). *Alaskozetes antarcticus* was the only species to show a bimodal distribution.

2.4.4.2. Effect of acclimation

In all three species, the SCPs of individuals acclimated at −2 °C for one month, and summer acclimatised individuals of *C. antarcticus* and *A. antarcticus*, were significantly lower than those of individuals maintained at +4 °C ($P < 0.05$ Kruskal–Wallis test). Conversely, the SCP of individuals after a +9 °C acclimation period was not significantly different to those maintained at +4 °C ($P > 0.05$ Kruskal–Wallis test). Summer acclimatised individuals of *C. antarcticus* also had significantly lower SCPs than individuals acclimated at −2 °C ($P < 0.05$ Kruskal–Wallis test).

Table 2.1. *SCP of C. antarcticus, A. antarcticus and M. arctica, following acclimation at 4, 10 and -2°C, and individuals acclimatised to the Antarctic summer (C. antarcticus and A. antarcticus only). Means ± S.E.M. are presented for 32 individuals (24 for summer acclimatised individuals). Asterisks indicate a treatment significantly different from 4°C acclimated individuals for each species at P < 0.05 (Kruskal-Wallis test).*

Species	4°C	9°C	-2°C	Summer acclimatised
C. antarcticus	-6.31	-7.71	-8.9 *	-14.9 *
A. antarcticus	-7.42	-7.8	-15.9 *	-11.9 *
M. arctica	-6.13	-5.9	-8.1 *	

2.5. Discussion

2.5.1. Activity at low temperatures

Temperate and tropical invertebrates, such as the peach-potato aphid, *Myzus persicae*, the predatory mirid, *Nesidiocoris tenuis*, and the brown planthopper, *Nilaparvata lugens*, lose the ability to coordinate movement (CTmin) at temperatures above 0 °C, and more usually above +3 °C (Chidwanyika and Terblanche, 2011; Clusella-Trullas *et al.*, 2010; Hazell *et al.*, 2010; Hughes *et al.*, 2010; Nyamukondiwa and Terblanche, 2010; Piyaphongkul pers. comm.). These CTmin values are not compatible with polar summer microhabitat temperatures, which regularly fall below 0 °C and average less than +3 °C in the maritime and continental Antarctic, and only a little more in the High Arctic (Davey *et al.*, 1992; Block *et al.*, 2009; Coulson *et al.*, 1993; Strathdee and Bale, 1998). It is not surprising, therefore, that polar terrestrial invertebrates have lower thermal thresholds than their temperate and tropical counterparts, and have been observed performing activity at temperatures as low as −13.3 °C (Sinclair *et al.*, 2006), including attempts to fly at −4 °C (Hågvar, 2010). Other examples of sub-zero activity are found in high altitude environments and include the Himalayan *Diamesa* sp., which has been observed walking at −16 °C (MacMillan and Sinclair, 2010). In the current study, the CTmin and chill coma of the two Collembola, *M. arctica* and *C. antarcticus*, and the mite, *A. antarcticus*, were below −0.6 and −3.8 °C, respectively. Locomotion analysis also showed that the invertebrates walked in a coordinated manner at +4 and 0 °C, and that they were capable of movement at −4 °C, but at a reduced speed (Figs. 2.3-2.5).

In the two collembolan species, the CTmin of individuals maintained at +4 °C was low, averaging between −3.5 and −4 °C. Conversely, the CTmin of the mite only averaged −0.6 °C, even though its chill coma was similar to both Collembola (Fig. 2.1). Observation revealed that the mites tended to aggregate or stop moving early in the cooling regime and moved little thereafter. *Alaskozetes antarcticus* is well known to aggregate in the field, and has been observed aggregating in numbers of tens, hundreds and even many thousands of individuals (Richard *et al.*, 1994; Strong, 1967; Tilbrook, 1973). Block and Convey (1995) and other authors suggest that, due to the reduced surface area to volume ratio of the aggregation, this behaviour may buffer the mite against low temperatures and reduce water loss. The reason that mites may aggregate so early on during the cooling regime at temperatures near to 0 °C, rather than attempting to select for more "optimal" thermal conditions, may be a consequence of their relatively restricted mobility. Unlike Collembola, which are more capable of moving rapidly to habitats in their preferred temperature range (Figs. 2.3-2.5), restricted mobility leaves non-acclimated mites susceptible to a sudden cold exposure. Hence, it may be better for mites to select sub-lethal low temperatures and acclimate. Hayward *et al.* (2003) have demonstrated such a preference for low temperatures in *A. antarcticus* using a thermal gradient. The high CTmin value of the mite may therefore be a product of "choice" rather than an inability to coordinate movement.

2.5.2. Activity at high temperatures

Deutsch *et al.* (2008) suggested that, with increasing distance away from the equator, the thermal sensitivity of terrestrial invertebrates to a temperature rise decreases. Many studies, including that of Piyaphongkul *et al.* (2012), have shown tropical insects to

have upper lethal temperatures (ULTs) very close to the highest temperatures they experience in their natural habitat, while Everatt *et al.* (2013a), Deere *et al.* (2006), Sinclair *et al.* (2006) and Slabber *et al.* (2007) have shown the converse in polar Collembola and mites. The current study also supports the suggestion of Deutsch *et al.* (2008), and shows the CTmax of three polar species to be above 30 °C, and even as high as 34.1 °C in *A. antarcticus* (Fig. 2.2). In addition, each species exhibited their fastest movement at 25 °C (data not shown for Collembola), a temperature rarely experienced in the High Arctic or maritime Antarctic habitats typical for these species. While some polar microhabitats may already briefly exceed 30 °C (Everatt *et al.*, 2013a; Smith, 1988), these instances are rare and of very restricted physical extent. Even if such extremes become more frequent as a result of climate warming, it is unlikely that an individual invertebrate would be present in such a location, and even if so, it could quickly move to a more suitable microhabitat. Based on predicted microhabitat temperature increases of around 5 °C over the next 50–100 years (Convey *et al.*, 2009; Turner *et al.*, 2009), the heat tolerance of these polar invertebrates certainly suggests scope for them to endure future warming.

2.5.3. Thermal activity windows

While the polar terrestrial invertebrates of this study showed little sensitivity to a temperature rise, their thermal range of activity is similar to that of temperate and tropical species. The activity of *M. arctica* ranged from −4 (CTmin) to 31.7 °C (CTmax), a thermal activity window of 35.7 °C. Likewise, *C. antarcticus* and *A. antarcticus* showed activity windows of 33.6 °C and 34.7 °C, respectively. These windows of activity are comparable to the temperate aphid, *Myzus persicae*, in which

the CTmin was between 4 and 9.4 °C, and the CTmax between 39.6 and 40.7 °C, but are shifted towards lower temperatures (Alford *et al.*, 2012). Other temperate species such as the predatory mirid, *Nesidiocoris tenuis*, the mite, *Tetranychus urticae*, and moth, *Cydia pomonella*, and tropical species such as the seed harvester ant, *Messor capensis*, show somewhat broader thermal activity windows of around 40 °C or more (Chidwanyika and Terblanche, 2011; Clusella-Trullas *et al.*, 2010; Hughes *et al.*, 2010). Invertebrates native to locations slightly further north in the sub-Antarctic, such as the spiders, *Myro kerguelenensis* and *Prinerigone vegans*, also show thermal activity windows above 40 °C (Jumbam *et al.*, 2008).

2.5.4. The effect of low temperature acclimation on thermal activity thresholds

The role of acclimation on thermal activity thresholds has only been explored infrequently. Most studies have been carried out on the fruit fly, *Drosophila*, and have shown a clear relationship between the acclimation temperature and the CTmin (Hori and Kimura, 1998; Hoffmann *et al.*, 2005; Kelty and Lee, 2001; Mellanby, 1939; Rako and Hoffmann, 2006). Gibert and Huey (2001) showed that the CTmin of several *Drosophila* species decreased by 1 °C for every 4 °C drop in development temperature. This result is in line with the Beneficial Acclimation Hypothesis (BAH), which suggests that the performance of individuals is improved at temperatures close to those which they have previously experienced (Leroi *et al.*, 1994). Frazier *et al.* (2008) provided further evidence supporting the BAH in *D. melanogaster* by demonstrating greater flight performance at cool temperatures in individuals acclimated at 15 rather than 28 °C. More recent work in other invertebrates, including the cricket, *Acheta*

domesticus, the moth, *C. pomonella*, and the spiders, *M. kerguelenensis* and *P. vegans*, also support the BAH with respect to low temperature activity (Chidwanyika and Terblanche, 2011; Jumbam *et al.*, 2008 and Lachenicht *et al.*, 2010). There are exceptions, however, such as in the ant, *M. capensis*, in which individuals acclimated at an intermediate temperature performed best under the coolest conditions tested, this instead supporting the Optimal Acclimation Hypothesis (OAH = individuals acclimated at an intermediate temperature will perform better at all temperatures) (Clusella-Trullas *et al.*, 2010; Huey and Berrigan, 1996). The acclimatory ability of the three polar species examined here was in agreement with the former hypothesis, BAH. A period of one month at −2 °C lowered chill coma onset significantly in all three species, and lowered the CTmin in the two Antarctic invertebrates, compared with individuals maintained at +4 °C (Fig. 2.1). Further evidence of beneficial acclimation was seen for the CTmax and heat coma, with both showing a considerable downward shift following time at −2 °C, as well as following summer acclimatisation (averaging approximately + 1 °C) in the two Antarctic species (Fig. 2.2). While these findings are consistent with the reports in *Drosophila* and other aforementioned species, they contrast with those of Young (1979), who reported that the chill coma temperature of *A. antarcticus* was unaffected by acclimation.

An ability to depress their lower thermal thresholds of movement and hence remain active at lower temperatures would be of great benefit to polar terrestrial invertebrates. Currently, polar summers can last for as little as 1–3 months of the year (Convey, 1996). By acclimatising their thresholds of activity to lower temperatures, polar terrestrial invertebrates would be better able to forage and reproduce during the spring and autumn, as well as during cooler periods in summer.

The maximisation of activity and adaptation to the low temperature environment was also seen in relation to the SCP. When the body fluids of an invertebrate are frozen, the invertebrate is no longer considered capable of movement and the SCP is seen as the absolute limit of mobility. In many temperate and tropical species, the lower lethal thresholds, and thus also the CTmin and chill coma, are well above the SCP (Bale, 2002). However, in the current study, prior to acclimation, the chill coma temperature of all three species, and the CTmin of the two Collembola, were within 2–3 °C of the SCP (Fig 2.1; Table 2.1). Likewise, the continental Antarctic collembolan, *Isotoma klovstadi*, was observed to be capable of walking at all temperatures down to its SCP, with an average chill coma onset temperature of −11.9 to −13.3 °C over the summer season (Sinclair *et al.*, 2006). These organisms are consequently able to search for more preferable habitats as the temperature falls, and possibly perform beneficial activities, such as foraging, very near to their SCP.

2.5.5. The effect of high temperature acclimation on thermal activity thresholds

Climate warming has resulted in a significant rise in polar temperatures, and will undoubtedly lead to future increases (Arctic Council, 2005; Convey *et al.*, 2009; Turner *et al.*, 2009). An advantage may therefore be gained by being able to acclimate to higher temperatures. However, the species examined here showed no acclimation ability allowing an increase in their upper activity thresholds following a two week period at 9 °C, and even showed a decline in both their CTmax and heat coma (Fig. 2.2). Everatt *et al.* (2013a) and Slabber *et al.* (2007) also found that acclimation to higher temperatures (9 and 15 °C, respectively) either resulted in no change in, or impaired,

survival at temperatures above 30 °C in both Collembola and Acari. Further, a number of studies have shown little plasticity in upper thermal tolerance traits in non-polar species, including in the cricket, *A. domesticus*, the fruit fly, *D. melanogaster*, dung beetles, and the tsetse fly, *Glossina pallidipes* (Gaston and Chown, 1999; Goto *et al.*, 2000; Hoffmann *et al.*, 2005; Lachenicht *et al.*, 2010; Terblanche *et al.*, 2011). There is now a general consensus that thermal tolerance shows less phenotypic plasticity at higher temperatures than at lower temperatures in invertebrates, and that this may be due to each involving a distinct suite of physiological and molecular mechanisms (Bowler and Terblanche, 2008). Even though the polar species of this study show a limited ability to acclimate their upper thermal thresholds to higher temperatures, the upper thermal tolerance they already possess (see Section 2.4.2.) gives these invertebrates sufficient capacity to cope with future climate warming.

Intriguingly, a subtle difference may exist between the locomotion speeds of the mite and the Collembola. In *A. antarcticus*, movement was greater between 0 and 25 °C in individuals which had received a 2 week acclimation at 9 °C, as compared to individuals reared at 4 °C. While in the Collembola, movement was impaired between 0 and 20 °C by the same acclimation treatment. *Alaskozetes antarcticus* is already known to have a greater capacity to survive higher temperatures than the Collembola (Everatt *et al.*, 2013a). It is therefore plausible that *A. antarcticus* is able to benefit physiologically from a period at 9 °C, while the Collembola may find the temperature damaging.

It should be noted that, while no acclimation response was exhibited for the CTmax and heat coma following two weeks at 9 °C, acclimation did occur in both −2 and +4 °C reared individuals, with all species showing significantly higher CTmax and heat coma

temperatures under +4 vs −2 °C treatments (Fig. 2.2). The ability to acclimate in response to these two temperature regimes perhaps illustrates the process of natural acclimatisation between winter and summer conditions. However, as the upper thresholds of activity in −2 °C acclimated individuals are already above the highest summer temperatures they experience, the observed change may simply reflect the acclimation of their lower activity thresholds, which are lowered following one month at −2 °C (Fig. 2.1). This further supports the consensus highlighted above, that greater plasticity is shown at lower temperatures but not at higher temperatures. Physiological changes that improve activity at low temperatures, such as increased membrane fluidity and subsequent improvement in the function of neurotransmitters, ATPases and ion channels (MacMillan and Sinclair, 2010), are likely to be to the detriment of higher temperature activity.

2.6. Conclusion

The current study has expanded on previous studies to show that the polar mite, *A. antarcticus*, and Collembola, *C. antarcticus* and *M. arctica*, are capable of sub-zero activity. These invertebrates also show plasticity in their CTmin and chill coma temperature following acclimation at lower temperatures, as well as being capable of activity at temperatures close to their SCPs. By depressing their lower thermal activity thresholds as temperature falls, these invertebrates are able to maximise the short growing season. At higher temperatures, these species are able to remain active above 30 °C, a temperature far higher than is experienced in their Antarctic or Arctic habitats. This indicates polar terrestrial invertebrates have a thermal activity window comparable to that of temperate and tropical insects and, in spite of their limited physiological

plasticity at higher temperatures, have thermal scope to tolerate future rises in temperature under climate change.

Chapter transition

In the following Chapter, acclimation to temperature is also assessed in polar terrestrial invertebrates, but is looked at over shorter timescales of hours and minutes (rapid cold hardening), and is investigated in an alien species – the midge, *E. murphyi*.

CHAPTER 3: PRE-ADAPTED TO THE MARITIME ANTARCTIC? – RAPID COLD HARDENING OF THE MIDGE, *ERETMOPTERA MURPHYI*

The work presented in this chapter has been published in the Journal of Insect Physiology (Everatt, M. J., Worland, M. R., Bale, J. S., Convey, P. and Hayward, S. A. L. (2012) Pre-adapted to the maritime Antarctic? – Rapid cold hardening of the midge, *Eretmoptera murphyi*. Journal of Insect Physiology. 58, 1104-1111)

3.1. Abstract

During the 1960s, the midge, *Eretmoptera murphyi*, was transferred from sub-Antarctic South Georgia (55^{o}S 37^{o}W) where it is endemic to a single location on maritime Antarctic Signy Island (60^{o}S 45^{o}W). Its distribution has since expanded considerably, suggesting that it is pre-adapted to the more severe conditions further south. To test one aspect of the level of its pre-adaptation, the rapid cold hardening (RCH) response in this species was investigated. When juvenile (L1–L2) and mature (L3–L4) larvae of *E. murphyi* were directly exposed to progressively lower temperatures for 8 h, they exhibited Discriminating Temperatures (DTemp, temperature at which there is 10–20% survival of exposed individuals) of −11.5 and −12.5°C, respectively. The mean SCP was above −7.5°C in both larval groups, confirming the finding of previous studies that this species is freeze-tolerant. Following gradual cooling (0.2°C min^{-1}), survival was significantly greater at the DTemp in both larval groups. The response was strong, lowering the lower lethal temperature (LLT) by up to 6.5°C and maintaining survival

above 80% for at least 22 h at the DTemp. RCH was also exhibited during the cooling phase of an ecologically relevant thermoperiodic cycle (+4°C to −3°C). Mechanistically, the response did not affect freezing, with no alteration in the supercooling point (SCP) found following gradual cooling, and was not induced while the organism was in a frozen state. These results are discussed in light of *E. murphyi*'s pre-adaptation to conditions on Signy Island and its potential to colonize regions further south in the maritime Antarctic.

3.2. Introduction

Over the last 200 years, human presence in the Antarctic has risen as a result of seal and whale hunting, scientific research and, more recently, tourism (Tin *et al.*, 2009; Chwedorzewska, 2009). Humans, via their cargo, vehicles and themselves, are a carrier of organisms (Hughes *et al.*, 2005; Hughes *et al.*, 2010). Consequently, species have been able to bypass geographical and environmental barriers and colonize the Antarctic at an increasing rate (Frenot *et al.*, 2005). Global warming trends are now also aiding this process. By raising the average temperature of parts of the Antarctic by at least 2.5°C in the last century (Convey *et al.*, 2009), warming has opened up areas which were previously too stressful for the organisms being transferred (Chwedorzewska, 2009; Frenot *et al.*, 2005). However, in the maritime and continental Antarctic, instances of establishment of alien (or introduced) species remain limited (Hughes and Convey, 2012), best explained by the severity and isolation of their habitats eclipsing the alleviation of recent warming. Thus, if an organism is to colonize, establish and spread in the maritime or continental Antarctic, it must first possess the requisite physiology (i.e. appropriate "pre-adaptation").

The freeze-tolerant midge, *Eretmoptera murphyi* (Diptera, Chironomidae), may be one such organism. As a likely result of plant transplant experiments in the 1960s, it was introduced onto Signy Island in the maritime Antarctic (60°S 45°W) from the sub-Antarctic island of South Georgia (55°S 37°W) (Block *et al.*, 1984; Convey and Block, 1996). The species has since spread widely and now covers an area >2000 m^2, with densities as high as 142 000 ind. m^{-2} (Worland and Hughes, 2010). This is particularly striking when considering the environmental differences between Signy Island and South Georgia. While South Georgia has a yearly average soil temperature of +1.8°C and winter values that rarely fall below −2°C (Heilbronn and Walton, 1984), temperatures below −10°C on Signy Island are not uncommon and the average is approximately 4.5°C lower than on South Georgia (Davey *et al.*, 1992).

This fly spends the majority of its biennial life cycle as a larva, with the non-feeding adults only emerging and being active for a short period in mid-summer on Signy Island (Convey and Block, 1996). The larvae are therefore exposed to the full range of environmental conditions on the island over the annual cycle. To determine the pre-adaptive capacity of *E. murphyi*, Worland (2010) examined the level of freeze-tolerance and long-term acclimatory ability of larvae. Prior to acclimation, larvae exhibited moderate freeze-tolerance, with an $LTemp_{50}$ of −13.19°C, ~7 °C lower than their SCP (−5.75 to −6.15°C). Following 12 d at −4°C, their $LTemp_{50}$ decreased to below −20°C. Such an increase in cold tolerance would allow larvae to survive temperature conditions at the soil surface on Signy Island at any time throughout the year. However, their capacity to survive over short time-scales while in an un-acclimated state, including their ability to rapidly cold harden, is unknown.

Rapid cold hardening (RCH) is defined as the rapid induction (minutes to hours) of tolerance to otherwise harmful low temperatures (Lee *et al.*, 2006b; Yi *et al.*, 2007). It was first described in the flesh fly, *Sarcophaga crassipalpis*, by Lee *et al.* (1987), and has since been observed in a wide range of organisms, including polar invertebrates such as the collembolan, *Cryptopygus antarcticus*, the mites, *Alaskozetes antarcticus* and *Halozetes belgicae* (Worland and Convey, 2001; Hawes *et al.*, 2007), and the midge, *Belgica antarctica* (Lee *et al.*, 2006b). The presence of RCH in Antarctic invertebrates is perhaps unsurprising given that it allows organisms to adjust rapidly to sharp changes in environmental temperatures, particularly those near to ecological and physiological thresholds, which are a hallmark of the Antarctic climate (Convey, 1997).

Although the ecological role of RCH is well established, relatively little is known about the mechanisms underlying the response. It was originally thought to involve cryoprotectants, such as glycerol, alanine and glutamine (Chen *et al.*, 1987), but, as increasing numbers of species were found to possess the response in the absence of these compounds (e.g. Kelty and Lee, 1999; Lee *et al.*, 2006b), the suggestion of cryoprotectants playing a universal role was abandoned. Now, RCH is thought to be involved more with protection against cold induced apoptosis, as shown in *Drosophila melanogaster* and *S. crassipalpis* (Yi *et al.*, 2007; Yi and Lee, 2011), and with maintenance of membrane fluidity, as shown in *B. antarctica* (Lee *et al.*, 2006a; Teets *et al.*, 2008). RCH therefore seems, in the limited number of organisms studied, to ameliorate chilling injury as opposed to freezing damage.

In the current study, we investigated the strength of the RCH response in *E. murphyi* and its relevance in the context of the maritime Antarctic climate, and examined

whether RCH has any effect on the whole body freezing temperature, commonly known as the supercooling point (SCP).

3.3. Materials and methods

3.3.1. Insect collection and storage conditions

Summer acclimatized larvae of *E. murphyi* were collected from soil and moss on Signy Island (60°S 45°W) near to the British Antarctic Survey Signy Research Station between January and March 2011. They were transported to the University of Birmingham under cool conditions (+4°C) and subsequently held in plastic boxes containing substratum from the site of collection at +4°C (0:24 L:D). For comparative purposes, experiments tested both juvenile larvae (L1 and L2 stages) and mature larvae (L3 and L4). These two groups were separated on the basis of size and colouration (Cranston, 1985). However, due to the limited number of juveniles, only mature larvae were used in the following experiments – 3.3.4 (ii), 3.3.5 and 3.3.7.

3.3.2. Determination of the Discriminating Temperature (DTemp)

The temperature at which 10–20% survival occurs (DTemp, Lee *et al.*, 1987) was determined by exposing larvae (3 × 10 replicates) to progressively lower sub-zero temperatures (−9 to −14°C) for 8 h, before being re-warmed to the rearing temperature (+4°C) at 0.2°C min^{-1}. Larvae were re-warmed from sub-zero temperatures to the rearing temperature at 0.2°C min^{-1}, as preliminary trials suggested that larvae experienced greater mortality if directly transferred (data not shown). Three replicates of 10 individuals were placed in Eppendorf tubes, inside glass test tubes plugged with sponge, in an alcohol bath (Haake Phoenix II C50P, Thermo Electron Corporation),

prior to each experimental treatment. Control groups were handled, and exposed, in the same way at +4°C. The temperature experienced by the larvae was measured by placing a thermocouple within an identical Eppendorf tube into one of the glass test tubes. At the end of experimental treatments, the larvae were rapidly transferred (over ice) from the Eppendorf tubes into plastic recovery capsules containing substratum and returned to the rearing conditions (+4°C, 0:24 L:D). Survival, defined by individuals moving either spontaneously or in response to gentle contact stimulus, was assessed 24 and 72 h after treatment. The highest temperature at which survival was between 10 and 20% after 72 h recovery was defined as the DTemp. Replicate collection, controls, thermocouple use, recovery and survival assessment were the same for all following experimental procedures unless stated otherwise.

3.3.3. Induction of RCH

In order to detect an RCH response, larvae (3 × 10 replicates) were subjected to the following treatments:

1) 1 h at 0 or −5°C, before being transferred to the DTemp for 8 h and then re-warmed to +4°C at 0.2°C min^{-1}.

2) Gradual cooling to the DTemp at 0.2°C min^{-1}, before being held for 8 h, and then re-warmed to + 4°C at 0.2°C min^{-1}.

3.3.4. Limits of the RCH response

The limits of RCH were determined by transferring larvae (3 × 10 replicates), via gradual cooling (0.2°C min^{-1}), to (i) progressively lower sub-zero temperatures (−12.5 to −19.5°C) below the DTemp for 8 h, before re-warming to +4°C at 0.2°C min^{-1}, and

(ii) progressively longer periods (10–48 h) at the DTemp, before re-warming to +4°C at 0.2°C min^{-1}.

3.3.5. Detection of RCH under a thermoperiodic cycle

Soil temperature data available from previous seasons at Signy Island and Anchorage Island (67°S 68°W) were used as a basis to establish two thermoperiods; one that *E. murphyi* currently experiences in summer on Signy Island, and one that might be experienced in summer on Anchorage Island. This was undertaken to assess the ability of *E. murphyi* larvae to survive at a more extreme, higher latitude, location. Using these models, an alcohol bath was programmed to cycle between +6 and −1°C, and between +4 and −3°C, representing Signy and Anchorage Islands respectively, over a 24 h period (Fig. 3.1). Larvae were transferred to each thermoperiod (beginning at 4°C). Three replicates of 10 individuals were removed at two points in the cycle (−1 and 6°C [Signy Island model] and −3 and 4°C [Anchorage Island model]) each day for 3 days during each thermoperiodic cycle and directly transferred to the DTemp for 8 h, before being re-warmed to +4°C at 0.2°C min^{-1}.

3.3.6. Effect of RCH on the supercooling point (SCP)

To determine the effect of RCH on the SCP, juvenile and mature larvae were cooled from +4 to −30°C at either 0.2°C min^{-1} (RCH treatment) or 1°C min^{-1} (mature larvae only). Controls were directly transferred to the DTemp. Juvenile and mature larvae (8 and 24 individuals) were placed in contact with a thermocouple, within Beem capsules, in glass test tubes plugged with sponge, inside an alcohol bath, prior to each cooling regime. SCPs, defined as the temperature at the onset of the freezing exotherm, were

identified using an eight channel datalogger interfaced to a computer and recorded using PicoLog Recorder Software (Pico Technology Limited, UK) (cf. Hawes *et al.*, 2006).

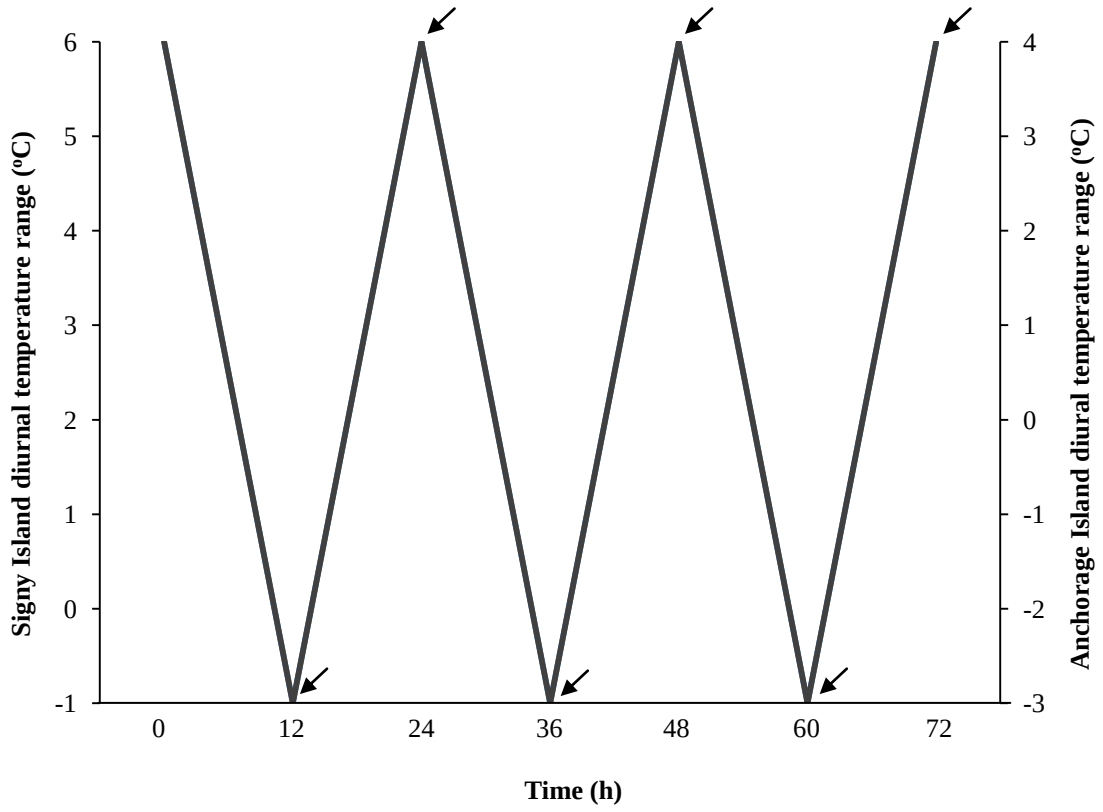

Fig. 3.1. *Three day simulated thermoperiodic cycle for Signy (between 6 and -1°C) and Anchorage (4and -3°C) Island. Arrows indicate the points at which 3 replicates of 10 mature larvae were removed from each thermoperiodic cycle and transferred directly to the DTemp (-12.5°C).*

3.3.7. Induction of RCH in a frozen organism

The time at which all mature larvae froze at −7°C, having been cooled at 1°C min^{-1} from +4°C, was calculated as 4 min using PicoLog Recorder Software (Pico Technology Limited, UK). Three groups of 10 mature larvae were subsequently cooled from +4 to −7°C at 1°C min^{-1}, held for 4 min or 1 h 4 min, and transferred to the DTemp for 8 h, before being re-warmed to +4°C at 0.2°C min^{-1}. Survival was assessed 24 and 72 h after each treatment.

3.3.8. Statistical analyses

The Kolmogorov–Smirnov test was used to confirm that all percentage survival and SCP data were normally distributed. The data were subsequently analyzed using analysis of variance (ANOVA) and Tukey's multiple range test.

3.4. Results

3.4.1. Determination of the DTemp

The mean survival of both juvenile and mature larvae decreased significantly following exposure to progressively lower sub-zero temperatures for 8 h (Fig. 3.2; $P < 0.05$ Tukey's multiple range test), declining from more than 80% at −9°C to 0% at −14°C. Juvenile larvae appeared more susceptible to sub-zero temperatures, showing lower survival at all temperatures tested, though the difference with mature larvae was not significant ($P > 0.05$ Tukey's multiple range test). Based on these data, −11.5 and −12.5 °C were designated as the DTemps for juvenile and mature larvae, respectively.

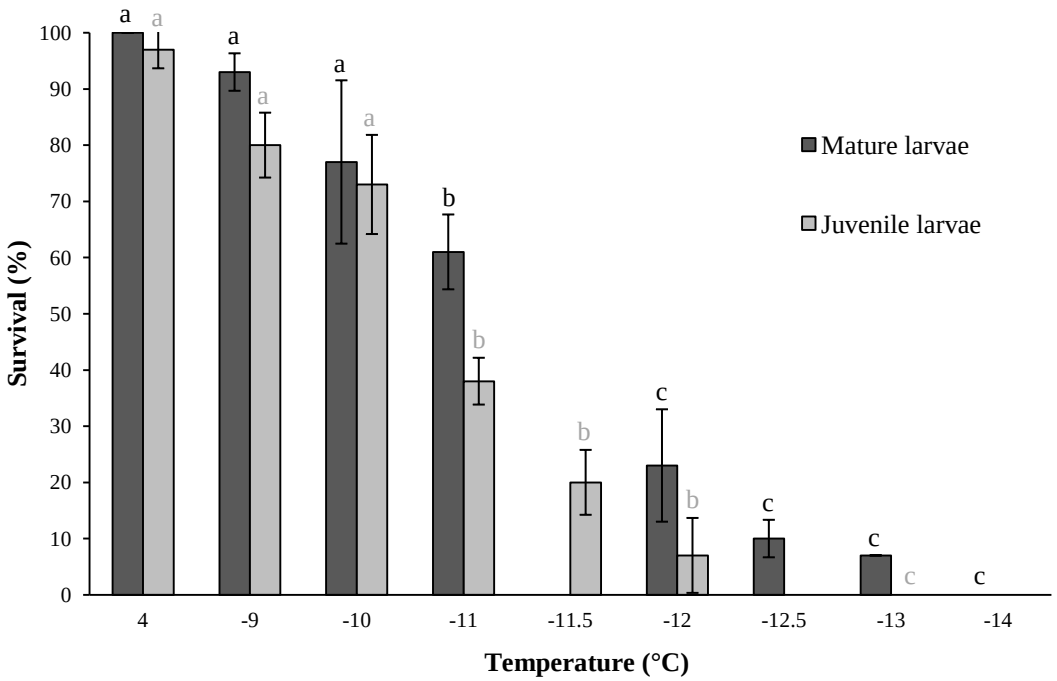

Fig. 3.2. *Survival of juvenile and mature larvae after exposure to progressively lower sub-zero temperatures (-9 to -14°C) for 8 h, before re-warming at 0.2°C min^{-1} to the rearing temperature (+4°C). Temperatures of -11.5 and -12.5°C were assessed for only juvenile and mature larvae, respectively, in order to attain a DTemp with between 10 and 20% survival. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each larval group at P < 0.05 (Tukey's multiple range test).*

3.4.2. Induction of RCH

Survival of larvae exposed to the DTemp for 8 h increased following prior acclimation to −5°C for 1 h, and gradual cooling (+4°C to the DTemp at 0.2°C min^{-1}), but not after acclimation for 1 h at 0°C (Fig. 3.3). The highest survival was seen after gradual cooling for both juvenile (74%) and mature (83%) larvae. This was significantly different from their survival after direct transfer to the DTemp ($P < 0.05$ Tukey's multiple range test). Under all treatments, the strength of the RCH response was not significantly different between juvenile and mature larvae ($P > 0.05$ Tukey's multiple range test).

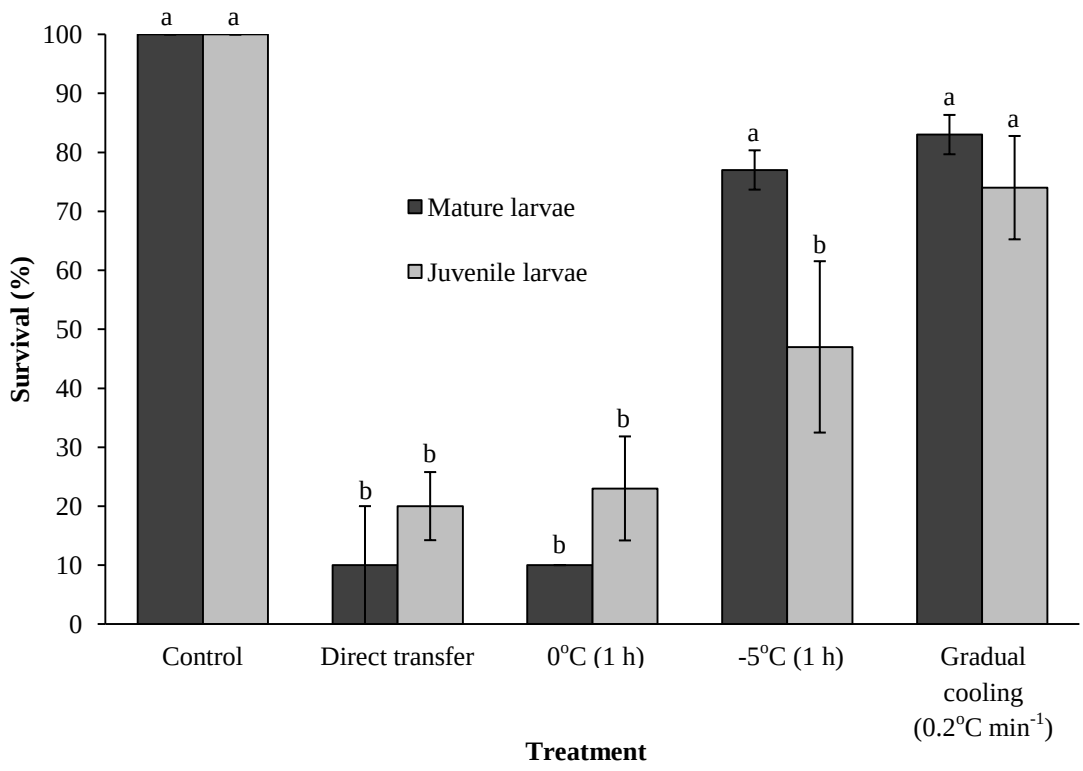

Fig. 3.3. *Survival of juvenile and mature larvae after exposure to the DTemp for 8 h (-11.5 and -12.5°C, respectively), following either direct transfer to the DTemp or 3 pre-treatments: 1 h at 0°C, 1 h at -5°C and gradual cooling (0.2°C min^{-1}) from +4°C to the DTemp. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed after 72 h. Means with the same letter are not significantly different within each larval group at P < 0.05 (Tukey's multiple range test).*

3.4.3. Limits of RCH

RCH lowered the lower lethal temperature (LLT) by 2.5 and 6.5°C in mature and juvenile larvae, respectively (Fig. 3.4). Survival ≥ 80% at the DTemp (−12.5 °C) was also extended by at least 14 h in mature larvae following RCH and some individuals even survived 48 h under the same treatment (Fig. 3.5).

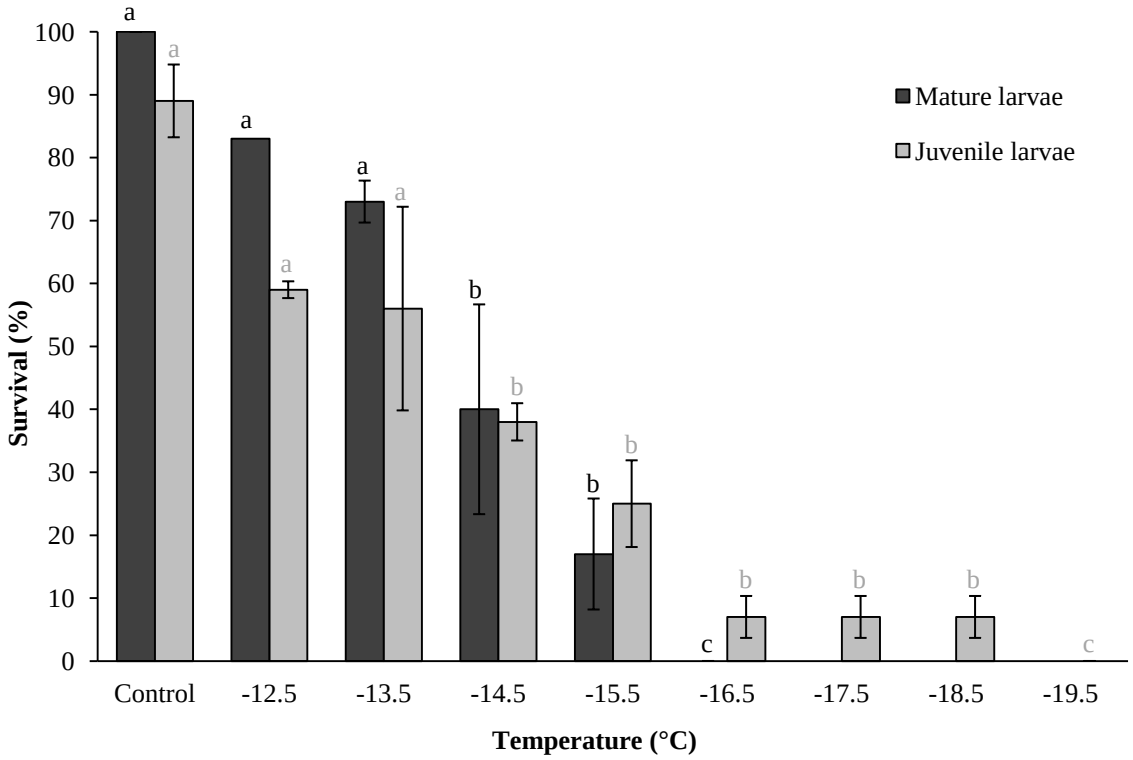

Fig. 3.4. *Survival of juvenile and mature larvae following gradual cooling (0.2°C min^{-1}) from the rearing temperature (+4°C) to progressively lower temperatures below the DTemp (-11.5 to -19.5°C) for 8 h at these temperatures. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each larval group at P < 0.05 (Tukey's multiple range test).*

3.4.4. RCH during a thermoperiodic cycle

Mature larvae acclimated to a model Signy Island thermoperiod (+6 to −1°C over a 24 h cycle) exhibited increased survival of the DTemp for 8 h (Fig. 3.6). However, this was not significant ($P > 0.05$ Tukey's multiple range test). Survival was also not significantly different within or between −1 and +6°C conditioned groups across all 3 days tested ($P > 0.05$ Tukey's multiple range test). In contrast, mature larvae acclimated to a model Anchorage Island thermoperiod (+4 to −3°C over a 24 h cycle) showed significantly higher survival of the DTemp for 8 h following removal at −3°C after 2 d ($F_{1,4} = 8.915$, $P < 0.05$) and 3 d ($F_{1,4} = 9.291$, $P < 0.05$) (Fig. 3.7). There was a significant decline in cold tolerance during the warming phase at +4°C on day 2, but

cold tolerance was regained during the subsequent cooling phase on day 3 (Fig 3.7) The tolerance accrued over 3 d was maintained during the day 3 warming phase, with significantly higher survival exhibited at the DTemp when larvae were removed at 4°C on day 3 ($F_{1,4}$ = 11.560, P < 0.05).

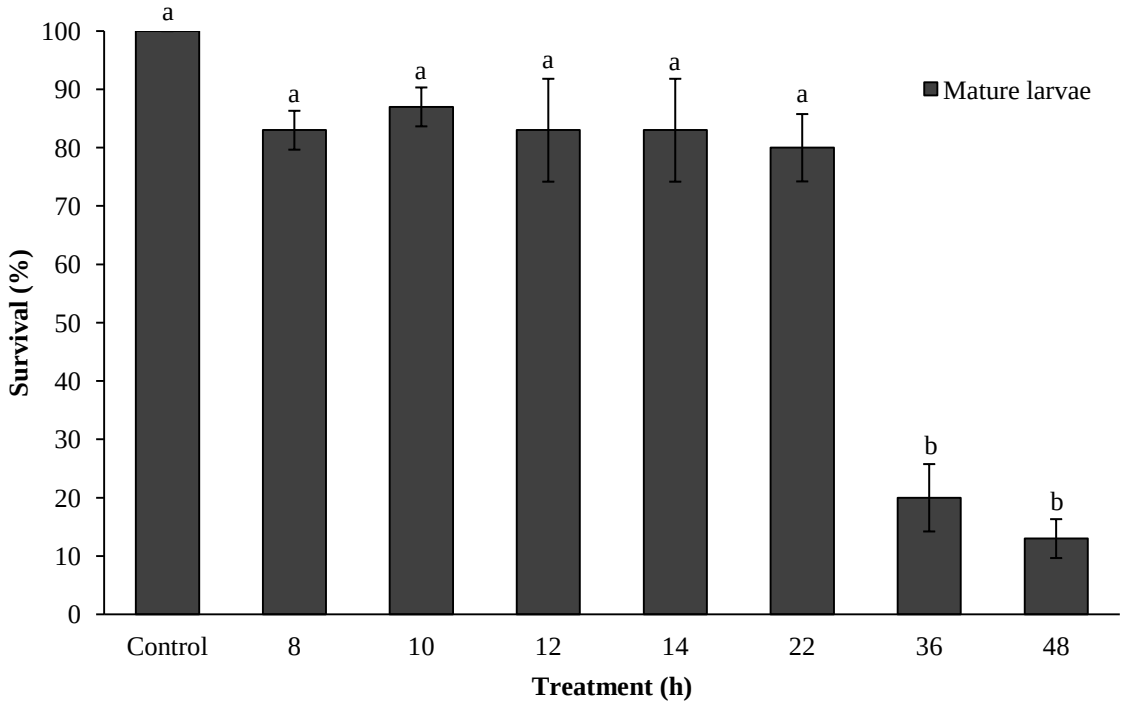

Fig. 3.5. *Survival of mature larvae after exposure to the DTemp for extended periods of time (8-48 h), following gradual cooling (0.2°C min^{-1}) from the rearing temperature (+4°C) to the DTemp. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each larval group at P < 0.05 (Tukey's multiple range test).*

3.4.5. Effect of RCH on the SCP

The mean SCP of mature larvae following RCH (0.2°C min^{-1}) was −5.54°C. While slightly lower, this was not significantly different to the mean SCP of larvae cooled at 1 °C min^{-1} (−5.07°C) and larvae directly transferred to the DTemp (−5.73°C) (Table 3.1, P > 0.05 Tukey's multiple range test). Juvenile larvae cooled at 0.2°C min^{-1} (SCP: −7.29°C) also showed no significant difference in their SCP when compared with those

directly transferred to the DTemp (SCP: −5.86°C) (Table 1, $P > 0.05$ Tukey's multiple range test).

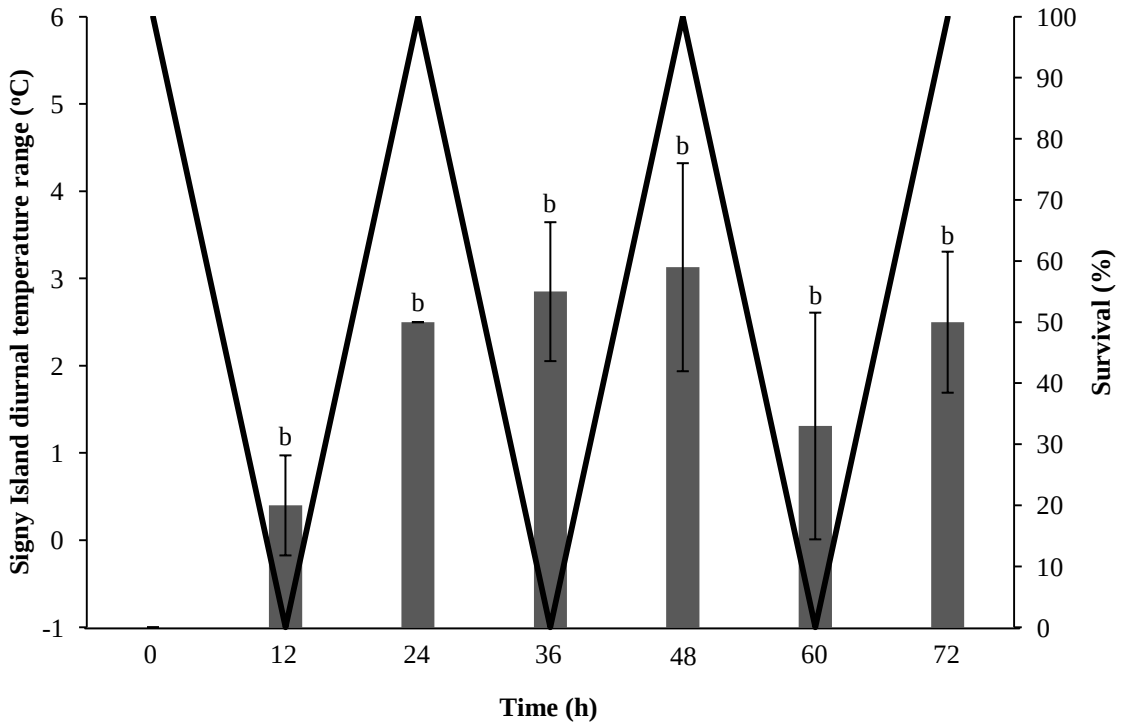

Fig. 3.6. *Survival of mature larvae (bars) after direct transfer to the DTemp (-12.5°C) or exposure to the DTemp at specific points (-1 and 6°C after 1, 2 and 3 d) during a thermoperiodic cycle. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each larval group at P < 0.05 (Tukey's multiple range test). The line denotes the simulated thermoperiodic cycle for Signy Island.*

3.4.6. Induction of RCH in a frozen organism

The difference in survival between mature larvae that were held frozen at −7°C for 4 min (20% survival) or frozen for 1 h 4 min (13% survival) was not statistically significant ($F_{1,4} = 0.308$, $P > 0.05$), indicating that RCH was not induced after the organisms froze.

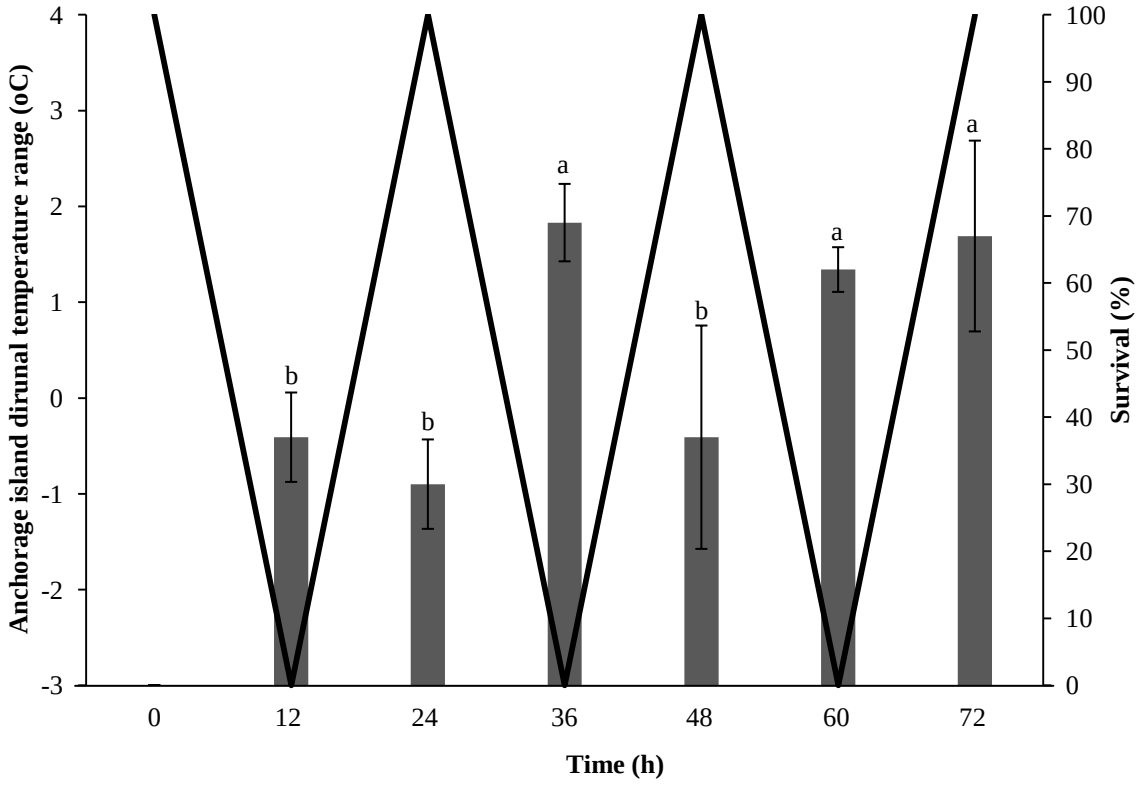

Fig. 3.7. *Survival of mature larvae (bars) after direct transfer to the DTemp (-12.5°C) or exposure to the DTemp at specific points (-3 and 4°C after 1, 2 and 3 d) during a thermoperiodic cycle. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each larval group at P < 0.05 (Tukey's multiple range test). The line denotes the simulated thermoperiodic cycle for Anchorage Island.*

Table 3.1. *SCP of mature larvae following cooling at 0.2°C min^{-1} (RCH) and 1°C (non-RCH) from 4 to -30°C, and following direct transfer to the DTemp (-12.5°C). Mean ± S.E.M. are presented for 8 and 24 replicates of single individuals (juvenile and mature larvae, respectively). Survival was assessed 72 h after treatment.*

	SCP (^{o}C) ± S.E.M.
0.2°C min^{-1} (mature larvae)	-5.42 ± 0.51
0.2°C min^{-1} (juvenile larvae)	-7.29 ± 0.93
1°C min^{-1} (mature larvae)	-5.00 ± 0.48
Direct transfer (-12.5°C, mature larvae)	-5.60 ± 0.60
Direct transfer (-11.5°C, juvenile larvae)	-5.86 ± 0.33

3.5. Discussion

As human activity increases and global warming intensifies, maritime Antarctic areas, which were previously inaccessible, are opening up for species originating from less extreme environments further north. This applies both to organisms not previously present anywhere in the Antarctic region, and to those whose occurrence or southern distributional limit already lie within the region. However, because of the severity of Antarctic terrestrial ecosystems, if organisms are to become established beyond their current range, they require tolerance physiology beyond that which is necessary in their native climate. Such organisms are said to be "pre-adapted".

There have been eight known establishment events in the maritime Antarctic to date (Hughes and Convey, 2012). These include the Collembola, *Folsomia candida* and *Protaphorura* sp., on Deception Island, the transfer of the collembolan, *Hypogastrura viatica*, onto the South Shetland and Léonie Islands, and the introduction of the enchytraeid worm, *Christensenidrilus blocki*, and the chironomid, *E. murphyi*, on Signy Island. Further species of Collembola have recently been recorded from Deception Island (Greenslade *et al.*, in review). As with the non-native species (>200) known from the sub-Antarctic islands, these organisms may have significant impacts on the native ecosystems (Frenot *et al.*, 2005). *H. viatica* is described as an aggressive invader on South Georgia and Macquarie Islands (Frenot *et al.*, 2005; Tin *et al.*, 2009). Likewise, *E. murphyi* has been shown by Hughes *et al.* (2013) as potentially contributing more to nutrient cycling on Signy Island than by that of all the native invertebrates combined. It is therefore important to gain an insight into the pre-adaptation of such organisms if a

full understanding of their establishment and impact, as well as the potential establishment and impact of other organisms, is to be realized.

3.5.1. Basal cold tolerance

Although this study centres on the RCH response of *E. murphyi*, the data obtained also confirm that both juvenile and mature larvae possess a marked basal cold tolerance (Worland, 2010). In both larval groups, the DTemp and the LLT fell below −11.5 and −13°C, respectively. This, in itself, is a good example of their pre-adaptation, as temperatures rarely, if ever, reach −10°C in summer (Davey *et al.*, 1992). Similarly, summer acclimatised larvae of the only other flightless midge of the maritime Antarctic, *B. antarctica*, showed 95% survival after 24 h at −10°C, a temperature lower than that which they experience in summer at Palmer Station (64°S 46°W) (Teets *et al.*, 2008).

Our data also indicated a subtle difference in cold tolerance between juvenile and mature larvae. Juveniles were more susceptible at all sub-zero temperatures tested, resulting in an LLT 1°C higher than that of mature larvae, which survived until −14°C. Possible explanations include a developmental effect as seen in tardigrades (Hengherr *et al.*, 2010) and the presence of a possible (though undescribed) diapause (or stress tolerant stage) in mature larvae prior to pupation (Bale and Hayward, 2010).

3.5.2. RCH in E. murphyi

Having been pre-treated at −5°C for 1 h, mature larvae exhibited a 67% increase in survival compared with those directly transferred to the DTemp, making *E. murphyi* just the second freeze-tolerant organism, alongside *B. antarctica* (Lee *et al.*, 2006b), to demonstrate an RCH response. Similar survivorship was not shown after a 0°C pre-

treatment, unlike many temperate species, such as the grain aphid, *Sitobion avenae* (Powell and Bale, 2004; Powell and Bale, 2005; Powell and Bale, 2006), *S. crassipalpis* (Lee *et al.*, 1987) and the western flower thrips, *Frankliniella occidentalis* (McDonald *et al.*, 1997). This is likely to be explained by the fact that 0, as compared to −5°C, is perhaps a poor indicator of ensuing stressful conditions in the Antarctic environment (Worland and Convey, 2001; Davey *et al.*, 1992).

While 1 h direct transfer to −5°C induced RCH, such a sharp decrease in temperature is unlikely to be ecologically relevant (Bale, 2002). It was therefore important to test for RCH following gradual cooling (0.2°C min^{-1}). The data thereby obtained ultimately proved analogous to the −5°C pre-treatment, with significantly higher survival shown in mature and juvenile larvae than when each group was directly exposed to the DTemp (Fig. 3.3). Such a response is supported by studies in a range of other organisms, including the fruit fly, *Drosophila melanogaster* (Kelty and Lee, 1999), *F. occidentalis* (McDonald *et al.*, 1997) and the migratory locust, *Locusta migratoria* (Wang and Kang, 2003).

To test the ecological relevance of the response further, mature larvae were assessed for RCH during an experimental imitation of naturally occurring thermoperiodic cycles on Signy (between + 6 to −1°C) and Anchorage (between + 4 and −3°C) Islands. For mature larvae exposed to the cooling regime of the Signy Island thermoperiod, survival was raised, but not significantly. This is likely to be because −1°C, the temperature at which larvae were removed from the cycle, was not sufficiently low to induce a strong RCH response. A lower subzero induction temperature for the RCH response in *E. murphyi* is supported by the survival of mature larvae following exposure to the Anchorage Island thermoperiod (Fig. 3.7). Following 2 and 3 d exposures to this

thermoperiod, larvae removed at −3°C exhibited RCH, indicating that the response can occur under diurnal cycles, as long as temperatures are sufficiently low. Cold tolerance was also assessed during the warming phase of the thermoperiod to discern whether the protection afforded during the cooling phase is maintained at higher temperatures (cf. Kelty and Lee, 2001). While cold tolerance was not retained during the warming phase of day 2 in the cycle, significantly greater survival (at the DTemp) was retained during the warming phase (+4°C) of day 3 (Fig. 3.7). This suggests that cold tolerance strategies are sustained even during warmer diurnal periods if successive subzero "night-time" conditions are encountered.

Further to exploring the induction of RCH under gradual cooling and model thermoperiodic cycle regimes, the limits of RCH were investigated. In juvenile and mature larvae, the LLT was lowered by 6.5 and 2.5°C, respectively, and in mature larvae alone, survival above 80% was exhibited even after 22 h at the DTemp (−12.5°C). It is therefore evident that the larvae of *E. murphyi* possess a very strong RCH response. This is in contrast to most other species, in which survival is extended for, at most, 10 h at the DTemp and to temperatures just 2–3°C below it (Bale, 2002). For example, RCH in the mite, *Euseius finlandicus*, lengthened the $LTime_{50}$ by only 1 h 15 min (Broufas and Koveos, 2001), whilst in *L. migratoria*, the change was similarly small, increasing the $LTime_{50}$ by just 2 h and reducing the $LTemp_{50}$ from −10 to −12°C (Wang and Kang, 2003).

3.5.3. Thresholds of RCH in a freeze-tolerant organism

While our data principally provide evidence of the occurrence and strength of RCH in *E. murphyi*, they also indicate the thresholds which govern the response. The first is

temperature. In mature larvae, RCH was not induced at 0°C (Fig. 3.3), and only slightly at −1°C (Fig. 6), while a much stronger response was induced at −3 (Fig. 3.7) and −5°C (Fig. 3.3). An even lower induction temperature was required by juvenile larvae, which failed to respond after a 0 or a −5°C pre-treatment (Fig. 3.3). It makes sense for the induction temperature of RCH in *E. murphyi* to be below 0°C, and therefore lower than that found in temperate species, as otherwise it would be continually induced in the Antarctic terrestrial environments, which would be energetically costly.

The second threshold is time. In mature larvae pre-treated at −5°C for 10 min (data not shown), survival was significantly lower than in those pre-treated at −5°C for 1 h. This is a clear indication that time is required for the protection afforded by RCH to increase (cf. Powell and Bale, 2004). The absence of a response after 1 d at −3°C, but presence after the following 2 days at this temperature also supports this hypothesis (Fig. 3.7).

The third and final threshold is freezing. It was already known from the Anchorage Island thermoperiod data that RCH was induced at −3°C, which is above the SCP of mature larvae, and is thus not dependent on the freezing event itself ("freeze-induced hardening"), but it was not known if RCH could be induced in a frozen organism. When the survival of mature larvae at the DTemp was compared between those just frozen and those an hour after freezing at −7°C, there was no significant difference between the two treatments. These data suggest that freezing defines the absolute limit of RCH accruement in *E. murphyi*. This is in contrast to a study by Teets *et al.* (2008), which showed RCH to occur in frozen *B. antarctica* at a cellular, and possibly also a whole organism, level. Hypothetically, because ice first forms in the extracellular fluid and the cytoplasm remains supercooled in a freeze-tolerant organism (Duman and Horwath, 1983), there is still potential for intracellular RCH to occur in a frozen insect. However,

as water is lost to the ice outside the cell, intracellular processes including those involved in RCH may become inactive (Danks, 2000). In the aforementioned study, *B. antarctica* was frozen inoculatively at -5°C over 1 h, but there was no indication of when the organism actually froze, and so it is possible that the RCH observed was accrued prior to the freezing event in this organism.

3.5.4. Evolutionary significance of RCH

In general, the capacity for RCH is a valuable ecophysiological response for invertebrates, by allowing them to adjust rapidly to sudden changes in temperature on a temporal and spatial scale (Powell and Bale, 2005; Sinclair and Chown, 2006). However, the temperatures which RCH protects against in summer acclimated *E. murphyi* are rarely, if ever, seen on Signy Island during the active season (Davey *et al.*, 1992). In addition, Worland (2010) has shown that, following long-term acclimation (4 d at −4°C), larvae can survive to −20°C, a temperature never experienced in their soil habitat on Signy Island. Thus, RCH may prove to be unnecessary even in winter. Accordingly, RCH may serve a greater purpose at sub-lethal temperatures, with the enhancement of survival under limiting conditions in this study simply denoting a by-product of the RCH response acting on sub-lethal characteristics (e.g. reproduction) at temperatures more frequently seen in nature. Sub-lethal effects have been recorded in a number of studies. For example, in *D. melanogaster*, Shreve *et al.* (2004) demonstrated an improvement in courting and reproduction at 16°C after RCH, while Kelty and Lee (1999) identified a lower critical thermal minimum (CTmin, temperature below which activity does not occur). A reduction in the CTmin was also noted in *S. avenae* after RCH (Powell and Bale, 2006). An analogous response in *E. murphyi* would clearly be

ecologically beneficial. For instance, by being able to feed and, subsequently, develop at lower temperatures, *E. murphyi* might be in a better position at the end of the short growing season (cf. Hawes *et al.*, 2007).

3.5.5. Physiological mechanisms of RCH

For the majority of animals, RCH is thought to ameliorate chilling injury, via the maintenance of membrane fluidity (Lee *et al.*, 2006a; Teets *et al.*, 2008; Overgaard *et al.*, 2005) and the inhibition of apoptosis (Yi *et al.*, 2007; Yi and Lee, 2011). This interpretation is supported, in part, by the current study. As there was no significant difference between the SCPs of rapidly cold hardened and non-rapidly cold hardened larvae, the mechanisms involved in the RCH response are unlikely to have been associated with freezing injury prevention processes that alter the SCP, such as the accumulation of antifreeze proteins (AFPs) and the augmentation of ice nucleating agents (INAs) (Bale, 2002). Worland (2010) also found no significant difference between the SCPs of *E. murphyi* cooled at rates ranging from 0.05 to 2°C min^{-1}. This null response is in contrast to a number of freeze-avoiding polar organisms, including *C. antarcticus*, *A. antarcticus* and *H. belgicae*, which track environmental temperatures with their SCPs (Worland and Convey, 2001). In these freezing intolerant species, where the SCP defines the limit of their survival, altering the SCP is imperative if they are to rapidly cold harden. It is therefore likely that they possess mechanisms which separate them from chill susceptible and freeze-tolerant organisms.

3.6. Conclusion

Eretmoptera murphyi is only the second freeze-tolerant insect found to possess RCH, the other being another midge from the Antarctic, *B. antarctica* (Lee *et al.*, 2006b) This

feature, along with its basal cold tolerance, means that *E. murphyi* is clearly pre-adapted for conditions on Signy Island and is able to accommodate all summer and winter temperatures experienced in its habitat there. This midge's cold tolerance physiology is very similar to that of *B. antarctica*, which is found as far south as 68^{o} latitude (Convey and Block, 1996; Allegrucci *et al.*, 2006), and indeed the latest molecular Phylogenetic study suggests that the two species are actually congeneric (Allegrucci *et al.*, 2012). It therefore appears that there is potential for *E. murphyi* to establish and spread, not just at the northern edge of the maritime Antarctic, but also to considerably higher southern latitudes.

Chapter transition

The first two results chapters of this thesis have focused on the responses of polar terrestrial invertebrates to temperature. In the following three chapters, the response of these animals to water stress is explored, beginning with an investigation of *C. antarcticus*'s tolerance to high salinity and associated desiccation.

CHAPTER 4: THE IMPACT OF SALINITY EXPOSURE ON SURVIVAL AND TEMPERATURE TOLERANCE OF THE ANTARCTIC COLLEMBOLAN *CRYPTOPYGUS ANTARCTICUS*

The work presented in this chapter has been published in Physiological Entomology (Everatt, M. J., Worland, M. R., Convey, P. Bale, J. S. and Hayward, S. A. L. (2013) The impact of salinity exposure on survival and temperature tolerance of the Antarctic collembolan *Cryptopygus antarcticus*. Physiological Entomology, 38, 202-210.)

4.1. Abstract

The collembolan *Cryptopygus antarcticus* Willem is potentially exposed to habitat salinities equal to (or greater than) sea water, as a result of sea spray, drying of littoral habitats, dispersal or temporary entrapment on the surface of sea water, or exposure to localized salt deposits from dense vertebrate populations on terrestrial habitats. To test the impact of this exposure on *C. antarcticus*, the tolerance of the collembolan to being placed on the surface of sea water and solutions of higher salt concentrations is investigated. The effects of acclimation to exposure to liquids of different salinities [44, 100 and 200 parts per thousand (ppt) sea salt] on cold and heat tolerance, as well as thermal activity thresholds, are also explored. *Cryptopygus antarcticus* shows > 75% survival after 10 days of exposure to both sea water and 100-ppt salt, whereas it exhibits significantly lower survival after 5 days (60% survival) and 10 days (40%) of exposure to a 200-ppt solution. Body water content also decreases after exposure to all salinities,

and particularly to the 200-ppt solution, in which > 50% of body water is lost after 10 days. Acclimation results in greater cold tolerance, although heat tolerance at 33, 35 and 37°C is either unaltered or reduced. The thermal activity thresholds of *C. antarcticus* at both high and low temperatures are also negatively affected by saline exposure. The data demonstrate the capacity of *C. antarcticus* to tolerate periods of exposure to saline conditions, and also show that this exposure can enhance cross-tolerance to low temperatures. The present study also demonstrates that salinity-associated stress at moderately low and high temperatures narrows the thermal range of activity, thus reducing the ability of collembolans to forage, develop and reproduce.

4.2. Introduction

The Antarctic presents potentially stressful environmental conditions for terrestrial fauna and flora. Winters are long, lasting for 6–9 months of the year (Convey, 1996). Air temperatures during these months regularly drop below −10°C in the maritime Antarctic and −40°C in the continental Antarctic (Block *et al*., 2009). Water is locked up as ice in winter and therefore is inaccessible to living organisms (Block *et al*., 2009). Further stressors in the Antarctic, or other polar high latitude environments, include anoxia (Lopez-Martinez *et al*., 2008), extremes of pH (Rinehart *et al*., 2006), the lack of photoperiodic cues (Strathdee *et al*., 1993), ultraviolet radiation (Strathdee and Bale, 1998), heat shock (Michaud *et al*., 2008), salinity (Elnitsky *et al*., 2009) and pollution (Ávila-Jiménez *et al*., 2010). Salinity can be a particular issue in ice-free areas near to the sea, where storms, transfer of spray, and associated tidal inundation of the shoreline during the summer result in the creation of supralittoral rock pools in which (or on the surface of which) terrestrial invertebrates may be temporarily trapped (Baust and Lee,

1987). The evaporation of these pools will then lead to increasing salinity (Elnitsky *et al*., 2009). Similarly, unfrozen soils exposed to marine influences (e.g. through spray transfer, or locally associated with dense marine vertebrate concentrations) may experience elevated salinity (Bokhorst *et al*., 2007; Zmudczyńska *et al*., 2012). Insufficient interstitial moisture to alter ionic concentration in frozen soils may also raise the salinity levels to which the resident invertebrate community is then exposed (Nkem *et al*., 2006).

High salinity is known to cause mortality in several invertebrates directly, including freshwater species, such as the mosquito *Aedes camptorhynchus* (Schie *et al*., 2009), the chironomid *Chironomus salinarius* (Cartier *et al*., 2011) and the mayfly *Centroptilum* sp. (Hassell *et al*., 2006), as well as terrestrial invertebrates, such as nematodes (Nkem *et al*., 2006) and the midge *Belgica antarctica* (Elnitsky *et al*., 2009). The level of salinity also influences sub-lethal stress consequences. For example, mayflies demonstrate reduced growth and a smaller body size when exposed to high salinities (Hassell *et al*., 2006). The cause of these injuries is explained in part by disrupted ion regulation; both the ions Na^{+} and Cl^{-} bind to and destabilize nucleic acids and proteins under saline conditions (Somero and Yancey, 1997; Hochachka and Somero, 2002; Yancey, 2005; Cartier *et al*., 2011). The destabilization of nucleic acids leads to DNA breaks in mammalian cells (Kültz & Chakravarty, 2001), nematode (*Caenhorhabditis elegans*) cells *in vivo* (Dmitrieva *et al*., 2005) and marine invertebrates (Dmitrieva *et al*., 2006). Desiccation, which occurs in association with high salinity, also causes DNA breaks, either directly or through encouraging the production of reactive oxygen species (Gusev *et al*., 2010). Additional desiccation-related injuries, such as protein

denaturation and unwanted macromolecular interactions (Benoit *et al*., 2009a), as well as crystalline to gel membrane phase transitions (Hazel, 1995), may also be involved.

Because of their high surface area to volume ratios and the fine hairs that coat their bodies, some Collembola are well adapted to float on water surfaces, including sea water (Hopkin, 1997; Hawes, 2011; McGaughran *et al*., 2011). *Cryptopygus antarcticus* Willem is a freeze-avoiding collembolan, which ranges throughout the maritime Antarctic and some sub-Antarctic islands (Block *et al*., 2009). It is commonly found living in low altitude terrestrial habitats near the coast and is frequently observed floating and 'rafting' (sometimes on top of moult exuviae) on fresh and sea water (Coulson *et al*., 2002; Hawes *et al*., 2008). Hawes *et al*. (2008) found the $LTime_{50}$ (i.e. the time at which survival is 50%) of *C. antarcticus* on sea water to be 75.38 days at 0°C, 64.47 days at 5°C and 34.26 days at 10°C. Furthermore, *C. antarcticus* is able to produce viable offspring when on sea water. This collembolan is therefore clearly capable of tolerating sea water exposure. However, details of the tolerance and physiological adaptation of *C. antarcticus* to exposure to higher salinities remain uncharacterized. How salinity exposure affects the temperature tolerance physiology and activity thresholds of this species has also received little attention. This question is pertinent when considering the chronic exposure of terrestrial invertebrates to low temperatures in the Antarctic (Block *et al*., 2009). Similarly, understanding the impact of exposure to increasing salinity on upper thermal physiology is important in an era of climate warming.

4.3. Materials and methods

4.3.1. Invertebrate collection and storage conditions

Summer acclimatized individuals of *C. antarcticus* were collected from moss and algae on Lagoon Island (67°35′S, 68°16′W), near to Rothera Research Station, Adelaide Island, Antarctic Peninsula, between January and March 2012. The general features of the terrestrial ecology and biodiversity of this location and region are described by Convey and Smith (1997). Samples were stored in an illuminated growth cabinet on station at 4°C (LD 24 : 0 h) in plastic bags containing substratum from the site of collection.

4.3.2. Tolerance of different salinity exposures and effect on water balance

Cryptopygus antarcticus individuals were exposed to fresh water and three salinity treatments [sea water (44 parts per thousand; ppt), 100 and 200 ppt (saturated solution)]. Fresh water was sourced from Rothera Research Station, produced using a Milli-Q water purifier (Merck Millipore, U.K.) and sea water (44 ppt) was sourced locally from Rothera South Cove. Higher salinity treatments (100 and 200 ppt) were produced via evaporative concentration of sea water, as might occur in rock pools. Solutions were not changed or added to during experimentation. Within each treatment, 3 × 10 replicates of *C. antarcticus* individuals were held for 6 h, 1, 2, 5 and 10 days at 4°C (LD 24 : 0 h). Individual springtails were transferred onto the surface of each solution (25 mL), inside small plastic containers with an open top covered with nylon gauze. After each treatment, individuals were transferred into recovery capsules (universal tubes with a base of moist Plaster of Paris) and placed at 4°C (LD 24 : 0 h). Survival, defined as

individuals that either moved spontaneously or in response to gentle contact stimulus, was assessed 24 and 72 h after treatment. Each replicate was also weighed (to nearest 10 μg) before fresh water or saline treatment, upon removal from each respective treatment, and after drying to a constant mass at 60°C for 24 h. From these values, initial water content and percentage water loss or gain were calculated (Hayward *et al*., 2007).

4.3.3. The effect of recovery on tolerance of salinity exposures and water balance

To test whether patchiness of salinity conditions has a bearing on survival and water loss, 3 × 10 replicates of *C. antarcticus* were exposed to each of the fresh water and saline treatments as described above for 2, 5 and 10 periods of 24 h. Each period was followed by 1 h of recovery in universal tubes with moist Plaster of Paris. Recovery, survival assessment and water balance analyses were carried out as described previously.

4.3.4. Salinity cross-tolerance

4.3.4.1. Effect of acclimation to salinity exposures on cold tolerance

Individuals were held at each fresh water and saline treatment for 3 days before experimentation. The supercooling point (SCP; i.e. the freezing point of body fluids) of 24 individuals (replicates) was subsequently determined by cooling from 4 to −30°C at 0.5°C min^{-1}. Individuals were placed in contact with a thermocouple within Eppendorf tubes, in glass test tubes plugged with sponge, and inside an alcohol bath (Haake Phoenix II C50P; Fisher Scientific Ltd, U.K.), before the cooling regime. SCPs, defined

as the temperature at the onset of the freezing exotherm, were identified using an eight channel datalogger interfaced to a computer and recorded using Picolog recorder software (Pico Technology, U.K.) (Hawes *et al*., 2006). The SCP is known to be the lower limit of survival, and equivalent to the lower lethal temperature, in *C. antarcticus* (Cannon and Block, 1988).

4.3.4.2. Effect of acclimation to salinity exposures on heat tolerance

Individuals were held at each fresh water and saline treatment for 3 days before experimentation. Three × 10 replicates were warmed subsequently from 4 to 33, 35 or 37°C at 0.2°C min^{-1}, held for 1 h, and cooled to 4°C at the same rate. Individuals were placed in Eppendorf tubes, inside glass test tubes plugged with sponge, and in an alcohol bath, before each warming regime. The temperature experienced by *C. antarcticus* was measured by placing a thermocouple within an identical Eppendorf tube into one of the glass test tubes. Recovery and survival assessment were carried out as described previously.

4.3.5. Effect of acclimation to salinity exposures on activity thresholds

4.3.5.1. Experimental conditions

Activity thresholds were assessed, after 3 days of acclimation to each salinity treatment, within an aluminium block arena, as described by Hazell *et al*. (2008). Ten individuals (30 individuals per treatment) were transferred into the arena (initially set to 4°C), and were allowed to settle for 5 min before video recording (Studio Capture DT; Studio86Designs, U.K.) and the alcohol bath programme began. This procedure was performed for each acclimation treatment.

4.3.5.2. Critical thermal minimum (CTmin) and chill coma

The temperature of the arena was reduced from 4 to −15°C at 0.2°C min^{-1}. The lowest temperatures at which each individual walked in a coordinated fashion (CTmin) and last moved its body, legs and/or antennae (chill coma) were recorded.

4.3.5.3. Critical thermal maximum (CTmin) and heat coma

The temperature of the arena was increased from 4 to 45°C at 0.2°C min^{-1}. The highest temperatures at which each individual walked in a coordinated fashion (CTmax) and last moved its body, legs and/or antennae (heat coma) were recorded.

4.3.6. Statistical analysis

The Kolmogorov–Smirnov test was used to confirm whether survival, SCP, activity threshold and percentage water loss data were normally distributed. Normally distributed data were analyzed using analysis of variance and Tukey's multiple range test, and non-normally-distributed data were analyzed using either the Mann–Whitney *U*-test or the Kruskal–Wallis test.

4.4. Results

4.4.1. Tolerance of salinity exposures

Springtail survival on the surface film of fresh water was high (≥ 93%) and remained constant over the course of 10 days (Fig. 4.1). For the first 2 days, survivorship on all salinity treatments also remained > 90%. After 10 days, survival declined to 83% on sea water and 77% on 100-ppt solution, although neither decrease was significantly

different from that on fresh water. Survivorship on 200-ppt solution was 60% after 5 days and 40% after 10 days, with both being significantly lower than on fresh water.

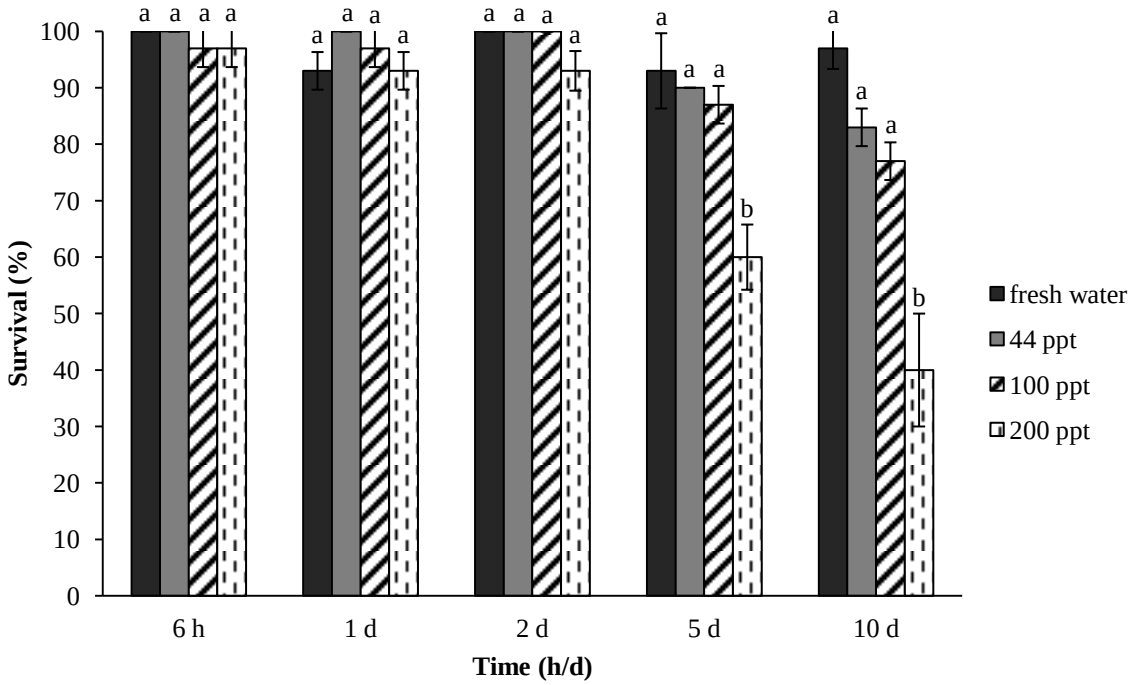

Fig. 4.1. *Survival (%) of C. antarcticus following exposure to one of four salinity treatments (fresh water, 44 ppt, 100 ppt and 200 ppt) for a range of time periods (6 h, 1 d, 2 d, 5 d, and 10 d). Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different to fresh water within each acclimation group at P < 0.05 (Tukey's multiple range test).*

4.4.2. Water balance

Cryptopygus antarcticus lost water in all treatments over 10 days (Fig. 4.2), and the extent of water loss increased significantly during exposure to 200 ppt. On fresh water, individuals lost over 20% of their body water content after 5 days, although some water was then regained by day 10, with an average loss of only 3%. Under the 200-ppt conditions, individuals steadily lost water, reaching an average loss of 52.8% of their original content after 10 days. In both 44- and 100-ppt treatments, water loss rates were similar and water content stabilized after 2 days.

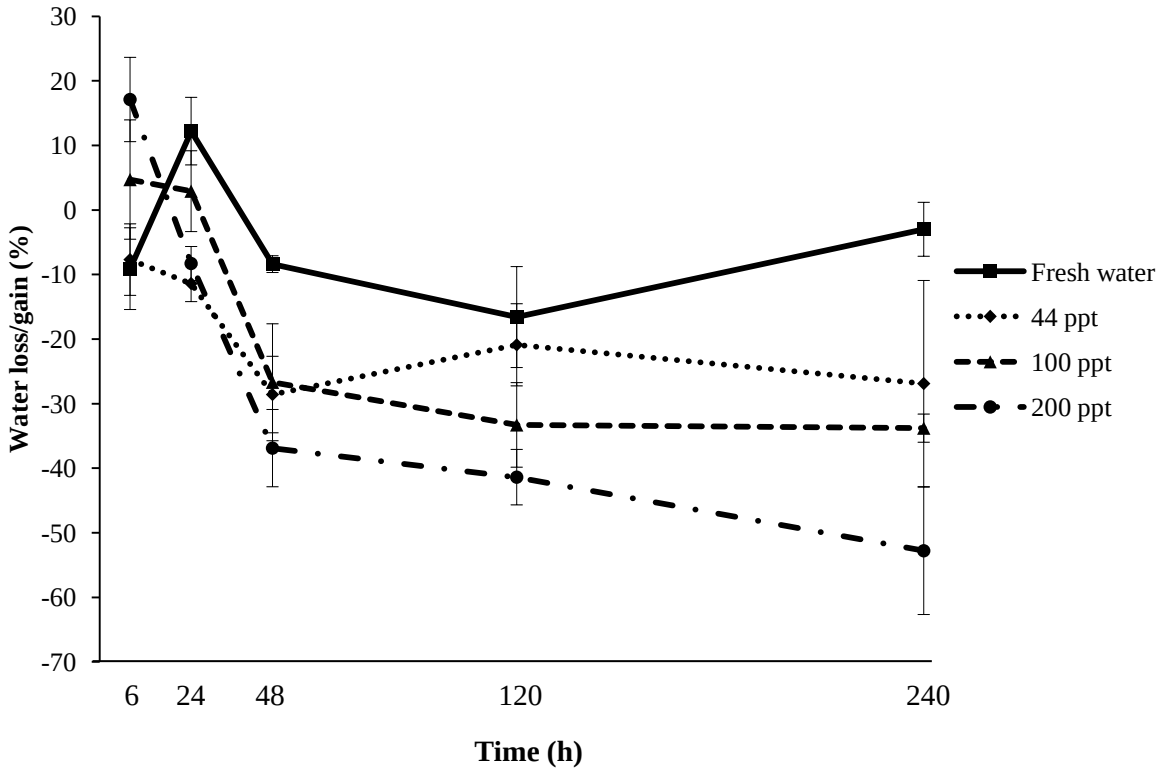

Fig. 4.2. *Percentage water loss or gain of C. antarcticus following exposure to one of four salinity treatments (fresh water, 44 ppt, 100 ppt and 200 ppt) for a range of time periods (6 h, 1 d, 2 d, 5 d and 10 d). Means ± S.E.M. are presented for three replicates of 10 individuals.*

4.4.3. The effect of recovery on tolerance of salinity exposures and water balance

After two 24-h exposure periods, survival of *C. antarcticus* was high across all treatments (Fig. 4.3). Survivorship also remained high after five exposure periods on fresh water, as well as the 44- and 100-ppt treatments, although it fell below 90% on the 200-ppt solution. Ten exposure periods resulted in a decrease in survival in all treatments, particularly exposure to the 200-ppt solution, in which there was only 43% survival. Survival after 2, 5 and 10 exposure periods at each salinity treatment was not significantly different from that obtained previously in the absence of recovery periods.

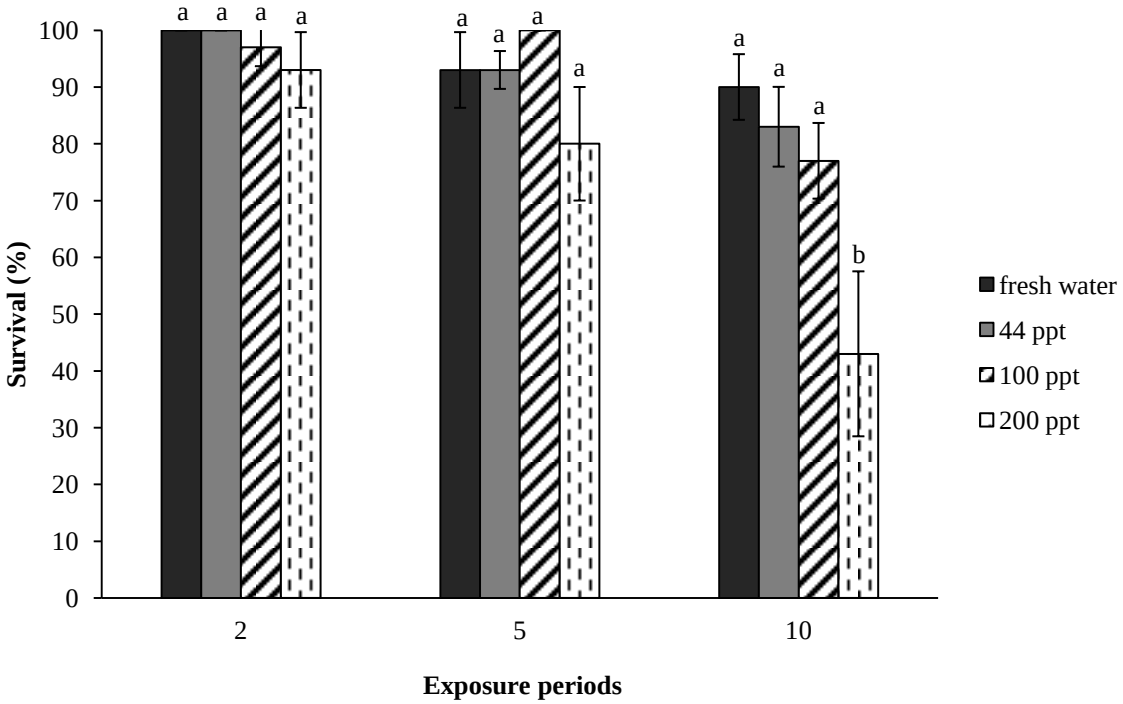

Fig. 4.3. *Survival (%.) of C. antarcticus following exposure to one of four salinity treatments (fresh water, 44 ppt, 100 ppt and 200 ppt) for two, five or ten 24 h periods. Each period was followed by 1 h recovery. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different to fresh water within each acclimation group at P < 0.05 (Tukey's multiple range test).*

Body water was lost after exposure to all treatments, even with 1-h recovery periods (Fig. 4.4). Water loss was again lowest on fresh water, and increased with increasing salinity. Compared with the water loss observed in the absence of a recovery period (Fig. 4.2), water loss was reduced across all treatments and time periods, except for 10 days of exposure to the 200-ppt solution (Fig. 4.4). These differences were statistically significant for the 44-ppt solution after two and five exposure periods, and the 200-ppt solution after five exposure periods.

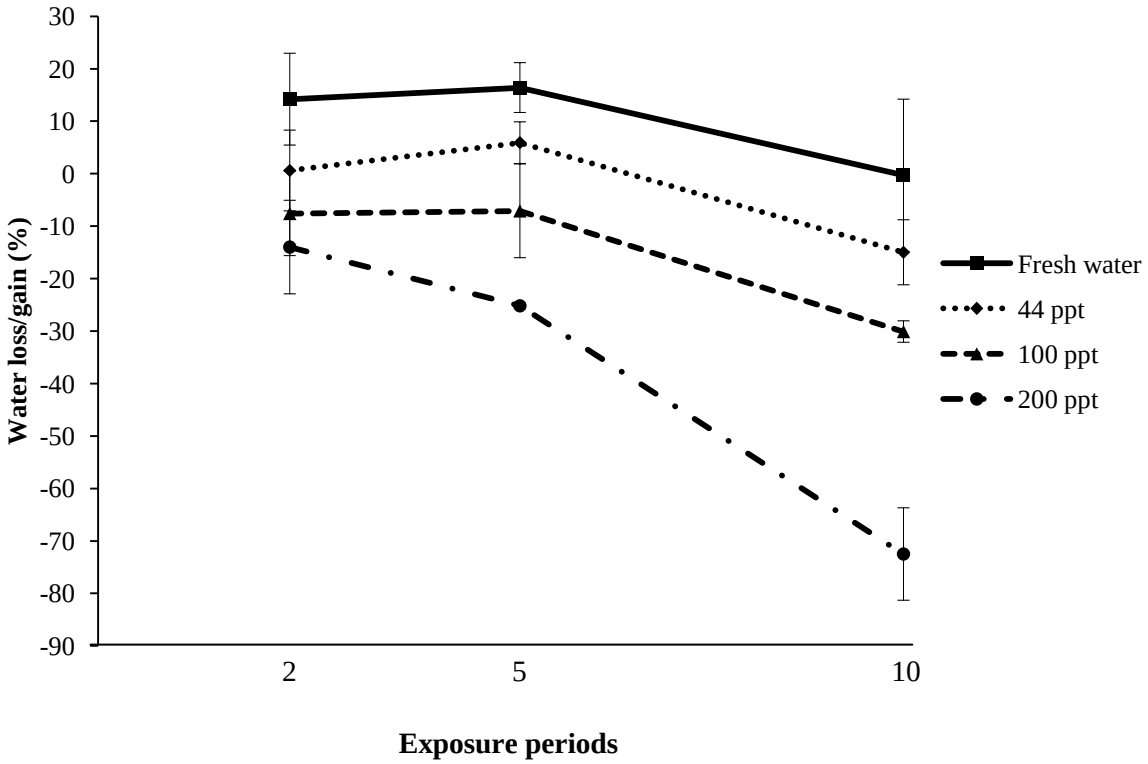

Fig. 4.4. *Percentage water gain or loss of C. antarcticus following exposure to one of four salinity treatments (fresh water, 44 ppt, 100 ppt and 200 ppt) for two, five or ten 24 h periods. Each period was followed by 1 h recovery (100% RH). Means ± S.E.M. are presented for three replicates of 10 individuals.*

4.4.4. Salinity cross-tolerance

4.4.4.1. Effect of acclimation to salinity exposures on cold tolerance (SCP)

Exposure to higher salinities lowered the SCP of *C. antarcticus*. After 3 days of exposure to fresh water, the mean SCP of *C. antarcticus* was −18.12°C. This value was very similar to that of individuals acclimated on 44-ppt (−18.34°C), although it was higher than those obtained on the other two treatments (100-ppt: −22.63°C, 200-ppt: −22.60°C). However, the lowered SCP of 100- and 200-ppt treatments was not significantly different from that of fresh water.

4.4.4.2. Effect of acclimation to salinity exposures on heat tolerance

Saline exposure resulted in no change in heat tolerance (Fig. 4.5). At 33°C, the lowest mean survival was noted in Collembola exposed to 100- and 200-ppt solutions (80%), although this was not significantly different from survival on fresh water. At 35°C, survival in all salinity treatments dropped below 50%, although, again, this was not significantly different from that of fresh water. There was no survival after exposure to 37°C across all treatments.

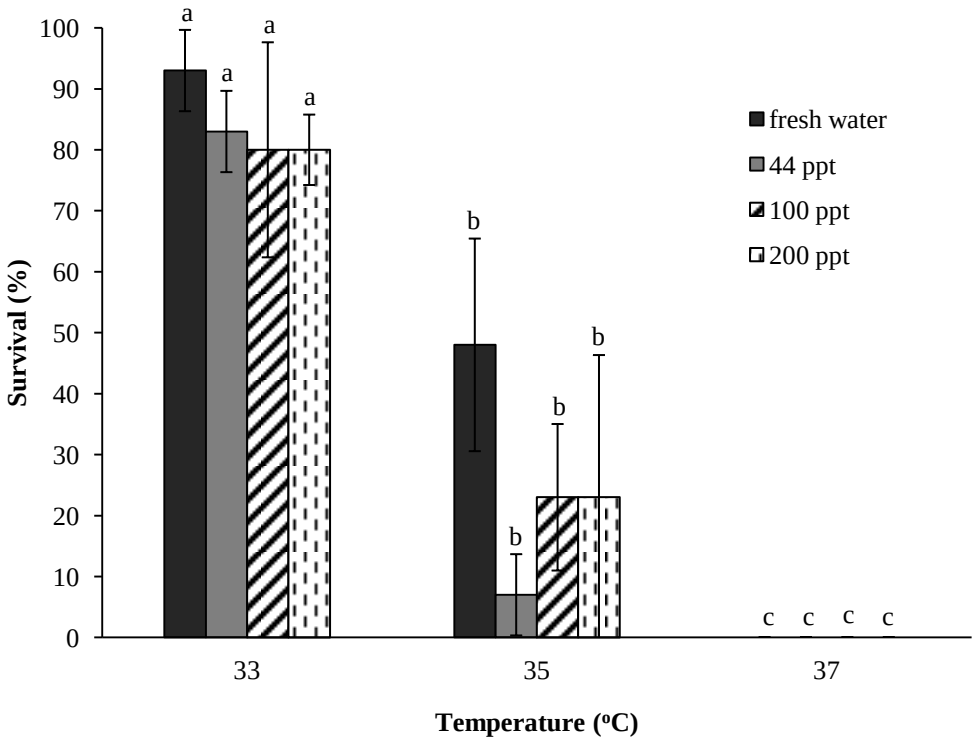

Fig. 4.5. *Survival (%) of C. antarcticus after exposure to 33, 35 and 37°C, following 3 d acclimation on fresh water, 44 ppt, 100 ppt or 200 ppt solutions. Means ± S.E.M. are presented for three replicates of 10 individuals. Means with the same letter are not significantly different within each acclimation group at P < 0.05 (Tukey's multiple range test).*

4.4.5. Effect of acclimation to salinity exposures on activity thresholds

As the salinity of the acclimation treatment was increased, the CTmin and chill coma

temperatures of *C. antarcticus* rose, whereas the CTmax (with the exception of the sea water treatment) and heat coma values fell (Table 4.1). The CTmin and chill coma temperature of *C. antarcticus* increased from −4.1 and −5.8°C, respectively, after 3 days of fresh water acclimation, to −1.2 and −2.4°C after 3 days of acclimation on the 200-ppt solution. The CTmax and heat coma temperatures decreased similarly, falling from 22.2 and 24.2°C after 3 days of exposure to fresh water to 18.9 and 21.2°C, respectively, after exposure to 200 ppt. When compared with fresh water, the differences were significant for the 200-ppt exposure across all measurements (CTmin, chill coma, CTmax and heat coma), and significant for the 100-ppt exposure for CTmin, chill coma and heat coma.

Table 4.1. *Activity threshold temperatures (CTmin, chill coma, CTmax and heat coma) (± S. E.) of C. antarcticus following a 3 d acclimation on fresh water, 44 ppt, 100 ppt and 200 ppt solutions. Means ± S.E.M. are presented for three replicates of 10 individuals. Asterisks indicate a treatment significantly different from fresh water at P < 0.05 (Kruskal-Wallis test).*

Treatment	CTmin (^{o}C)	Chill coma (^{o}C)	CTmax (^{o}C)	Heat coma (^{o}C)
Fresh water	-4.1 ± 0.14	-5.8 ± 0.49	22.2 ± 0.51	24.2 ± 0.29
Sea water	-3.8 ± 0.27	-4.2 ± 0.27	22.6 ± 0.38	23.6 ± 0.31
100g salt/L	-2.7 ± 0.21 *	-4.1 ± 0.44 *	21.4 ± 0.47	22.8 ± 0.30 *
Saturated salt	-1.2 ± 0.43 *	-2.4 ± 0.50 *	18.9 ± 0.76 *	21.2 ± 0.46 *

4.5. Discussion

4.5.1. Tolerance of different saline environments

Invertebrates such as springtails are regularly trapped in or on fresh water and littoral rock pools (Pryor, 1962; Tilbrook, 1967). Travelling on the sea surface may also be a viable means of dispersal (Hopkin, 1997; Coulson *et al*., 2002; Hawes *et al*., 2008). The

Antarctic collembolan *C. antarcticus* is therefore expected to possess a high level of tolerance to salinity exposure. In the present study, *C. antarcticus* survives well after 10 days of exposure to 44 ppt at 4°C, which is consistent with the findings of Hawes *et al*. (2008). Hawes *et al*. (2008) show that some individuals of *C. antarcticus* can survive > 120 days on the surface film of sea water. *Cryptopygus antarcticus* also shows high survival after 10 days on a 100-ppt solution in the present study, and only when exposed to a 200-ppt solution does mortality increase significantly (60% after 10 days). *Cryptopygus antarcticus* may therefore be at risk when sea water evaporates to leave higher salt concentrations in littoral pools. However, it is still able to tolerate a 200-ppt solution for up to 2 days and this may be sufficient for the collembolan to locate a less saline environment before injuries become too great.

The injuries that can result from exposure to saline conditions include disrupted ion regulation (Yancey, 2005), DNA breaks (Dmitrieva *et al*., 2006) and other cellular desiccation related injuries, such as macromolecular degradation and membrane solidification (Benoit *et al*., 2009a). Because *C. antarcticus* has a hydrophobic cuticle and dense setation, which means that its body does not contact the water or saline solution directly (Hopkin, 1997; Hawes, 2011; McGaughran *et al*., 2011), desiccation, rather than direct salinity damage, likely contributes to the mortality seen in the present study with respect to exposure to the 200-ppt solution, with 41.4% and 52.8% water loss after 5 and 10 days of exposure, respectively. Water is also lost, and mortality incurred, after exposure to sea water (44 ppt) and 100-ppt treatments. Elnitsky *et al*. (2008a) report similarly that *C. antarcticus* experiences mortality after desiccation at low relative humidities (RH), with the level of mortality being highly dependent on the rate of dehydration. At 0% RH, < 50% of individuals survive a 50% loss of water, whereas

at 93% and 98.2% RH, 50% of *C. antarcticus* are able to survive 58% and > 60% water loss, respectively.

Salinity tolerant invertebrates employ two tactics to reduce the risk of injury resulting from salinity: osmoregulation and osmoconformation. Osmoregulators maintain their haemolymph concentration at a level higher than (or hyperosmotic to) the environment by drinking the external medium and expelling excess water (Neumann, 1976; Bradley, 1987). In response to increased salt load, osmoregulators excrete more concentrated urine (Elnitsky *et al*., 2009). Osmoconformers, on the other hand, equilibrate their haemolymph concentration with that of the environment. The latter strategy is observed in the mosquito genera *Culiseta* and *Deinocerites* (Bradley, 1994), the dragonfly nymph *Enallagama clausam* (Stobbart and Shaw, 1974) and the Antarctic midge *B. antarctica* (Elnitsky *et al*., 2009). In the present study, the water content of *C. antarcticus* stabilizes between 2 and 10 days at approximately 60–75% of initial water content in both the 44- and 100-ppt treatments but not the 200-ppt treatment. Such stabilization suggests that *C. antarcticus* is able to osmoconform to some extent. Osmoconformation is generally associated with the enhanced concentration of osmolytes, such as sugars (e.g. trehalose), polyols (e.g. glycerol), amino acids (e.g. glycine) and methylamines (e.g. glycine betaine) (Yancey, 2005). This is also likely to be the case for *C. antarcticus* because both Hawes *et al*. (2008) and Elnitsky *et al*. (2008a) report an increase in osmolality in response to sea water and a desiccation treatment, respectively.

In the natural habitats of this springtail, exposure to increased salinity conditions is likely to be patchy and unpredictable, with intermittent opportunities to rehydrate. In the present study, *C. antarcticus* individuals are therefore exposed to periods of saline conditions followed by a return to nonsaline conditions to test whether such patchiness

would facilitate recovery. The data obtained from these tests suggest that *C. antarcticus* is able to recover at least partially. After five exposure periods, survival is enhanced in the sea water treatment, as well as the 100- and 200-ppt treatments, compared with 5 days of exposure in the absence of a recovery period. Water loss is also reduced after five exposure periods, compared with 5 days, at 44-ppt (26.8% reduction), 100-ppt (26.2%) and 200-ppt (16.2%), and after 10 exposure periods at 44-ppt (11.9%) and 100-ppt (3.7%). The benefits of short recovery periods are already known in connection with temperature stress; pulses at more favourable temperatures allow for the resumption of ion gradient homeostasis in the bug *Pyrrhocoris apterus* and the beetle *Alphitobius diaperinus* (Kostál *et al*., 2007), the induction of antioxidants in *Alphitobius diaperinus* (Lalouette *et al*., 2011) and the up-regulation of key proteins in the parasitic wasp *Aphidius colemani* (Colinet *et al*., 2007). Such mechanisms lead to elevated survival in a number of insects, including the flesh fly *Sarcophaga crassipalpis* (Dollo *et al*., 2010) and *Alphitobius diaperinus* (Renault *et al*., 2004; Colinet, 2011). An analogous response may be involved in the present study with respect to the recovery of *C. antarcticus* between saline exposures. For example, the re-establishment of ion gradient homeostasis allows for the intake of water during recovery, giving reduced water loss and the minimization of desiccation related injuries. It should be noted that, in the present study, only a 1-h recovery period is used in between exposure periods, and that a longer recovery period would have likely provided an even greater benefit to the collembolan.

4.5.2. Effect of salinity exposure on cold and heat tolerance

Cross-tolerance, which is defined as the enhanced ability to tolerate one stress after exposure to another, is observed in a number of invertebrate groups, including nematodes (Adhikari *et al*., 2010), chironomids (Elnitsky *et al*., 2009; Gusev *et al*., 2010) and Collembola (Holmstrup *et al*., 2002; Bahrndorff *et al*., 2007). In the present study, saline exposure enhances cold tolerance (but not heat tolerance) in *C. antarcticus*. Cross-tolerance between salinity and low temperatures is also documented in diving beetles (Sánchez-Fernández *et al*., 2010) and the Antarctic midge *B. antarctica*, where survival at −12 and −15°C is significantly greater after 3 days of exposure to soil water salinity of 1000 $mOsm\ kg^{-1}$ (Elnitsky *et al*., 2009). Elnitsky *et al*. (2009) suggest that much of the enhanced cold tolerance is a result of associated desiccation. Injuries resulting from low temperature and desiccation are similar and include the impaired folding of proteins (Hayward *et al*., 2007) and reduced membrane fluidity (Bayley *et al*., 2001). The physiological mechanisms induced in response to either stress are therefore analogous (Block *et al*., 1990). *Cryptopygus antarcticus* also experiences greater water loss after saline exposure, particularly in the 100- and 200-ppt treatments. It is thus plausible that the enhanced cold tolerance found in the present study after saline acclimation is the result of a physiological response to desiccation.

Additionally, cold tolerance in *C. antarcticus* is greatly enhanced after acclimation on fresh water compared with *C. antarcticus* held in vegetation (data not shown), lowering the SCP by 3.24°C. Worland *et al*. (2006) also find that the time spent on water lowers the SCP of the collembolan *Ceratophysella denticulata*. Such a phenomenon may be attributed to starvation. During starvation, the contents of the gut, including ice-

nucleating agents, are cleared, reducing the likelihood of ice formation (Sømme and Block, 1982; Cannon and Block, 1988). The mechanisms involved in starvation are also physiologically similar to those of moulting, which are known to lower the SCP of invertebrates (Worland, 2005; Worland and Convey, 2008).

Saline exposure does not provide any additional tolerance against high temperatures. Previous acclimation on sea water, 100- and 200-ppt solutions reduces the tolerance of *C. antarcticus* to exposure to 33 and 35°C. Hawes *et al*. (2008) also report that high temperature and salinity are compounding stresses for the collembolan. These results can be at least partially explained by the differing injuries after desiccation and high temperatures. Desiccation leads to the transition of the plasma membrane from a crystalline to gel phase (Elnitsky *et al*., 2008b), whereas high temperatures have the opposite effect of increasing the fluidity of the membrane (Hazel, 1995). Measures to counteract one of these injuries will therefore likely be to the detriment of the other. High temperature exposure also leads to further water loss. Reduced heat tolerance after desiccation is observed in a number of invertebrates, including in nematodes (Holmstrup and Zachariassen, 1996). Reduced heat tolerance is also observed after saline exposure in *B. antarctica* (Elnitsky *et al*., 2009). However, there are exceptions. *Nebrioporus* diving beetles exhibit a markedly greater heat tolerance after acclimation at 60 g salt L^{-1} but not after salinities in the range of 1–35 g salt L^{-1} (Sánchez-Fernández *et al*., 2010).

4.5.3. Effect of saline exposure on thermal activity thresholds

In the present study, previous exposure to a saline treatment is shown to influence survivorship at high and low temperatures. There is also evidence of salinity having an

influence on sub-lethal stress consequences, including growth and development, in a number of freshwater species, such as the chironomid *C. salinarius* (Cartier *et al*., 2011) and the mayfly *Centroptilum* sp. (Hassell *et al*., 2006). The present study further explores this phenomenon by testing the thermal activity thresholds of *C. antarcticus* after exposure to fluids of different salinities. As the concentration of sea water increases, the CTmin and chill coma temperatures rise and the CTmax and heat coma temperatures fall. Thus, saline exposure constrains the thermal activity window of *C. antarcticus*. This impact could impede the collembolan's reproduction, foraging and subsequent development, both at high and low temperatures. Macmillan and Sinclair (2010) provide a summary of the known physiological mechanisms underpinning chill coma, noting the importance of managing cation concentrations for the excitability of neurones and muscle cells, and that a failure to do so will lead to chill coma. This failure is suggested to be a result of disrupted ion regulation. Disrupted ion regulation is already well known to be associated with saline exposure and desiccation in insects (Holmstrup *et al*., 2002; Yancey, 2005) and plants (Grattan and Grieve, 1999), and thus it is unsurprising that exposure to higher salinity fluids leads to the decline of activity observed in the present study. For example, membrane-bound ion pumps and channels are negatively affected by the transition of the plasma membrane from a crystalline to gel phase that results from a loss of water (Holmstrup *et al*., 2002).

4.6. Conclusion

The present study examines the salinity stress tolerance of *C. antarcticus* in greater detail than previously attempted and addresses whether acclimation to saline conditions affects temperature cross-tolerance or thermal activity thresholds. In the Antarctic, *C.*

antarcticus is not exposed to one stress alone but, instead, to a range of stresses, in particular low and high temperature, salinity and desiccation. After previous acclimation to saline conditions, the cold tolerance of *C. antarcticus* is improved. However, activity at low temperatures is impaired by the same acclimation. A negative effect is also seen at higher temperatures and may put pressure on this collembolan under changing environmental conditions resulting from climate warming. Further study of the underpinning molecular mechanisms is required to fully understand the physiological changes of *C. antarcticus* under different salinities.

Chapter transition

In the following Chapter, the physiological tolerance to desiccation is investigated for two polar midges, *E. murphyi* and *H. borealis*, though this time under arid, rather than saline, conditions. Cross-tolerance between low water availability and temperature will also be further explored.

CHAPTER 5: CONTRASTING STRATEGIES OF RESISTANCE VS. TOLERANCE TO DESICCATION IN TWO POLAR DIPTERANS

The work of this chapter has been accepted for publication by Polar Research.

5.1. Abstract

Low water availability is one of the principal stressors for terrestrial invertebrates in the polar regions, determining the survival of individuals, the success of species, and the composition of communities. The Arctic dipteran, *Heleomyza borealis*, and the Antarctic dipteran, *Eretmoptera murphyi*, spend the majority of their biennial life cycles as larvae, and so are exposed to the full range of environmental conditions, including low water availability, over the annual cycle. In the current study, the desiccation resistance and desiccation tolerance of larvae were investigated, as well as their capacity for cross-tolerance to temperature stress. Larvae of *H. borealis* showed high levels of desiccation resistance, only losing 6.9% of their body water after 12 d at 98.2% relative humidity (RH). In contrast, larvae of *E. murphyi* lost 46.7% of their body water after 12 d at the same RH. Survival of *E. murphyi* larvae remained high in spite of this loss (> 80% survival). Following exposure to 98.2% RH, larvae of *E. murphyi* showed enhanced survival at -18^{o}C for 2 h. The supercooling point of larvae of both species was also lowered following prior treatment at 98.2% RH. Cross-tolerance to high temperatures (37 or 38.5^{o}C) was not noted following desiccation in *E. murphyi*, and survival even fell at 37^{o}C following a 12 d pre-treatment. The current study

demonstrates two different strategies of responding to low water availability in the polar regions, and indicates the potential for cross-tolerance, a capacity which is likely to be beneficial in the ever changing polar climate.

5.2. Introduction

Insects, which are largely of small size, have a high surface area to volume ratio and are vulnerable to water loss (Gibbs *et al.*, 1997). Injuries resulting from the loss of water include protein denaturation and unwanted macromolecular interactions, crystalline to gel membrane phase transitions, oxidative damage and mechanical stress (Danks, 2000). In order to protect against these injuries, invertebrates generally adopt one of two strategies, desiccation resistance or desiccation tolerance. The capacity to prevent water loss from the body (desiccation resistance) varies greatly among invertebrates and has led to three species classifications, namely hygric species, which have little or no control over their water loss, and transitional and mesic species, which are increasingly able to regulate the loss of body water (Danks, 2000). The mesic status of invertebrates like the Antarctic mite, *Alaskozetes antarcticus*, is largely achieved through lowered cuticular permeability (e.g. Benoit *et al.*, 2007b), though the regulation of water is also achieved in other invertebrates using methods of freezing (Convey, 1992), membrane alteration and metabolic suppression (Michaud *et al.*, 2008), and/or specialised respiration (Danks, 2000). In hygric species, the loss of water is tolerated. *Dendrobaena octaedr*a (earthworm) cocoons (Holmstrup and Zachariassen, 1996) and larvae of the Antarctic dipteran, *Belgica antarctica* (Hayward *et al.*, 2007), are able to endure > 75% loss of their water content, while some insects, such as the dipteran, *Polypedilum vanderplaanki*, and many nematodes and tardigrades are able to survive the loss of

virtually all their osmotically active water (OAW) employing the tactic of anhydrobiosis (Crowe and Madin, 1975; Wharton, 1993, 2003, 2011; Watanabe *et al.*, 2002; Hengherr *et al.*, 2010). Molecular mechanisms underpinning desiccation tolerance include the accumulation of polyhydric alcohols and sugars (Benoit *et al.*, 2007a; Hengherr *et al.*, 2008), the utilisation of HSP and LEA proteins (Bahrndorff *et al.*, 2009; Lopez-Martinez *et al.*, 2009; Popovic *et al.*, 2011), shifts in metabolism (Danks, 2000; Li *et al.*, 2009), membrane remodelling (Lopez-Martinez *et al.*, 2009), oxidative damage repair (Lopez-Martinez *et al.*, 2008) and cytoskeletal reorganisation (Li *et al.*, 2009; Lopez-Martinez *et al.*, 2009).

Low water availability is seen as being one of two principal stresses to terrestrial invertebrates in the polar regions, with the other being low temperature (Cannon and Block, 1988; Convey, 1996; Strathdee and Bale, 1998; Block *et al.*, 2009). In winter, water is locked up as ice and is inaccessible to invertebrates (Block *et al.*, 2009) while, in summer, evaporation of meltwater can lead to drought (Kennedy, 1993). In some areas, such as the McMurdo Dry Valleys, soil water content can be as little as 2% (Treonis and Wall, 2005). The Antarctic dipteran, *Eretmoptera murphyi*, and the high Arctic dipteran, *Heleomyza borealis*, also experience aridity in their respective habitats. The larval stages of these two species comprise the majority of the life cycle duration, and thus experience the full spectra of environmental conditions over the annual cycle (Convey and Block, 1996; Worland *et al.*, 2000). *Eretmoptera murphyi* is locally highly abundant in the sub-Antarctic island of South Georgia and, since its introduction onto Signy Island (maritime Antarctic) in the 1960s, it has spread to occupy an area > 30000 m^2, with densities as high as 410000 ind. m^{-2} (Hughes and Worland, 2010). Worland (2010) has shown that *E. murphyi* larvae possess good desiccation tolerance, but low

desiccation resistance. *Heleomyza borealis* is also abundant at certain High Arctic sites when found in association with bird colonies, but its desiccation tolerance has not been assessed to date.

Because injuries to invertebrates, such as protein impairment and loss of membrane fluidity, result from desiccation as well as from other stresses like low temperature, the physiological adaptations induced in response to these stresses are analogous, or at least complementary (Ring and Danks, 1994; Bayley *et al.*, 2001). For example, the desaturation of membranes, up-regulation of heat shock proteins and the accumulation of polyols and sugars occur after both desiccation and low temperature treatments (Bayley *et al.*, 2001; Holmstrup *et al.*, 2002; Bahrndorff *et al.*, 2007; Benoit *et al.*, 2009a). It therefore follows that a sub-lethal desiccation exposure can potentially afford protection for an invertebrate subsequently exposed to low temperatures. This phenomenon is termed cross-tolerance and has been observed in a number of organisms, though primarily in Diptera and Collembola (e.g. Holmstrup *et al.*, 2002; Elnitsky *et al.*, 2008a, b; Levis *et al.*, 2012). In the collembolan, *Megaphorura arctica*, desiccation in the presence of ice lowers the supercooling point (SCP) to such an extent that the collembolan is able to survive the low temperatures of the Arctic winter (Worland *et al.*, 1998). This strategy, termed cryoprotective dehydration, is now seen to be fairly common, having been described in a substantial number of invertebrates (e.g. Pedersen and Holmstrup, 2003; Elnitsky *et al.*, 2008a, b; Smith *et al.*, 2008; Sørensen and Holmstrup, 2011). Cross-tolerance also works independently of the SCP. In the freeze-tolerant dipteran, *B. antarctica*, survival was improved by 90% at -10^{o}C following 48 h at 98 % RH and by 60% at -15^{o}C following the loss of 50% of its body water (Benoit *et al.*, 2009a). Invertebrates which experience complete desiccation or anhydrobiosis are

also conferred improved low temperature tolerance, and the extent to which it is improved is usually greater than in partially desiccated animals like *B. antarctica* (e.g. Ramløv and Westh, 1992; Sømme and Meier, 1995; Shuker, 2001).

Climate change is leading to warmer summers in the polar regions, with evidence of increasing exposure to drought (Convey *et al.*, 2003, 2009; Turner *et al.*, 2009). Exploration of cross-tolerance between desiccation and low and high temperature in two additional dipteran species, therefore, provides further insight into how polar terrestrial invertebrates tolerate extreme conditions currently and may indicate how they will cope with climate warming in future.

5.3. Materials and methods

5.3.1. Insect collection and storage conditions

Summer acclimatised individuals of *H. borealis* were collected from the moss-covered slopes at Krykkeffellet and Blomstrandhalvøya, near Ny-Ålesund, Spitsbergen, Svalbard (78^{o}55'N, 11^{o}56'E) in August 2011. Summer acclimatised individuals of *E. murphyi* were collected from soil and moss on Signy Island (60^{o}S, 45^{o}W) near to the British Antarctic Survey Signy Research Station between January and March 2012. They were transported to the University of Birmingham under refrigerated conditions and then held in plastic boxes containing substratum from the site of collection at 4^{o}C (0:24 L:D). The duration of travel was approximately 2 d from the Arctic and two months from the Antarctic. Numbers of *H. borealis* were limited and hence it was not possible to assess for cross-tolerance to high and low temperatures, except with respect to their supercooling points (SCPs).

5.3.2. Water balance and desiccation tolerance

Bayley and Holmstrup (1999) highlighted the importance of performing desiccation experiments at more ecologically relevant RH values, and in particular those close to the wilting point of plants (~98.9% RH). The specific relative humidity of 98.2% used here was produced using 150ml of NaCl solution (31.60g NaCl L^{-1}) in a plastic container. The relative humidity was verified as being stable using a Hygrochron temperature/humidity logger iButton (Maxim, San Jose). Controls were maintained at 100% RH using purified water, and were given access to water. Dipteran larvae were placed in small glass containers, covered with nylon gauze, which were then placed inside the plastic containers, and sealed with a tight fitting lid. Following Raoult's law, the air inside the closed system quickly equilibrated with the aqueous solution used.

Three replicates of 10 individuals of each species were removed from 98.2% RH at set intervals over a 12 d period (6 h, 2 d, 4 d, 8 d and 12 d). Dipteran larvae were subsequently transferred into plastic universal tubes containing moist Plaster of Paris and given substrate and water. Survival, defined as larvae which either moved spontaneously or in response to gentle contact stimulus, was assessed 72 h after treatment. The larvae were also weighed prior to desiccation, upon removal from each desiccation treatment, and following drying to constant mass at 60^{o}C over 24 h. From these values, initial water content and percentage water loss or gain were calculated (cf. Hayward *et al.*, 2007).

5.3.3. Desiccation induced low temperature tolerance

5.3.3.1. Effect of desiccation on the supercooling point (SCP)

Individuals of *H. borealis* and *E. murphyi* were held at 98.2% RH for either 6 h, 2 d, 4 d, 8 d or 12 d (only 12 d for *H. borealis*) prior to experimental treatment. Fifteen larvae were placed in contact with a thermocouple, within Beem capsules, in glass test tubes plugged with sponge, inside an alcohol bath (Haake Phoenix II C50P, Fisher Scientific UK Ltd, Loughborough, U.K.). Larvae were subsequently cooled from 4 to -30^{o}C at 0.5 min^{-1}. SCPs, defined as the temperature at the onset of the freezing exotherm, were identified using an eight channel datalogger interfaced to a computer and recorded using Picolog Recorder software (Pico Technology Limited, U.K.) (cf. Hawes *et al.*, 2006).

5.3.3.2. Lower discriminating temperature

The temperature at which 10-20% survival occurred (Lee *et al.*, 1987) was determined by cooling three replicates of 10 larvae at 0.2^{o}C min^{-1} to progressively lower sub-zero temperatures (-15 to -19^{o}C) for 2 h, before being re-warmed to the rearing temperature (4^{o}C) at the same rate. Larvae were placed in Eppendorf tubes, inside glass test tubes plugged with sponge, in an alcohol bath prior to each experimental treatment. Control groups were handled, and exposed, in the same way at 4^{o}C. The temperature experienced by the larvae was measured by placing a thermocouple within an identical Eppendorf tube into one of the glass test tubes. At the end of each experimental treatment, the larvae were rapidly transferred (over ice) from the Eppendorf tubes into plastic universal tubes containing moist Plaster of Paris and substratum, and returned to the rearing conditions. Survival was assessed as described previously. The highest

temperature at which survival was between 10 and 20% after 72 h recovery was defined as the discriminating temperature.

5.3.3.3. Effect of desiccation on low temperature tolerance

Larvae of *E. murphyi* only were held at 98.2% RH for 6 h, 2 d, 4 d, 8 d or 12 d prior to experimental treatment. Three replicates of 10 larvae were subsequently cooled at 0.2^{o}C min^{-1} to the discriminating temperature and held for 2 h before being re-warmed to the rearing temperature at the same rate. Larvae collection and handling, controls, thermocouple use, recovery and survival assessment were as described previously.

5.3.4. Desiccation-induced heat tolerance

5.3.4.1. Higher discriminating temperature

The temperature at which 10-20% survival occurred was determined by warming three replicates of 10 individuals at 0.2^{o}C min^{-1} to progressively higher temperatures (30 to 40^{o}C) for 2 h, before being re-cooled to the rearing temperature at the same rate. Larvae collection and handling, controls, thermocouple use, recovery and survival assessment were as described previously. The lowest temperature at which survival was between 10 and 20% after 72 h recovery was defined as the discriminating temperature. The lowest temperature at which survival was between 80 and 90% was also used to assess whether survival was lowered by a prior desiccation exposure.

5.3.4.2. Effect of desiccation on high temperature tolerance

Larvae of *E. murphyi* were held at 98.2% RH for 6 h, 2 d, 4 d, 8 d or 12 d prior to experimental treatment. Three replicates of 10 larvae were subsequently warmed at

$0.2^{o}C\ min^{-1}$ to the discriminating temperature, and the 80-90% survival temperature. Larvae were held for 2 h, and cooled to the rearing temperature at the same rate. Larvae collection and handling, controls, thermocouple use, recovery and survival assessment were as described previously.

5.3.5. Statistical analysis

The Kolmogorov-Smirnov test was used to check for normality in the survival, SCP and percentage water loss data. Normally distributed data were analysed using analysis of variance (ANOVA) and Tukey's multiple range test; data that were not normally distributed were analysed using the Kruskal-Wallis test.

5.4. Results

5.4.1. Water balance and desiccation tolerance

Larvae of *H. borealis* were significantly more desiccation resistant than those of *E. murphyi* (Fig. 5.1). After 12 d, larvae of *H. borealis* had lost only 6.9% of their water content, as compared with 46.7% in larvae of *E. murphyi*. Water loss rate was not constant in larvae of *E. murphyi*; between 2 and 4 d, they regained 13.6% of their initial water content, before losing water rapidly again thereafter. Survival following 6 h, 2 d, 4 d, 8 d and 12 d at 98.2% RH was high in larvae of both *H. borealis* and *E. murphyi*, and was not significantly different from survival in the control (Fig. 5.2).

5.4.2. Desiccation-induced cold tolerance

5.4.2.1. Effect of desiccation on the SCP

Prior exposure to 98.2% RH significantly lowered the SCP in larvae of both species (Table 5.1). In larvae of *H. borealis*, the SCP fell by 1.6°C after 12 d at 98.2% RH, while in larvae of *E. murphyi*, the SCP fell by up to 2.5°C.

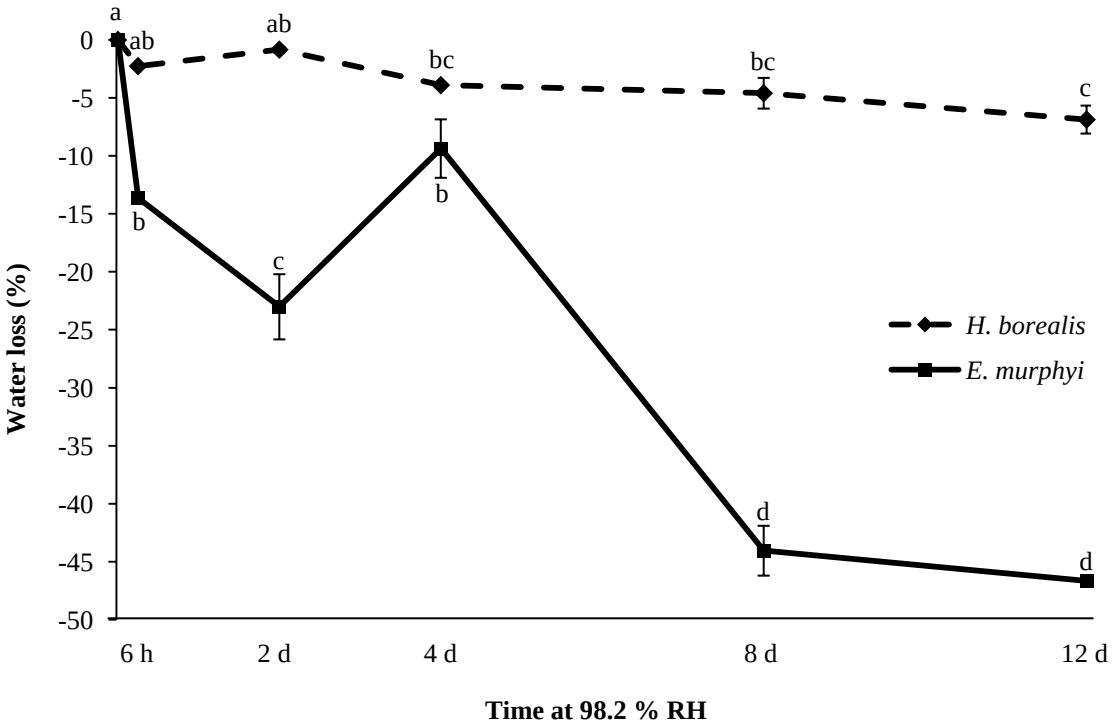

Fig. 5.1. *Percentage water loss or gain of larvae of H. borealis and E. murphyi following exposure to 98.2% RH for 6 h, 2 d, 4 d, 8 d and 12 d. Means ± S.E.M. are presented for three replicates of 10 individuals. Means with the same letter are not significantly different within each species group at P < 0.05 (Tukey's multiple range test).*

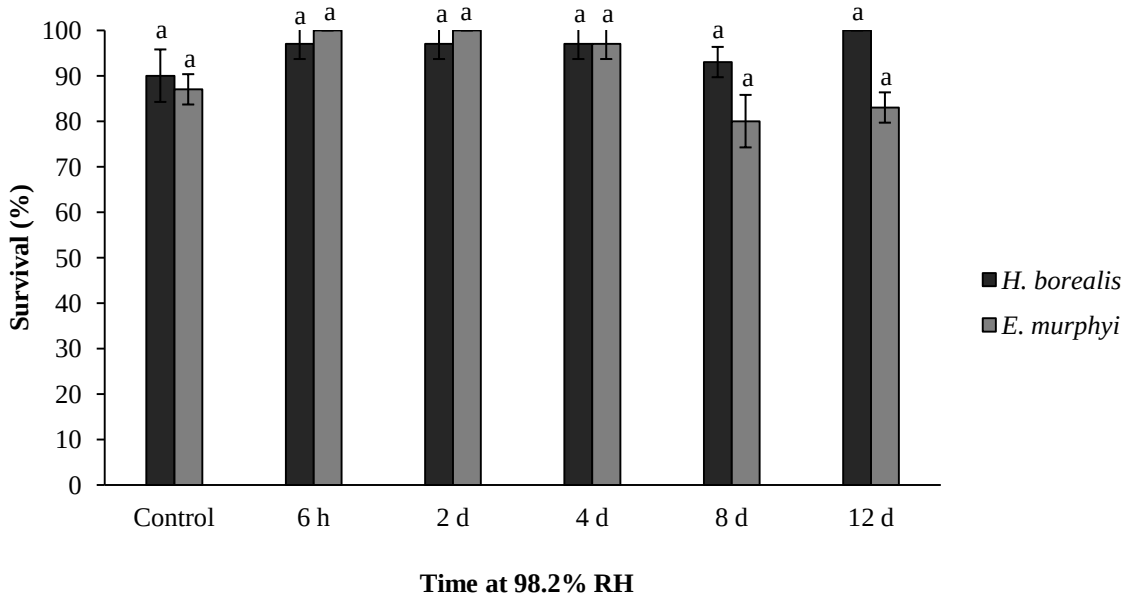

Fig. 5.2. *Survival (%) of larvae of H. borealis and E. murphyi following exposure to 98.2% RH for 6 h, 2 d, 4 d, 8 d and 12 d. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different from the control within each species group at P < 0.05 (Tukey's multiple range test).*

Table 5.1. *H. borealis and E. murphyi larval SCPs following exposure to 98.2% RH for 6 h, 2 d, 4 d, 8 d and 12 d (only 12 d for H. borealis). Means ± S.E.M. are presented for 16 replicates. Asterisks indicate a treatment significantly different from the control (0 h) at P < 0.05 (Tukey's multiple range test).*

Species	**SCP (°C)**					
	0 h	6 h	2 d	4 d	8 d	12 d
H. borealis	-7.70 ± 0.28	-	-	-	-	-9.29 ± 0.38*
E. murphyi	-5.05 ± 0.29	-6.16 ± 0.11	-6.21 ± 0.29	-5.36 ± 0.40	-7.52 ± 0.22*	-6.74 ± 0.46*

5.4.2.2. Lower discriminating temperature and the effect of desiccation on low temperature tolerance

Survival of *E. murphyi* larvae declined gradually following exposure to progressively lower temperatures (Fig. 5.3a). The discriminating temperature (20% survival) was determined to be -18^{o}C. At this temperature, survival of *E. murphyi* larvae was raised following all acclimation treatments (6 h, 2 d, 4 d, 8 d and 12 d) at 98.2% RH (Fig. 5.3b). The increase in survival was significant following 4 d.

5.4.3. Desiccation-induced heat tolerance

5.4.3.1. Higher discriminating temperature and the effect of desiccation on high temperature tolerance

Survival of *E. murphyi* larvae remained at 100% up to 35^{o}C, but declined rapidly at temperatures near to the upper lethal temperature (ULT), falling by 80% between 37 and 40^{o}C (Fig. 5.4a). The discriminating temperatures were determined to be 38.5 and 37^{o}C, giving 20% and 80% survival, respectively. At 37^{o}C, survival of *E. murphyi* larvae was unchanged at around 80% following 2, 4 and 8 d at 98.2% RH, but declined to 55% after 12 d (Fig. 5.4b). There was also no significant difference between non-acclimated and acclimated larvae of *E. murphyi* at 38.5^{o}C.

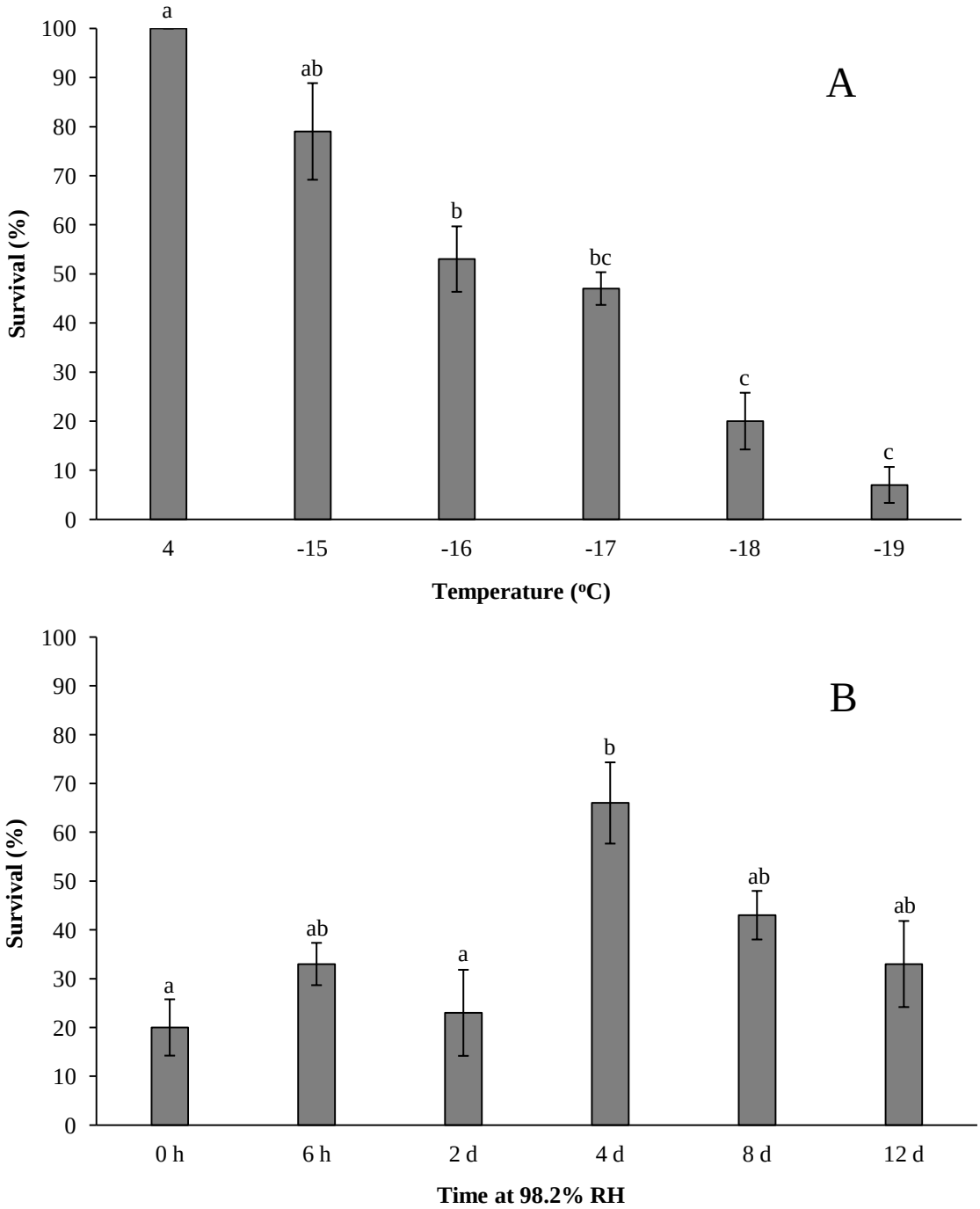

Fig. 5.3. *Survival (%) of larvae of E. murphyi after exposure to progressively lower sub-zero temperatures (-15 to -19°C) for 2 h (A), and after exposure to -18°C, following prior exposure to 98.2% RH for 6 h, 2 d, 4 d, 8 d and 12 d (B). Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different at P < 0.05 (Tukey's multiple range test).*

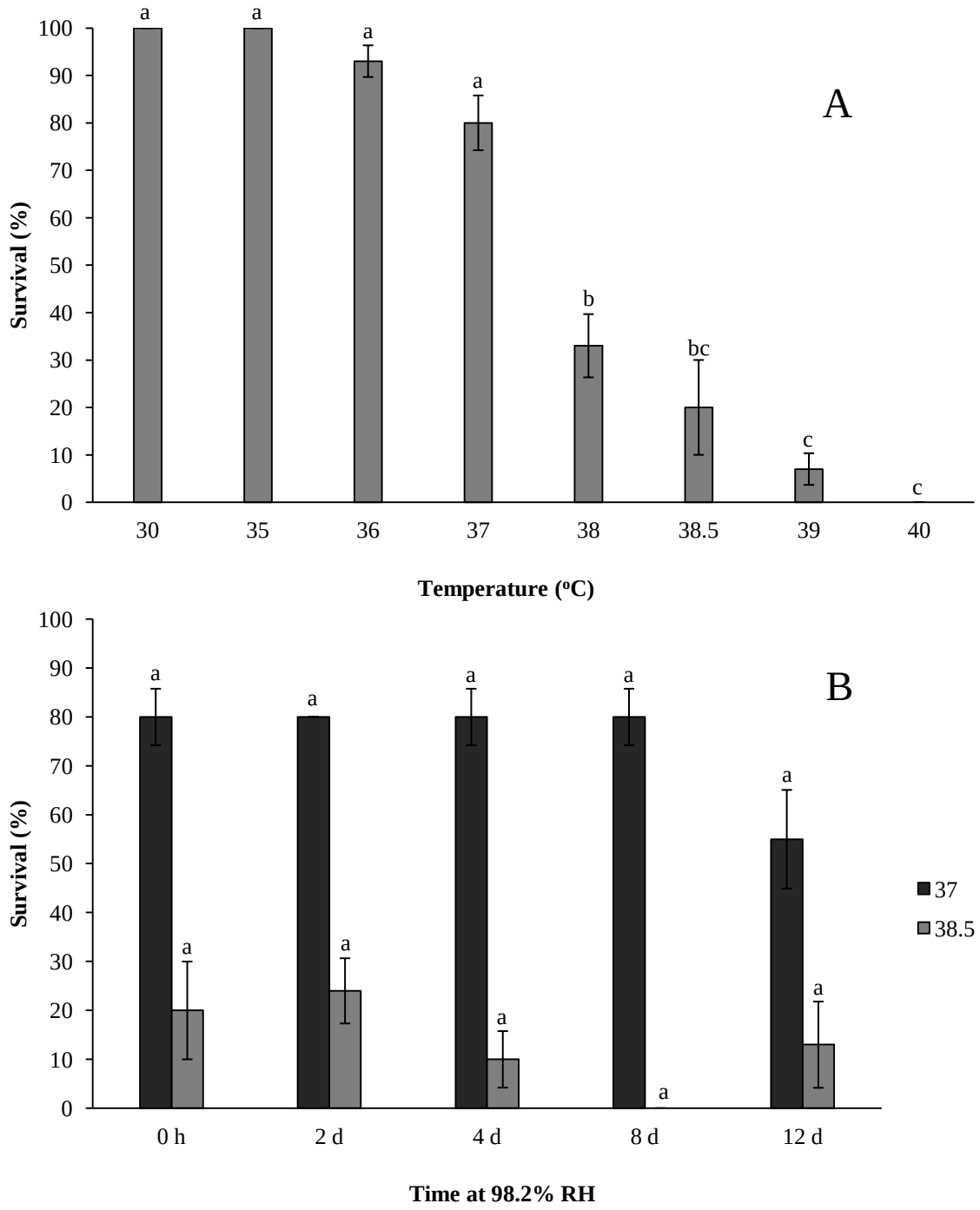

Fig. 5.4. *Survival (%) of larvae of E. murphyi after exposure to progressively higher temperatures (30 to 40°C) for 2 h (A) and after exposure to 37 or 38.5°C, following prior exposure to 98.2% RH for 6 h, 2 d, 4 d, 8 d and 12 d (B). Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different are across temperature treatments (a) and between temperature treatments (b) at P < 0.05 (Tukey's multiple range test).*

5.5. Discussion

5.5.1. Desiccation resistance

Water availability is limited for much of the year in the Arctic and Antarctic (Strathdee and Bale, 1998; Block *et al.*, 2009), and polar terrestrial invertebrates respond with one of two strategies, desiccation resistance or desiccation tolerance. Our data suggest that the Arctic dipteran, *H. borealis*, responds through desiccation resistance, while the Antarctic dipteran, *E. murphyi,* tolerates substantial desiccation (Fig. 5.1, 5.2). The rate of water loss in *E. murphyi* was seven times more rapid than in *H. borealis* and was similar to that reported by Worland (2010) at 88% RH. The closely related dipteran, *B. antarctica*, which also inhabits the maritime Antarctic, likewise shows a high rate of water loss. When exposed to 98% RH, larvae of this dipteran took around five days to lose 50%, and eight to ten days to lose over 60%, of their body water (Benoit *et al.*, 2007a).

There is therefore a clear difference in the level of desiccation resistance between *H. borealis* and the Antarctic Diptera, *E. murphyi* and *B. antarctica*. The physiology of the cuticular layer between these species provides a possible, albeit unexplored, explanation for the difference, and lowered cuticular permeability is a widespread adaptation that invertebrates use to raise their desiccation resistance (Danks, 2000). This reduced permeability is largely achieved through the modulation of the wax layer which coats the cuticle, and consists of bipolar molecules with hydrophobic and hydrophilic ends (Speight *et al.*, 2008). In the majority of species, the hydrophobic ends face outward and limit the rate of water loss and, while this is true of mesic species, these organisms tend to either accumulate or increase the length of hydrocarbons or hydrophobic molecules,

resulting in tighter packing and a greater reduction of water loss rate (Gibbs *et al.*, 1997; Benoit *et al.*, 2007b). This is exemplified in the mesic mite, *Alaskozetes antarcticus*, which lost water at a rate of 0.21-0.36% h^{-1} at 0% RH, around 5-6% less than in the mites *Hydrogamasellus antarcticus* and *Rhagidia gerlachei*, which had two to three times less hydrocarbons in their wax layer (Benoit *et al.*, 2007b). Benoit *et al.* (2007b) have shown that the length of hydrocarbons increases in desiccated larvae of *B. antarctica*, but this change was only slight and there was no change in the number of hydrocarbons. *Eretmoptera murphyi* is closely related to *B. antarctica* (Allegrucci *et al.*, 2006, 2012) and may possess similar physiological adaptations. We speculate that the initial composition and change in the cuticle layer is more biased towards a greater number and length of hydrocarbons in *H. borealis* than either Antarctic dipteran.

Body melanisation is a common source of phenotypic variation among insects, including Diptera. Recent studies on species of *Drosophila* have shown a correlation between body melanisation and desiccation resistance, which corresponds with the aridity of the flies' local climate. Parkash *et al.* (2008a, b) demonstrated greater desiccation resistance in darker morphs of *Drosophila melanogaster* and *D. immigrans* that were predominantly found in drier, high altitude habitats, as compared with their lighter counterparts found at lower altitudes. Likewise, lower desiccation resistance of *D. melanogaster* and *D. ananassae* during the rainy season was correlated with lower melanisation (Parkash *et al.*, 2009, 2012). Greater desiccation resistance in strains of *D. melanogaster* selected for higher levels of melanisation, and the reverse in those selected for lower levels, has now provided direct evidence of the phenomenon (Ramniwas *et al.*, 2013). Differences in melanisation between larvae of *H. borealis* and *E. murphyi* may also offer an explanation for the differing levels of resistance in the

current study, but this was not accounted for. Juvenile forms of *E. murphyi* are clear in appearance, while mature stages are more opaque and yellow in colour (Cranston, 1985). In contrast, *H, borealis* larvae all have an opaque, off white appearance (pers. obs. of individuals used). Future studies may benefit from separating the different stages of larvae of the Antarctic midge.

It should also be noted that 98.2% RH may be a sufficiently high humidity that larvae of *H. borealis* are able to absorb water from the atmosphere. This may also underlie our observation that, between two and four days' exposure at 98.2% RH, larvae of *E. murphyi* exhibited reduced water loss and even rehydration. Rehydration has also been observed in other species. In the collembolan, *Folsomia candida*, nearly all of the water lost initially at 98.2% RH was recovered within 5-7 d, despite being continually held at 98.2% RH (Bayley and Holmstrup, 1999). As confirmed by microarray, this recovery was supplemented by accumulating and synthesising myo-inositol, glucose and trehalose, which allowed the collembolan to become hyperosmotic to the environment and absorb moisture (Timmermans *et al.*, 2009). An analogous response may be present in *E. murphyi*. However, such a response has not been observed in the closely related *B. antarctica*, which was unable to absorb water from the atmosphere at any RH, except complete saturation (100% RH) (Benoit *et al.*, 2007a; Hayward *et al.*, 2007).

5.5.2. Desiccation tolerance

Heleomyza borealis showed greater than 90% survival following 12 d of desiccation (Fig. 5.2). However, because water loss was so slight, even after 12 d at 98.2% RH, larvae cannot be said to have tolerated desiccation. Instead, it suggests that larvae of *H. borealis* are able to survive well under ecologically relevant relative humidities using a

desiccation resistance strategy. Conversely, larvae of *E. murphyi* tolerated desiccation, having shown considerable water loss, but also survival, following 12 d at 98.2% RH (Fig. 5.2). *Belgica antarctica* also principally uses a desiccation tolerance strategy, and has been shown to survive well following a 75% loss of initial water content (Benoit *et al.*, 2007a; Hayward *et al.*, 2007).

One means of tolerating desiccation is through possessing high initial water content, as an organism must subsequently lose more water before reaching a point at which damage occurs or energy intensive mechanisms are induced (cf. Hayward *et al.*, 2007). This argument is reinforced by the increased water content observed in selected desiccation tolerant lines of *Drosophila melanogaster* (Gibbs, 2002). In *E. murphyi* larvae, the initial water content was high, averaging 74.3% (73.28-75.40%) of body mass (cf. Benoit *et al.*, 2009a). We did not assess osmotically active water (OAW) in this study, though *B. antarctica* is known to have very high OAW content relative to temperate species (Hayward *et al.*, 2007). Once considerable water loss does occur, as was the case in the current study, the potential for injury is great and an organism must adapt accordingly. Injuries that result from desiccation include protein denaturation and unwanted macromolecular interactions (Benoit *et al.*, 2009a), crystalline to gel phase transitions (Hazel, 1995), oxidative damage (Lopez-Martinez *et al.*, 2008), and mechanical stress (Li *et al.*, 2009). The responses of *B. antarctica* in this regard have been particularly well studied. Larvae accumulate glycerol and trehalose, which are suggested as being replacements for lost water and/or an aid to the production of amorphous sugar glasses (Danks, 2000; Benoit *et al.*, 2007a; Bahrndorff *et al.*, 2009; Benoit *et al.*, 2009a; Hengherr *et al.*, 2009; Michaud *et al.*, 2008; Clarke *et al.*, 2013). Protein denaturation is also ameliorated via the up-regulation of HSPs in response to

desiccation (Lopez-Martinez *et al.*, 2009; Teets *et al.*, 2012), and the fluidity of the membrane maintained using enzymes such as Δ9 FAD desaturase (Lopez-Martinez *et al.*, 2009). Further physiological mechanisms induced in response to desiccation include oxidative damage repair through the accumulation of antioxidants (Lopez-Martinez *et al.*, 2008), the minimisation of mechanical stress via the restructuring of the cytoskeleton (Li *et al.*, 2009), the inhibition of apoptosis through the regulation of autophagy, and the suppression of metabolism (Teets *et al.*, 2012). Larvae of *B. antarctica* therefore possess a suite of physiological responses against injuries resulting from desiccation. It is possible that *E. murphyi* possesses similar physiological adaptations that underlie its high level of desiccation tolerance. Indeed, the capacity to which they respond to temperature is very similar (Lee *et al.*, 2006b; Everatt *et al.*, 2012).

5.5.3. Desiccation-induced cross-tolerance

5.5.3.1. Low temperatures

Survival of *E. murphyi* at -18^{o}C was significantly raised following desiccation at 98.2% RH (Fig. 5.3b). Greater survivorship at low temperatures, following pre-exposure to unsaturated conditions, has also been observed for a number of other invertebrates, including *B. antarctica* (Benoit *et al.*, 2009a), and the springtails *Cryptopygus antarcticus* (Elnitsky *et al.*, 2008a; Everatt *et al.*, 2013b) and *Folsomia candida* (Holmstrup *et al.*, 2002). Cross-tolerance is thought to occur between desiccation and low temperature because injuries that result from the two stresses are similar. Consequently, the physiological mechanisms induced in response to desiccation and low temperatures are often analogous (e.g. Bayley *et al.*, 2001), and act in concert to

give greater protection. Even a mild desiccation treatment resulting in 6-10% water loss has been shown to confer significant gains in cold tolerance in the goldenrod gall fly, *Eurosta solidaginis* (Levis *et al.*, 2012). Interestingly, survival of -18°C was highest after 4 d at 98.2% RH, and not after longer durations of 8 and 12 d (Fig. 5.3b). This corresponds with the time period at which larvae of *E. murphyi* exhibited rehydration (Fig. 5.1), suggesting physiological processes associated with rehydration provide cold tolerance, and that these are additive to the protection provided by those solely concerned with desiccation tolerance.

An effect on the supercooling point (SCP) was also observed in the current study for both *H. borealis* and *E. murphyi*. Following a pre-exposure to 98.2% RH, the SCP was significantly reduced (Table 5.1). Both dipteran species are freeze-tolerant and it is therefore preferable for extracellular ice formation to take place at higher sub-zero temperatures, as it occurs more slowly and decreases the chance of tissue damage (Worland and Block, 1999). The lowering of the SCP in *H. borealis* and *E. murphyi* was therefore more likely a by-product of, rather than an adaptation to, desiccation. Water loss passively increases the concentration of solutes already present and results in the colligative lowering of the SCP (e.g. Holmstrup and Zachariassen, 1996). The dipteran larvae were also starved during the desiccation treatments and, during periods of starvation, ice-nucleating gut contents may be removed, reducing the likelihood of ice formation (Sømme and Block, 1982; Cannon and Block, 1988). Worland *et al.* (2006) also reported that time spent without access to food on water surfaces lowered the SCP of the collembolan, *Ceratophysella denticulata*.

5.5.3.2. High temperatures

Prior exposure to desiccation at 98.2% RH had either no effect or a negative effect on the heat tolerance of *E. murphyi* larvae (Fig. 5.4b). Unlike low temperatures, injuries incurred as a result of high temperatures are dissimilar to those of desiccation, and physiological defences mounted in response to desiccation are therefore also different, and could even be conflicting. Consequently, little protection is afforded by prior acclimation to desiccation. A similar response has been observed in nematodes (Holmstrup and Zachariassen, 1996) and Collembola (Everatt *et al.*, 2013b). However, improved heat tolerance has been noted in other invertebrates, particularly those which are anhydrobiotic (e.g. Hinton 1951, 1960; Sakurai *et al.*, 2008). It is speculated that anhydrobiotic organisms, because of their tendency to vitrify, are less susceptible to injuries in general and that conflicting injuries are therefore less important. While this explanation is appropriate for anhydrobiotic organisms, the same is not true of partially desiccated organisms, which tend not to vitrify. At 30^{o}C, heat tolerance was improved in partially desiccated larvae of *B. antarctica* following pre-exposure to 0, 75 and 98% RH (Benoit *et al.*, 2009a). In this instance, the up-regulation of heat shock proteins and the accumulation of trehalose were suggested as being possible explanations for the enhanced heat tolerance by overcompensating for any opposing injuries.

The heat tolerance of *E. murphyi* has been little explored, except in a study by Everatt *et al.* (in review), which showed larval survival up to 39°C for 1 h. In the current study, larvae of *E. murphyi* showed 100% survival up to 35°C, and also survived temperatures as high as 39^{o}C for the longer period of 2 h (Fig. 5.4a). Larvae of *B. antarctica* are likewise able to survive temperatures above 30^{o}C (Benoit *et al.*, 2009a). Although these

organisms rarely, if ever, experience temperatures nearing 30 or even 25^{o}C, their heat tolerance is not surprising. A number of other studies, including those by Deere *et al.* (2006), Everatt *et al.* (2013a), Sinclair *et al.* (2006) and Slabber *et al.* (2007) have similarly shown appreciable heat tolerance in polar invertebrates. Such findings are consistent with the 'thermal sensitivity hypothesis', which states that the thermal sensitivity of invertebrates to a temperature rise declines with increasing latitude (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008).

5.6. Conclusion

The Arctic and Antarctic are similar in that they include both cold and arid landscapes. However, the Diptera of these regions, based on the evidence presented here, have not adapted similarly. Two strategies of living in a dry environment have been identified. The Arctic dipteran, *H. borealis*, utilises desiccation resistance, while the Antarctic dipteran, *E. murphyi*, principally uses desiccation tolerance. Divergence between Antarctic and Arctic invertebrates has also been shown between *B. antarctica* and *M. arctica*, which utilise distinct molecular mechanisms in response to desiccation (Teets *et al.*, 2012). Desiccation was found to induce cross-tolerance to low temperatures, but not high temperatures, in *E. murphyi*. An ability to acclimate in this way would likely be beneficial in the variable climates typical of polar terrestrial habitats, where low temperature and low water availability are commonly encountered simultaneously.

Chapter transition

Unlike this Chapter and Chapter 4, the next Chapter will be looking at the capacity of a polar terrestrial invertebrate to tolerate an inundation of water, a further water stress experienced in the polar regions.

CHAPTER 6: CAN THE TERRESTRIAL MIDGE, *ERETMOPTERA MURPHYI*, TOLERATE LIFE IN WATER

The work of this chapter has been submitted to Ecological Entomology.

6.1. Abstract

Early season flooding and ice entrapment at sub-zero temperatures pose significant challenges to any terrestrial invertebrate. The chironomid midge, *Eretmoptera murphyi*, is native to the sub-Antarctic island of South Georgia and has been introduced to maritime Antarctic Signy Island. The majority of its two year life cycle is spent as a terrestrial larva. The current study explored the tolerance of the larvae to extended submergence. We demonstrate extended survival (28 d) in water and that this is likely underlain by their ability to respire whilst submerged, an ability so far not shared by any other terrestrial midge. Larvae also demonstrated notable anoxia tolerance whilst encased in ice, surviving for up to 28 d. The results observed indicate a capacity of the midge to survive ecologically-relevant periods of submergence and/or ice entrapment, such as may typically occur in their natural habitats.

6.2. Introduction

Eretmoptera murphyi is a freeze-tolerant, parthenogenetic midge that is endemic to the sub-Antarctic island of South Georgia (55°S, 37°W). Probably as a result of plant transplant trials in the 1960s, the midge was introduced to Signy Island in the maritime Antarctic (60°S, 45°W) (Block *et al.*, 1984; Convey and Block, 1996), and has since

spread to occupy an area of more than 2000 m^2, with densities of up to 400000 individuals m^{-2} (Worland and Hughes, 2010). The midge has a biennial life cycle which is largely spent as a detritivorous larva, with the non-feeding and flightless adult only active for a short period in mid-summer (Block *et al.*, 1984; Convey, 1992; Convey and Block, 1996). On South Georgia, larvae are commonly found in vegetation adjacent to meltwater streams (Cranston, 1985), while on Signy Island they are found within a sloping soil/moss habitat (Convey, 1992; Worland, 2010).

During the summer in sub- and maritime Antarctic habitats, freshwater arising from snow and ice melt often saturates the soil. This enforced switch between a terrestrial and an aquatic lifestyle for the invertebrates of these habitats poses a number of challenges to organisms which predominantly respire, feed and develop in a terrestrial environment. These challenges are exacerbated further under sub-zero conditions, when invertebrates are at risk from ice entrapment and subsequent anoxia (Hodkinson and Bird, 2004; Sømme and Block, 1982). For larvae of *E. murphyi*, this may mean tolerating anoxia for hours, days or weeks in spring and autumn, and possibly for months over the winter (Convey, 1996). An ability to tolerate hypoxic and anoxic conditions may also be used by larvae of *E. murphyi* to survive submergence in freshwater. Alternatively, it is possible that the larvae are capable of respiration whilst submerged, in common with most non-biting midge larvae (also members of the Chironomidae) which are fully aquatic (Brodersen *et al*., 2008). If so, *E. murphyi* may be capable of utilising an ancestral trait of this midge family.

Oxygen is a limiting factor in freshwater habitats and is an important regulator of chironomid population structure and abundance (Brodersen *et al.*, 2008). Understanding the respiratory adaptations of chironomids can offer insight into this regulation

(Brodersen and Quinlan, 2006). By measuring respiration under depleting levels of oxygen, it is also possible to identify the strategy used under low oxygen conditions (Berg *et al.*, 1962), and in turn assess the potential impact of climate change, as the solubility of oxygen diminishes at higher temperatures (Verbek and Bilton, 2013). There are three known respiration strategies - oxy-regulation, oxy-conformation and oxy-stressor. Organisms which oxy-regulate maintain a constant level of oxygen consumption as the level of ambient oxygen decreases until they reach a critical point of oxygen saturation, when regulation fails and respiration switches from aerobic to anaerobic (Berg *et al.*, 1962; Bridges and Brand, 1980; Tschischka *et al.*, 2000). Organisms of this group are generally found in low oxygen environments, in which the maximisation of oxygen consumption is critical (Brodersen and Quinlan, 2006). In contrast, organisms which are found in well-oxygenated environments tend to oxy-conform and show rates of oxygen consumption that parallel the level of ambient oxygen until a critical point is reached (Berg *et al.*, 1962). Oxy-stressors are less common and are characterised by an initial increase in oxygen consumption at low ambient levels prior to the critical point, as reported in the chironomid, *Micropsectra* sp. (Brodersen *et al.*, 2008).

In this study, we investigated (i) the tolerance of *E. murphyi* to submergence in water, (ii) its capacity for aquatic respiration, and (iii) survival of ice entrapment and hence of anoxia.

6.3. Materials and methods

6.3.1. Insect collection and storage conditions

Summer acclimatised larvae of *E. murphyi* were collected from soil and moss on Signy Island (60°S, 45°W) near to the British Antarctic Survey's Signy Research Station between January and March 2013. They were transported to the University of Birmingham under cool conditions (4-6°C) in plastic boxes containing substratum from the site of collection and subsequently held at 5°C (L:D 0:24) upon arrival. The duration of transport was approximately two months.

6.3.2. Tolerance of water submergence

Larvae were submerged in groups of 10 individuals in tap water inside small plastic containers and held at 5°C (L:D 0:24). Survival was assessed after 7, 14 and 28 d in water, either immediately following removal from water, or following 72 h recovery in plastic containers with a base of moist Plaster of Paris and a small amount of substratum. Larvae were also held in deionised water as a comparison, but limited samples only permitted assessment after 7 d (one replicate N=10). Survival was defined by individuals moving either spontaneously or in response to gentle contact stimulus. Three replicates of 10 individuals were used for each treatment duration.

6.3.3. Respirometry

Oxygen consumption of individual midge larvae was measured using the Unisense respirometry system (MicroResp, Unisense, Denmark) (Brodersen *et al.*, 2008). Five larvae were each transferred into separate 0.3 mL glass respiration chambers containing an OECD medium, and fitted with glass stoppers to prevent the entry of additional

medium but to give access to an oxygen microelectrode through a capillary hole. The experiment was carried out at 10^{o}C. Mixing of the OECD medium was achieved using a circulating glass-coated magnet, separated from the larvae by mesh on top of a plastic ring within each respiration chamber. Each respiration chamber had a magnetic stirrer (set to 120 rpm) implanted underneath to allow for the circulation of the glass coated magnets. Mixing was necessary as the terrestrial larvae only showed minimal movement when placed in the OECD medium. Oxygen level within each respiration chamber was monitored at set time points (regularly over 3 d) using an oxygen microelectrode and recorded using MiCox software from a multi-channel pA meter through an a/d converter. The equipment was regulated using a negative control (anoxic, sodium hydroxide and sodium ascorbate mixture) and a positive control (saturated, OECD medium alone). Following respiration in the OECD medium, larval dry mass (DM) was determined by weighing individual larva on a microbalance after drying on paper and then for 36 h at 50°C to constant mass in a drying oven. Oxygen consumption data were subsequently corrected for each larva based on their dry masses. Respiration rates (R) were expressed as µmol O_2 g DM^{-1} h^{-1}.

6.3.4. Ice entrapment

Larvae of *E. murphyi* were submerged in tap water inside Eppendorf tubes and held at -2°C (L:D 24:0) for up to 28 d. Within a few hours of being placed at -2°C, the water froze and the larvae became encased in ice. Encasement within ice was assumed equivalent to an anoxic environment, though metabolism at -2°C would have been slow and tissues may not have experienced anoxia immediately. Survival was assessed 72 h after each treatment (7, 14 and 28 d) as described above except that, to allow the ice to

melt, survival was first recorded after 7-8 h at 5°C. Three replicates of 10 individuals were used for each time duration.

6.3.5. Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether activity threshold, water submergence, ice entrapment and respirometry data were normally distributed. Normally distributed data were analysed using analysis of variance (ANOVA) and Tukey's multiple range test, and non-normally distributed data were analysed using the Kruskal-Wallis test.

6.4. Results

6.4.1. Tolerance of water submergence

Larvae tolerated submergence in tap water for up to 28 d (Fig. 6.1). After 7 d, survival declined to 53%, but did not drop any further by 14 d (57%). Survival was lowest after 28 d exposure (31%). Survival time in each time treatment did not differ significantly ($P > 0.05$, Tukey's multiple range test). Larval survival was 100% after 7 d in deionised water.

6.4.2. Respiration during submergence in water

As oxygen saturation decreased, the oxygen consumption of the larvae was maintained, until oxygen saturation was 0%, with one exception, which showed a downward trend with decreasing oxygen (Fig. 6.2).

6.4.3. Ice entrapment

Survival of *E. murphyi* larvae declined to 70% after 7 d of ice entrapment, and to 50% after 14 d. A further significant decline to 13% was seen after 28 d (Fig. 6.3). Recovery following ice entrapment was delayed, with movement shown by some individuals after 72 h recovery at 4°C but not when assessed immediately after melting of the ice (data not shown).

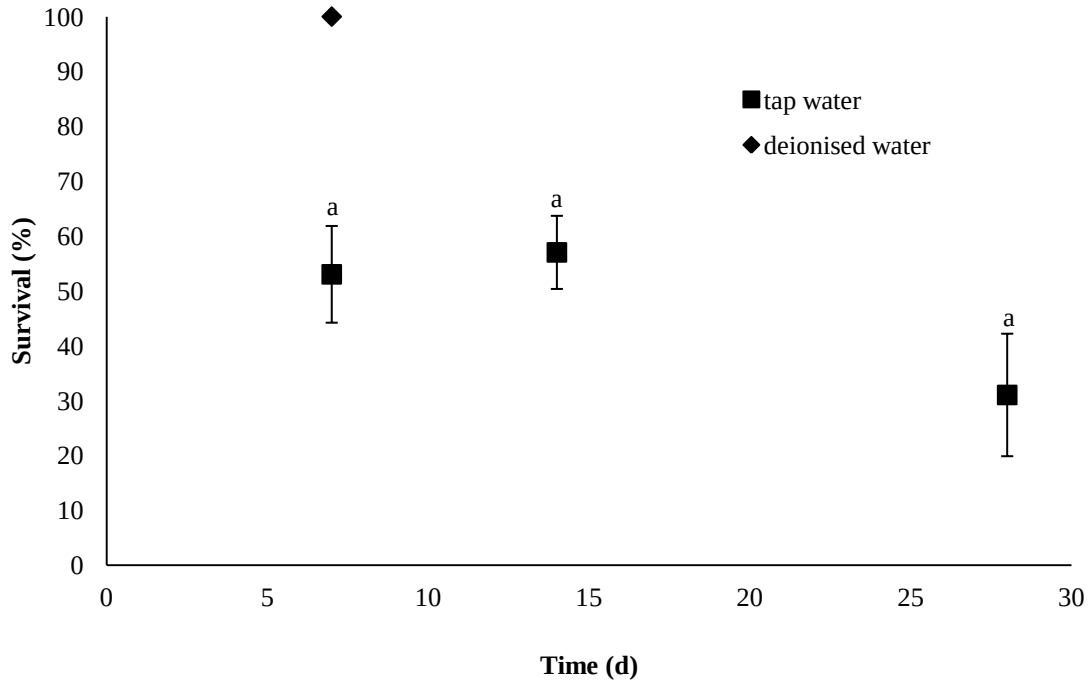

Fig. 6.1. *Mean percentage survival of larvae of E. murphyi, following 7, 14 and 28 d submergence in tap water, and 7 d submergence in deionised water. Means ± S.E.M. are presented for three replicates of 10 individuals (one replicate of 10 individuals for deionised water). Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each species group at P < 0.05 (Tukey's multiple range test).*

6.5. Discussion

6.5.1. Submergence in water

Many polar terrestrial habitats are vulnerable to large seasonal fluctuations in water availability, often but not only timed with the spring melt. The terrestrial habitats of South Georgia and Signy Island, in which the midge *E. murphyi* occurs, are vulnerable to flooding during the summer months (Sømme and Block, 1982). The current study shows *E. murphyi* to be capable of tolerating submergence in water for at least 28 d. The ability to survive such a prolonged exposure suggests that the midge is either able to tolerate anoxia or is able to respire while submerged. The respirometry approach used here demonstrated that the larvae can respire in an aquatic environment (Fig. 6.2), and that rates are comparable with wholly aquatic midge larvae (e.g. Brodersen and Quinlan, 2006; Brodersen *et al.*, 2008; Lencioni *et al.*, 2008). Our demonstration of respiration in the terrestrial midge *E. murphyi* (Convey, 1992) is quite unique, and as far as we know has not been demonstrated in any other polar terrestrial invertebrate or midge species.

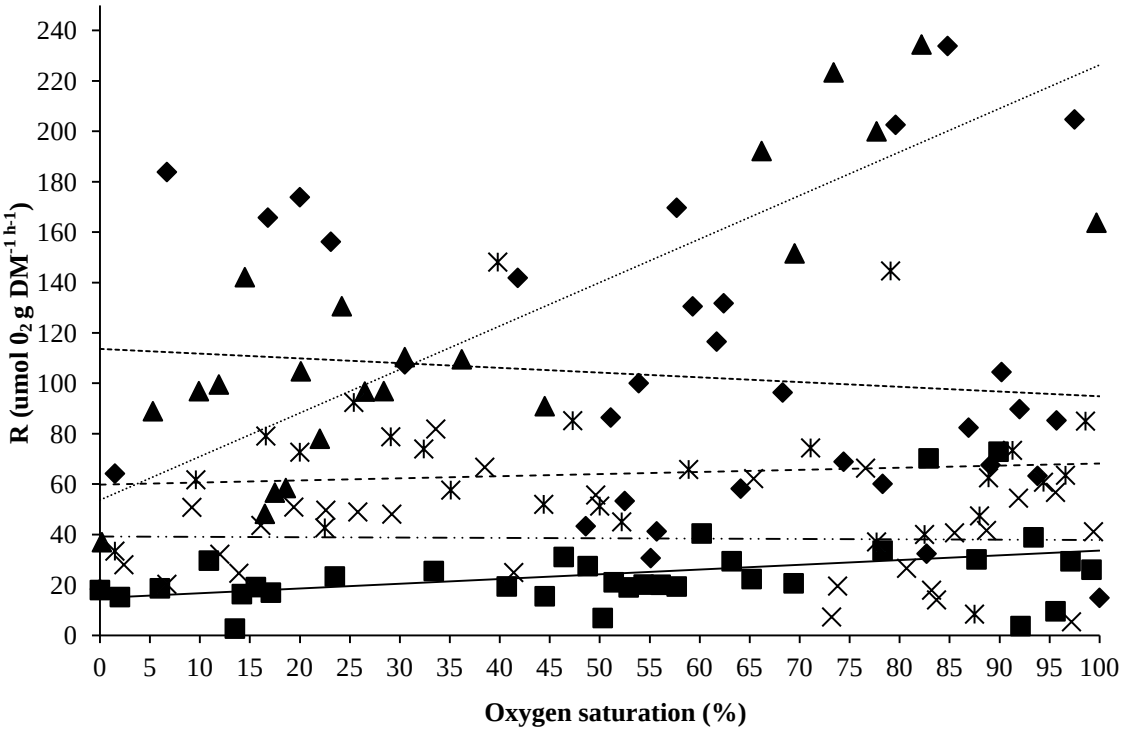

Fig. 6.2. *Respiration rate (R) curve for larvae of E. murphyi at 10°C. R (umol O_2 g DW^{-1} h^{-1}) is plotted in relation to oxygen saturation. Data points and trend lines are shown for five larvae: 1 – triangle data points with small dotted trend line, 2 – diamonds with short dashed line, 3 – stars with medium dashed line, 4 – crosses with long dashed line, and 5 – squares with full line. Negative values and values exceeding 250 umol O_2 g DW^{-1} h^{-1} were considered outliers and were removed (<15). These may have been due to the probe not being given sufficient time to settle prior to measurement.*

Very few insects are currently considered to be amphibious (Miller *et al.*, 2007; Saunders, 2010; Swennan and Buatip, 2009; Rubinoff, 2008; Rubinoff and Schmitz, 2010), and there is only one example of a terrestrial genus (*Hyposmocoma*, Lepidoptera) whose larvae have evolved an ability to forage and possibly pupate underwater as well as in the terrestrial environment (Rubinoff 2008; Rubinoff and Schmitz 2010). To our knowledge, there are no examples reported of midge larvae that can respire in both air and water. In the light of our study, there are other closely-related Antarctic chironomid species which may show this potential, including *Belgica antarctica* (Jacobs), which is endemic to the maritime Antarctic, and *B. albipes* (Seguy), which is endemic to the sub-

Antarctic Îles Crozet (Allegrucci *et al.*, 2013). *Belgica antarctica* larvae are known to survive well (~ 100% survival) for at least 10 d in 'field' water (Elnitsky *et al.*, 2009), though whether this represents anoxia tolerance or an ability to respire under water remains unexplored. *Eretmoptera murphyi* larvae the maintained consistent levels of oxygen consumption with decreasing oxygen saturation (Fig. 6.2), which is indicative of oxy-regulation (Berg *et al.*, 1962; Bridges and Brand, 1980; Tschischka *et al.*, 2000). This ability to regulate oxygen consumption may be critical to their fitness underwater, especially as they are likely to inhabit oxygen-poor, stagnant water. *Hyposmocoma* moths, in contrast, are thought to possess poor respiratory capabilities and are only found in fast flowing, oxygen-rich waters (Rubinoff and Schmitz, 2010).

While larvae of *E. murphyi* were able to survive for 28 d in tap water, only 31% did so and there was considerable mortality after 7 d (Fig. 6.1). The data for de-ionised water, 100% survival after 7 d submergence, suggests tap water may have had a negative effect on larvae, and future studies should use water acquired from their environment. The 100% survival of *B. antarctica* after 10 d submergence in 'field' water (Elnitsky *et al.*, 2009) further supports the idea that Antarctic midge larvae are able to tolerate flooding of their usually terrestrial environments. The maximum extent of this submergence tolerance is yet to be determined in *E. murphyi*, and should ideally be conducted under semi-field conditions.

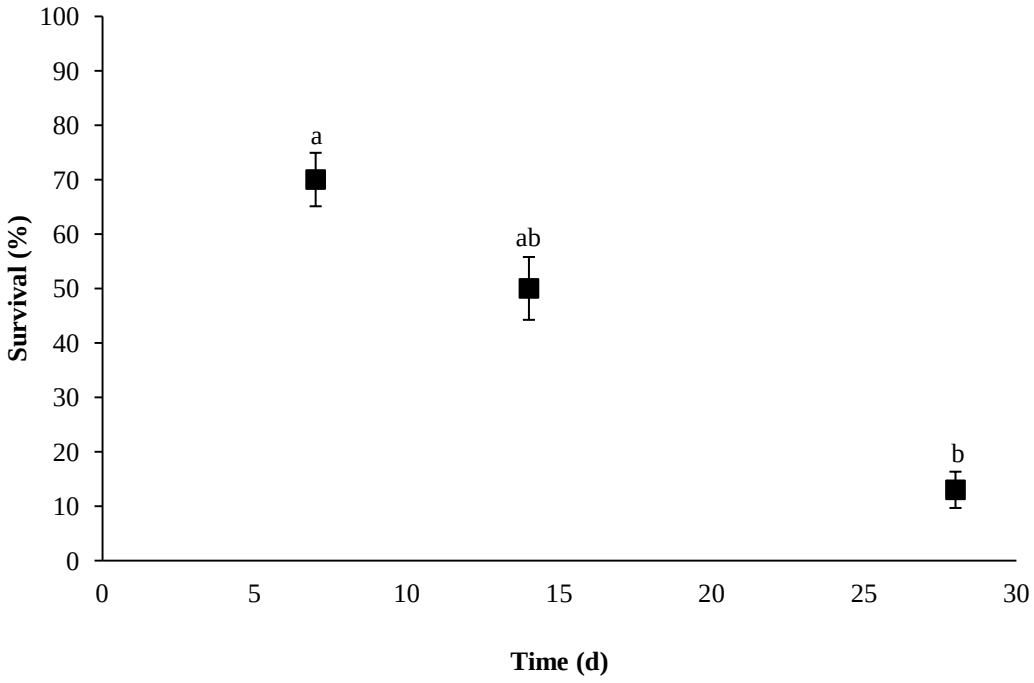

Fig. 6.3. *Mean percentage survival of larvae of E. murphyi, following 7, 14 and 28 d encasement in ice. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each species group at P < 0.05 (Tukey's multiple range test).*

6.5.2. Ice entrapment

As a result of encasement in ice at sub-zero temperatures, periods of hypoxia and/or anoxia are not uncommon to terrestrial invertebrates in the polar regions (Hodkinson and Bird, 2004; Sømme and Block, 1982). Some polar terrestrial invertebrates have consequently evolved considerable capacity for anoxia tolerance. Studies of both Antarctic (Block and Sømme, 1982, 1983; Sømme and Block, 1982, 1984), and Arctic arthropods (Hodkinson and Bird, 2004) have demonstrated that anoxia tolerance in these organisms can permit survival over days and sometimes weeks. Anoxia tolerance in alpine arthropods is similarly strong (Conradi-Larsen and Sømme, 1973; Leinaas and Sømme, 1984; Meidell, 1983; Sømme and Conradi-Larsen, 1977; Sømme, 1974). Some

beetles, such as *Pelophila borealis*, can survive for greater than 120 d under anoxic conditions (e.g. Sømme, 1974).

Larvae of *E. murphyi* are able to respire in water and are therefore unlikely to be exposed to anoxia while submerged during early season flooding. However, there is a clear possibility that larvae may become trapped, and experience anoxia, during periods of ice formation, both in short-term freezing events and at the onset of winter conditions. Our data indicate that larvae were able to survive anoxia for at least 28 d (Fig. 6.3). This level of tolerance is comparable with that reported in other invertebrates from Signy Island, including the collembolan, *Cryptopygus antarcticus*, and mite, *Alaskozetes antarcticus* (Block and Sømme, 1982; Sømme and Block, 1982).

Under anoxic conditions, invertebrates lower their metabolism and switch from aerobic to anaerobic respiration, producing ATP by conversion of glycogen to lactate and alanine (Hoback *et al.*, 2000; Wegener, 1993; Rutherford and Thuesen, 2005). Another advantageous characteristic of small invertebrates such as mites, springtails and midge larvae, may be their mode of gas exchange, utilising a one-step tracheal system which does not require the expenditure of ATP and is, instead, governed by diffusion. In contrast, a number of invertebrates utilise a two step system of ventilation and subsequent circulation of oxygen. Significantly, the circulation of oxygen requires the use of ATP (Schmitz and Harrison, 2004).

While larvae of *E. murphyi* are likely to be able to tolerate the short periods of anoxia of hours or days which may occur during the spring, summer and autumn, based on our data they would struggle to survive months of anoxia, as may occur in winter. It should also be noted that these lab cultures of larvae were in a more impoverished condition

than field samples, and so may possess better basal anoxia tolerance than observed. Acclimation to low temperatures, as occurs in the approach to winter (Worland, 2010), may also confer anoxia tolerance. The physiological symptoms and injuries resulting from low temperature and anoxia are similar, and any response to low temperature may also be similar to that to anoxia. Indeed, cross-tolerance between these two stresses in an invertebrate has been demonstrated (Yoder *et al.*, 2006).

6.6. Conclusion

To date, the success of *E. murphyi* in its native and introduced Antarctic terrestrial habitats has been attributed to its life cycle traits (Convey, 1992), low temperature tolerance (Block *et al.*, 1984; Everatt *et al.*, 2012; Worland, 2010), desiccation tolerance and cross-tolerance (Everatt *et al.* in press). We provide evidence here that this terrestrial midge also possesses a unique ability to respire in water and tolerate prolonged submergence. This, combined with anoxia tolerance under ice entrapment, further enhance its ability to survive the multiple stresses posed by polar terrestrial environments.

Chapter transition

Over Chapters 2 to 6 the capacity of polar terrestrial invertebrates to tolerate current environmental conditions was explored. In the following two Chapters, the capacity of these animals to tolerate future changes, with respect to climate warming, is investigated for four invertebrates, first with *C. antarcticus* and *A. antarcticus*, and secondly with *M. arctica* and *E. murphyi*.

CHAPTER 7: HEAT TOLERANCE AND PHYSIOLOGICAL PLASTICITY IN THE ANTARCTIC COLLEMBOLAN, *CRYPTOPYGUS ANTARCTICUS*, AND MITE, *ALASKOZETES ANTARCTICUS*

The work of this chapter has been published in the Journal of Thermal Biology (Everatt, M. J., Convey, P., Worland, M. R., Bale, J. S. and Hayward, S. A. L. (2013) Heat tolerance and physiological plasticity in the Antarctic collembolan, *Cryptopygus antarcticus*, and mite, *Alaskozetes antarcticus*. Journal of Thermal Biology. 38, 264-271.)

7.1. Abstract

Polar amplification of global warming has led to an average 2 °C rise in air temperatures in parts of the polar regions in the last 50 years. Poikilothermic ectotherms that are found in these regions, such as Collembola and mites, may therefore be put under pressure by changing environmental conditions. However, it has also been suggested that the thermal sensitivity of invertebrates declines with higher latitudes and, therefore, that polar ectotherms may not be at risk. In the current study, the heat tolerance and physiological plasticity to heat stress of two well-studied Antarctic invertebrates, the collembolan, *Cryptopygus antarcticus*, and the mite, *Alaskozetes antarcticus*, were investigated. Both species showed considerable heat tolerance, with each having an Upper Lethal Temperature (ULT) above 35 °C (1 h exposure). These species were also able to survive for over 43 d at 10 °C and for periods of 5–20 min at 40 °C. Across all experimental procedures, *A. antarcticus* possessed a somewhat greater

level of heat tolerance than *C. antarcticus*. Water loss during short duration exposures did not differ between the two species at 30, 35 and 40 °C, suggesting that the greater tolerance of *A. antarcticus* over this timescale was not due to higher desiccation resistance. Physiological plasticity was investigated by testing for Rapid Heat Hardening (RHH) and long-term acclimation. RHH was observed to a small degree in both species at a warming rate of 0.5 °C min^{-1}, and also 0.2 °C min^{-1} in *A. antarcticus* alone. Longer-term acclimation (1 week at 10 °C) did not enhance the heat tolerance of either species. Even with this limited physiological plasticity, the results of this study indicate that *C. antarcticus* and *A. antarcticus* have capacity in their heat tolerance to cope with current and future environmental extremes of high temperature.

7.2. Introduction

Over the last century, the mean surface temperature of the Earth has increased by 0.85 °C (IPCC, 2013). However, the rate of warming has been amplified at higher latitudes, with an average 2 °C rise in parts of the polar regions in the last 50 years (Arctic Council, 2005, Convey *et al.*, 2009; Turner *et al.*, 2009). The northern and western parts of the Antarctic Peninsula have been particularly affected; over the period 1951–2006, data from Vernadsky (Faraday) station in the Argentine Islands recorded a 0.53 °C rise in temperature per decade. A further consequence of this warming at a global scale has been a decrease in snow and ice cover of over 10% since the 1960s (Walther *et al.*, 2002). These trends are set to continue, with general circulation models predicting further warming across the planet, and especially rapid warming in the polar regions.

Invertebrates are poikilothermic ectotherms, meaning that their body temperature is highly influenced by, and varies markedly with, the external environment (Speight *et al.*, 2008). In essence, they are unable to regulate their body temperature as do birds and mammals, and are therefore susceptible to injuries, and developmental and reproductive impairment, resulting from temperature changes (Bale and Hayward, 2010). Invertebrates can respond to these changes through alterations in their behaviour, phenology, physiology and genetic make-up, with these responses acting within or between generations (Lachenicht *et al.*, 2010). Behaviourally, they can track favourable temperatures by moving towards either higher latitudes or altitudes (Walther *et al.*, 2002; Sinclair *et al.*, 2003; Gobbi *et al.*, 2006). Several alpine spiders, for instance, have been shown to remain in their preferred temperature range by tracking the recession of the Forni Glacier in Italy (Gobbi *et al.*, 2006). Invertebrates can also adapt behaviourally on a smaller scale, via microhabitat selection. Habitats, such as the Antarctic fellfields, are host to a diversity of microclimates and invertebrates select those which are the least stressful (Hodkinson *et al.*, 1999; Holmstrup and Zachariassen, 1996; Hoshikawa *et al.*, 1988; Spaull, 1973). Hayward *et al.* (2000), Hayward *et al.* (2003) and Hayward *et al.* (2004) have gone on to show thermal and hygric preferences that are suggestive of this type of behavioural selection in a laboratory setting. A further response identified is a shift of spring and autumn phenology with the changing of the growing season (Ibanez *et al.*, 2010; Walther *et al.*, 2002).

Within generations, physiological adaptation is demonstrated through experimental acclimation or natural acclimatisation—permitting an organism to adapt to changing conditions via a change in form, movement or rate of physiological activity (Lachenicht *et al.*, 2010). In the context of climate change, acclimatisation may involve the

improvement of heat tolerance and upper thermal sub-lethal characteristics, such as physical activity, as temperatures rise. This form of adaptation has been shown in a number of organisms, including plants (Meyer and Santarius, 1998), nematodes (Jagdale and Grewal, 2003) and insects (Lachenicht *et al.*, 2010). Over generations, invertebrates can adapt their physiology through the process of natural selection (Somero, 2010).

The thermal sensitivity of terrestrial invertebrates to temperature change has been reported to decline from the tropics to the poles (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008). Some tropical species live very close to their upper thermal limits and, in some cases, at temperatures that exceed their physiological optima (Somero, 2010). Polar species, in contrast, may live chronically below their temperature optima, and are suggested to have sufficient scope to tolerate higher temperatures. Warming might even help to alleviate the stress associated with low temperatures in the polar regions. Climate warming simulation studies using screens, solar domes and other controlled environmental systems (Bokhorst *et al.*, 2008; Bale and Hayward, 2010) suggest a rise in temperature will indeed lead to greater invertebrate numbers in Antarctic communities (Convey *et al.*, 2002; Convey and Wynn-Williams, 2002; Day *et al.*, 2009). However, some manipulation studies also suggest the opposite outcome, with responses depending both on the detailed changes at micro-environmental level associated with the manipulation, and also on the group of invertebrates being considered (Convey *et al.*, 2002; Convey *et al.*, 2003; Bokhorst *et al.*, 2011). Studies into upper thermal thresholds are also used in conjunction with climate manipulation studies and support the view that polar terrestrial invertebrates have low sensitivity to temperature change. Slabber *et al.* (2007), for example, showed that five Collembola

species from a sub-Antarctic island, including *Cryptopygus antarcticus*, possessed Upper Lethal Temperatures (ULT_{50}s) above 30 °C, far higher than the mean summer temperature in the Antarctic.

In the current study, the capacity of the collembolan, *Cryptopygus antarcticus*, and the mite, *Alaskozetes antarcticus*, to tolerate exposure to high temperatures was investigated, and their physiological plasticity to heat stress explored. In particular, this study addressed the ability of each species to respond to rapid increases in temperature, as might occur as a result of solar insolation of their microhabitats during diurnal cycles, and their tolerance to more prolonged exposures to high temperatures based on climate warming predictions. These species were selected as they represent two of the most successful arthropod groups in the maritime Antarctic and are considered 'model' organisms in polar research (Block and Convey, 1995; Block *et al.*, 2009), reaching numbers of up to 1.5×10^6 individuals m^{-2} (Burn, 1986; Convey and Smith, 1997; Tilbrook, 1967). Consequently, any effect warming may have on them will likely be reflected throughout the community.

7.3. Materials and methods

7.3.1. Invertebrate collection and storage conditions

Naturally occurring summer-acclimatised individuals of *C. antarcticus* and *A. antarcticus* were collected from algae, moss and rocks on Léonie Island (67°36′S, 68°21′W), near to the British Antarctic Survey's Rothera Research Station, Adelaide Island between January and March 2012. Samples were stored at 4 °C (24:0 L:D) in plastic buckets containing substratum from the site of collection. For water loss experiments (sub-section 7.3.3.1), samples were transported to the University of

Birmingham under cool conditions (4 °C to 6 °C), taking approximately two months, before being stored at 4 °C (0:24 L:D). All other experiments described were carried out at Rothera Research Station.

7.3.2. Microhabitat temperatures

The temperature range on Léonie Island on the soil surface underneath a rock was measured between 24 January and 12 March 2012. To illustrate the extremes of temperature potentially experienced by an animal on an exposed surface, temperature was also recorded every 5 min on a rock between 5 and 21 February 2012 at Rothera Research Station, using a Tinytag Transit 2 Datalogger (Gemini Data Loggers, Chichester, UK). Data were uploaded using Tinytag Explorer Software (Gemini Data Loggers, Chichester, UK).

7.3.3. Upper Lethal Temperatures (ULTs)

The upper temperature at which invertebrates no longer survived was determined by warming individuals of *C. antarcticus* and *A. antarcticus* at 0.2 °C min^{-1} from 4 °C to progressively higher temperatures (30 to 37 °C for *C. antarcticus* and 30 to 40 °C for *A. antarcticus*). Individuals were subsequently held at the target temperature for 1 h, before being cooled back to 4 °C at the same rate. Three replicates of 10 individuals of each species were placed in Eppendorf tubes, which were packed inside glass test tubes plugged with sponge and placed in an alcohol bath (Haake Phoenix II C50P, Fisher Scientific UK Ltd, Loughborough, U.K.) prior to each experimental treatment. Control groups were handled, and exposed, in the same way at 4 °C. The temperature experienced by the invertebrate was measured by placing a thermocouple within an identical Eppendorf tube into one of the glass test tubes. At the end of experimental

treatments, individuals were rapidly transferred (over ice) from the Eppendorf tubes into plastic universal tubes containing moist Plaster of Paris, and returned to the rearing conditions (4 °C, 0:24 L:D). Survival, defined by individuals moving either spontaneously or in response to gentle contact stimulus, was assessed 72 h after treatment. Replicate collection, controls, thermocouple use, recovery and survival assessment were the same for all following experimental procedures unless stated otherwise.

7.3.3.1. Water loss following high temperature exposure

For both species, five replicates of 10 individuals were exposed to three temperatures (30, 35 and 40 °C) as described in sub-Section 7.3.3. Individuals were weighed prior to and upon removal from each treatment, then following drying to constant mass at 60 °C for 24 h. From these values, initial water content and percentage water loss or gain were calculated (cf. Hayward *et al.*, 2007). The relative humidity was not controlled for during heat exposure.

7.3.4. Rapid Heat Hardening (RHH)

7.3.4.1. Determination of the discriminating temperature

In rapid cold and heat hardening experiments the discriminating temperature is defined as the temperature at which there is 10–20% survival after an exposure time of e.g. 1 h (Lee *et al.*, 1987). This temperature was determined here by exposing individuals (three replicates of 10 individuals) of *C. antarcticus* and *A. antarcticus* directly (i.e. without ramping from 4 °C) to progressively higher temperatures (30 to 36 °C for *C. antarcticus*

and 36 to 40 °C for *A. antarcticus*) for 1 h, before returning to the rearing temperature (4 °C) at 0.2 °C min^{-1}.

7.3.4.2. Induction of RHH

To investigate the RHH response, individuals of *C. antarcticus* and *A. antarcticus* (3 replicates of 10 individuals for each species) were warmed to the discriminating temperature at three different rates (0.5 °C min^{-1}, 0.2 °C min^{-1} and 0.1 °C min^{-1}). As before, individuals were held for 1 h at the discriminating temperature and then cooled back to the rearing temperature (4 °C) at 0.2 °C min^{-1}.

7.3.5. Long-term heat tolerance

Five replicates of 10 individuals of *C. antarcticus* and *A. antarcticus* were transferred to either 4 or 10 °C for up to 49 d. Individuals were held in universal tubes with a base of moist Plaster of Paris and a small amount of substratum within an incubator. Survival was assessed every 7 d for the first four weeks and then every 3 d thereafter. The temperature inside the incubator was measured using a Tinytag Transit 2 Datalogger.

7.3.6. Acute heat exposure

Three replicates of 10 individuals of *C. antarcticus* and *A. antarcticus* were exposed directly to three temperatures: 40, 45 and 50 °C. At each temperature, individuals were held for 5, 10 or 20 min. Following high temperature treatment, they were transferred directly to recovery conditions (4 °C, 24:0 L:D).

7.3.7. Effect of acclimation on heat tolerance

Stock cultures of *C. antarcticus* and *A. antarcticus* were held for one week at 10 °C prior to experimental treatments. Three replicates of 10 individuals of each species were subsequently warmed at 0.2 °C min^{-1} to three temperatures (33, 34 and 35 °C for *C. antarcticus* and 39, 39.5 and 40 °C for *A. antarcticus*), and held there for 1 h, before being cooled to the rearing temperature (4 °C) at 0.2 °C min^{-1}. Because one replicate was unviable and could not be used, only two replicates of 10 individuals of *C. antarcticus* were used for the 33 °C treatment.

7.3.8. Statistical analysis

The Kolmogorov–Smirnov test was used to check for normal distribution of survival and percentage water loss data. Normally distributed data were analysed using analysis of variance (ANOVA) and Tukey's multiple range test; data that were not normally distributed were analysed using the Kruskal-Wallis test or Mann-Whitney U test.

7.4. Results

7.4.1. Microhabitat temperatures

Soil surface temperatures beneath a rock on Léonie Island ranged from 13.5 to −6.1 °C, and averaged 1.9 °C, between 24 January and 12 March 2012 (Fig. 7.1), whereas the temperature on the rock surface ranged between 31.2 and −8.7 °C (Fig. 2). The diurnal temperature range on the rock surface was high, regularly exceeding 20 °C (with temperature changing at rates >2.5 °C/h), and on seven occasions the temperature ranged from below 0 °C to above 20 °C within 12 h.

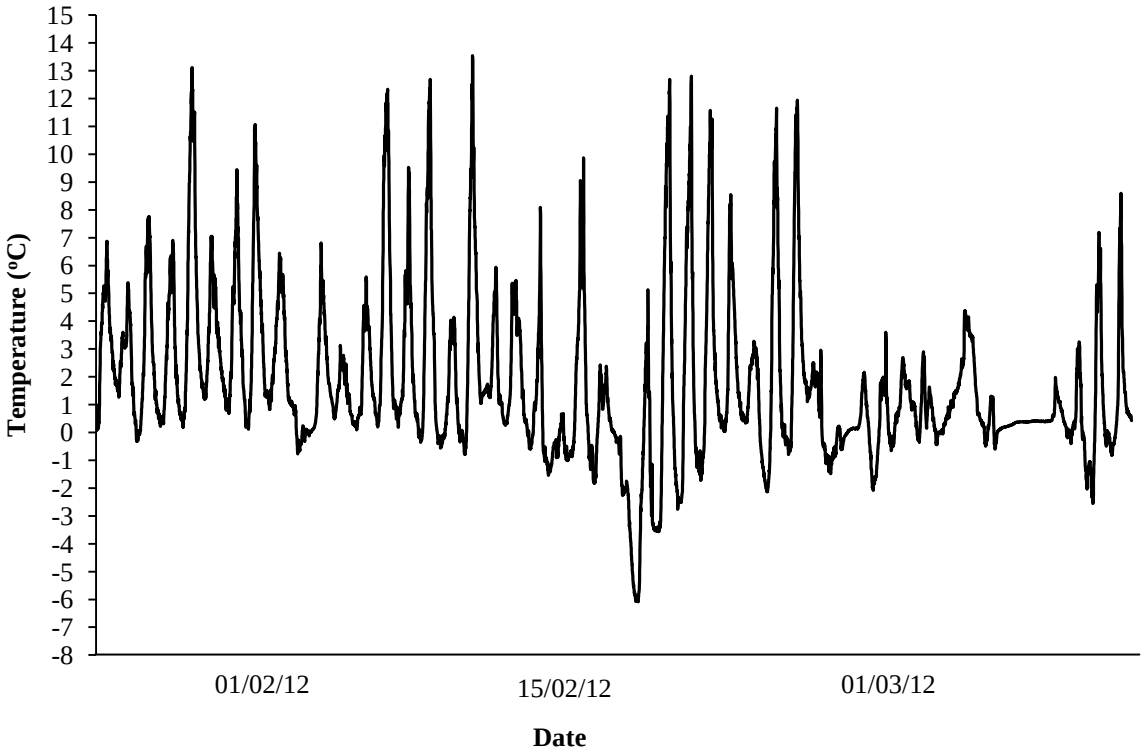

Fig. 7.1. *Surface temperature beneath a rock (roughly 5 inches in circumference) on Léonie Island, near Rothera Research Station, Adelaide Island, between 24th January and 12th March 2012.*

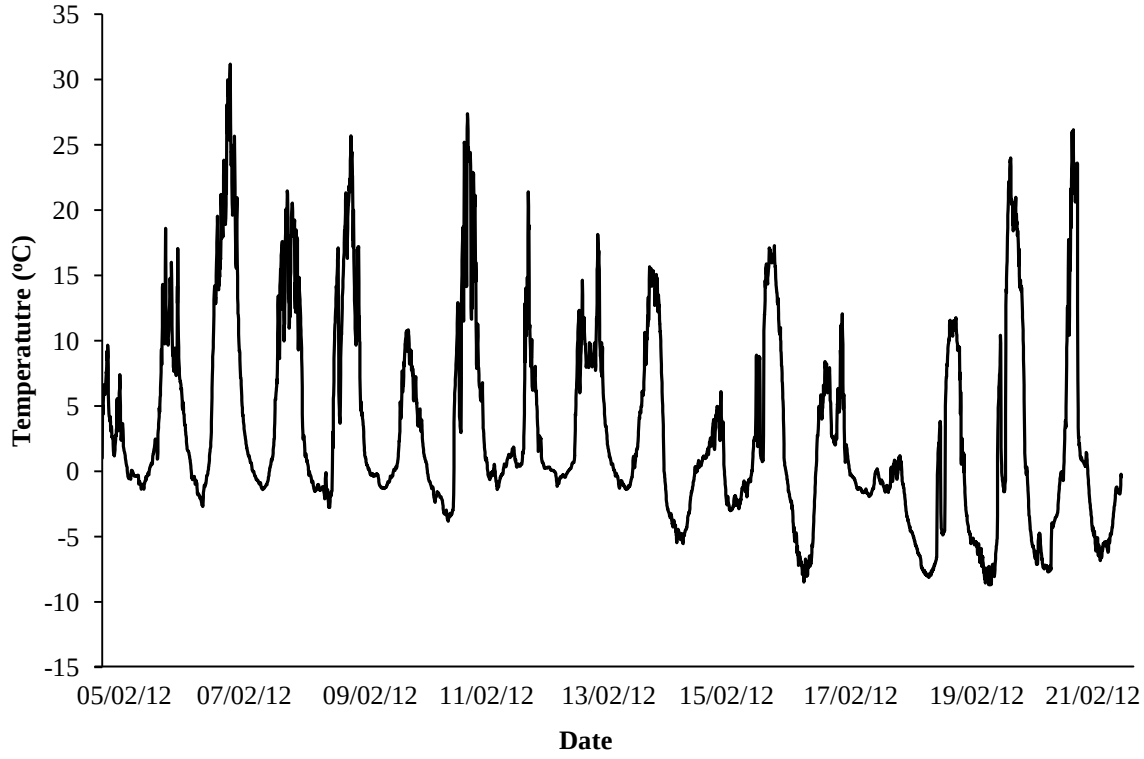

Fig. 7.2. *Temperature on a rock surface outside the Bonner Laboratory at Rothera Research Station, Adelaide Island, between 5th and 21st February 2012.*

7.4.2. Upper Lethal Temperatures (ULTs)

Survival declined dramatically at temperatures close to the ULT for both species (Fig. 7.3). After 1 h at 34 °C, almost 90% of *C. antarcticus* survived, while only 3% survived 1 h at 36 °C, and none survived at 37 °C. *Alaskozetes antarcticus* had greater heat tolerance than *C. antarcticus*, with 100% survival of 1 h at 37 °C, 81% survival at 39 °C, but 0% survival at 40 °C. The difference between species was not significant at 35, 36 and 37 °C (P>0.05 Mann–Whitney U test).

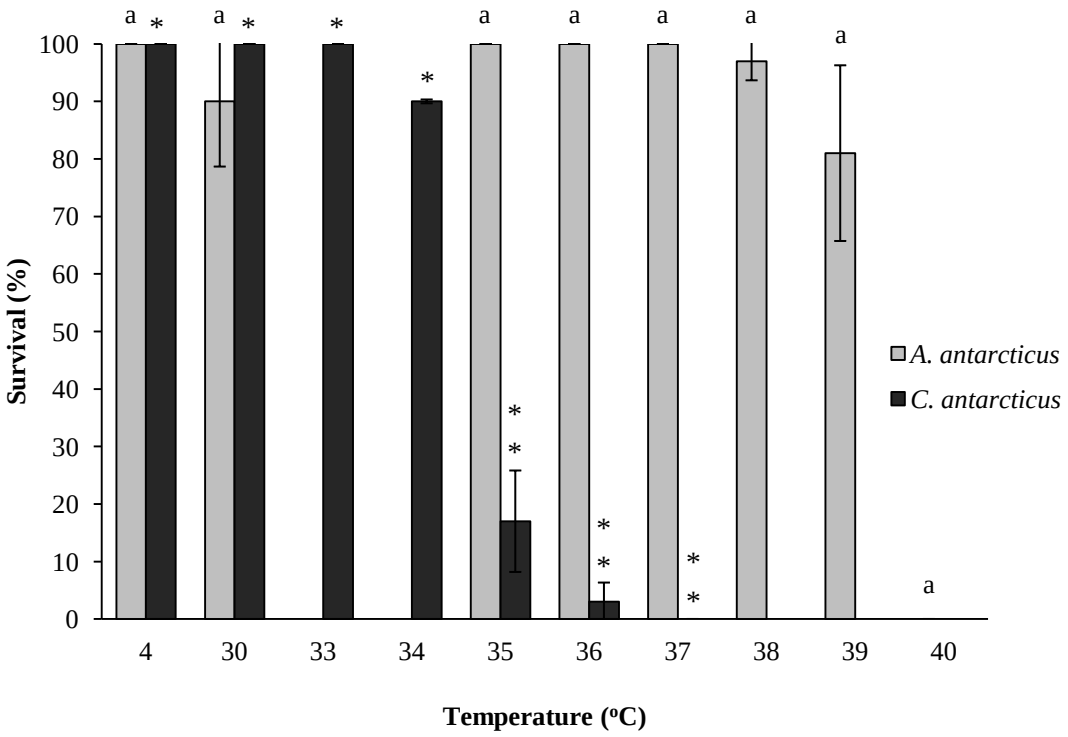

Fig. 7.3. *Mean percentage survival of C. antarcticus and A. antarcticus, following exposure to progressively higher temperatures (30 to 37°C – C. antarcticus, 30 to 40°C – A. antarcticus) for 1h, before cooling at 0.2°C min^{-1} to 4°C. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter (A. antarcticus) and same number of * symbols (C. antarcticus) are not significantly different within each species group at P < 0.05 (Kruskal-Wallis test and Tukey's multiple range test, respectively). A. antarcticus was not tested at 33 or 34°C.*

7.4.2.1. Water loss following high temperature exposure

Water loss was minimal following a 1 h exposure to 30, 35 and 40 °C in both species (Table 7.1). The amount lost did not differ significantly from the control (1 h at 4 °C) in all treatments, except for a 1 h exposure at 40 °C in *C. antarcticus* ($P<0.05$ Tukey's multiple range test). There was no significant difference between the amount of water lost in *C. antarcticus* and *A. antarcticus* across each of the three treatments ($P>0.05$ Tukey's multiple range test).

Table 7.1. *Mean percentage water loss of C. antarcticus and A. antarcticus, following exposure to 30, 35 and 40°C for 1 h, prior to cooling at 0.2°C min^{-1} to 4°C. Water content of control sample held at 4°C for 1 h also given. Means ± S.E.M. are presented for five replicates of 10 individuals.*

Temperature (°C)	**Water Content change (%)**	
	C. antarcticus	*A. antarcticus*
4	3.19 ± 2.86	-0.02 ± 1.82
30	-1.58 ± 1.76	0.12 ± 0.38
35	0.88 ± 3.65	-3.82 ± 1.61
40	-6.68 ± 0.81	-2.08 ± 0.45

7.4.3. Rapid Heat Hardening (RHH)

7.4.3.1. Determination of the discriminating temperature

The discriminating temperature was determined to be 35 °C for *C. antarcticus* (10% survival), and 39.5 °C for *A. antarcticus*, a temperature which although resulting in 0% survival, was chosen because it was closer to the 10–20% survival required than the 37% value obtained at 39 °C (Fig. 7.4).

7.4.3.2. RHH induction

In both species, all three warming treatments (0.5, 0.2 and 0.1 °C min^{-1}) gave greater survival compared to direct exposure to the discriminating temperature (Fig. 7.5). The increase in survivorship was significant for 0.5 °C min^{-1} in *C. antarcticus* ($P<0.05$ Tukey's multiple range test), and for 0.5 and 0.2 °C min^{-1} in *A. antarcticus* ($P<0.05$ Tukey's multiple range test). For *A. antarcticus*, survival declined as the rate of warming was lowered, from 73% at 0.5 °C min^{-1} to 30% at 0.1 °C min^{-1}. The rate of 0.5 °C min^{-1} also gave the greatest survival in *C. antarcticus*.

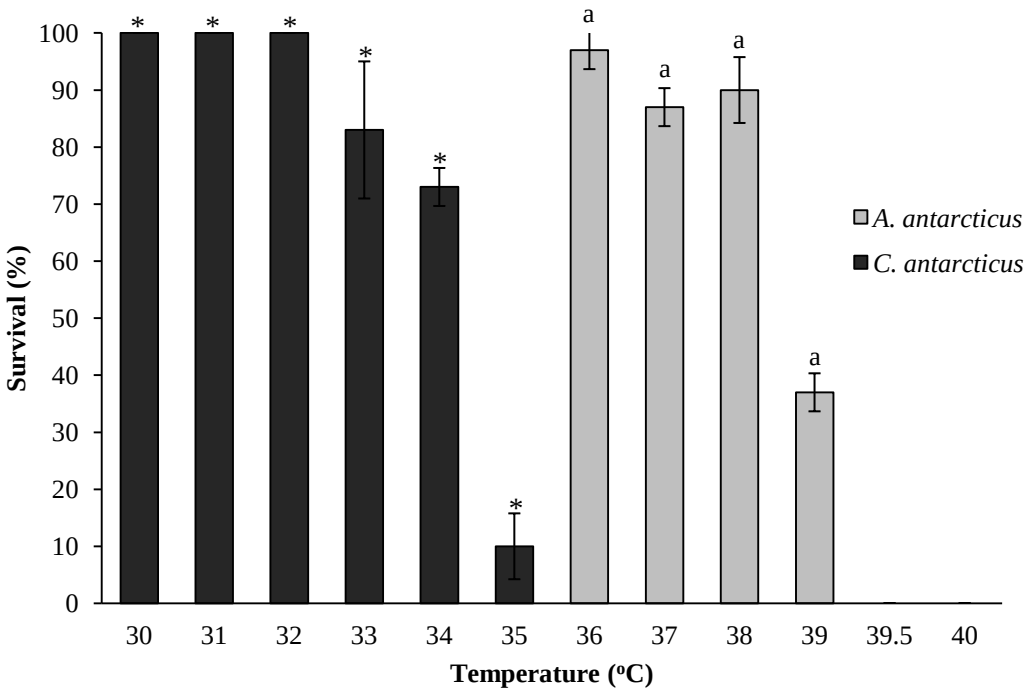

Fig. 7.4. *Mean percentage survival of C. antarcticus and A. antarcticus, following direct exposure to progressively higher temperatures (30 to 36°C for C. antarcticus and 36 to 40°C for A. antarcticus) for 1 h, before cooling at 0.2°C min^{-1} to 4°C. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter (A. antarcticus) and same number of * symbols (C. antarcticus) are not significantly different within each species group at P < 0.05 (Kruskal-Wallis test).*

7.4.4. Long-term heat tolerance

C. antarcticus was more susceptible at both 4 and 10 °C than *A. antarcticus* (Fig. 7.6). Survival of *C. antarcticus* decreased significantly at 4 °C to 70% after 46 d ($P<0.05$ Tukey's multiple range test), and to 0% at 10 °C ($P<0.05$ Kruskal–Wallis test) (Fig. 7.6). *Alaskozetes antarcticus* survival also decreased significantly at 10 °C ($P<0.05$ one-way ANOVA), but only to 63% after 49 d, and was not significantly lowered at 4 °C (80% survival, $P>0.05$ Kruskal–Wallis test).

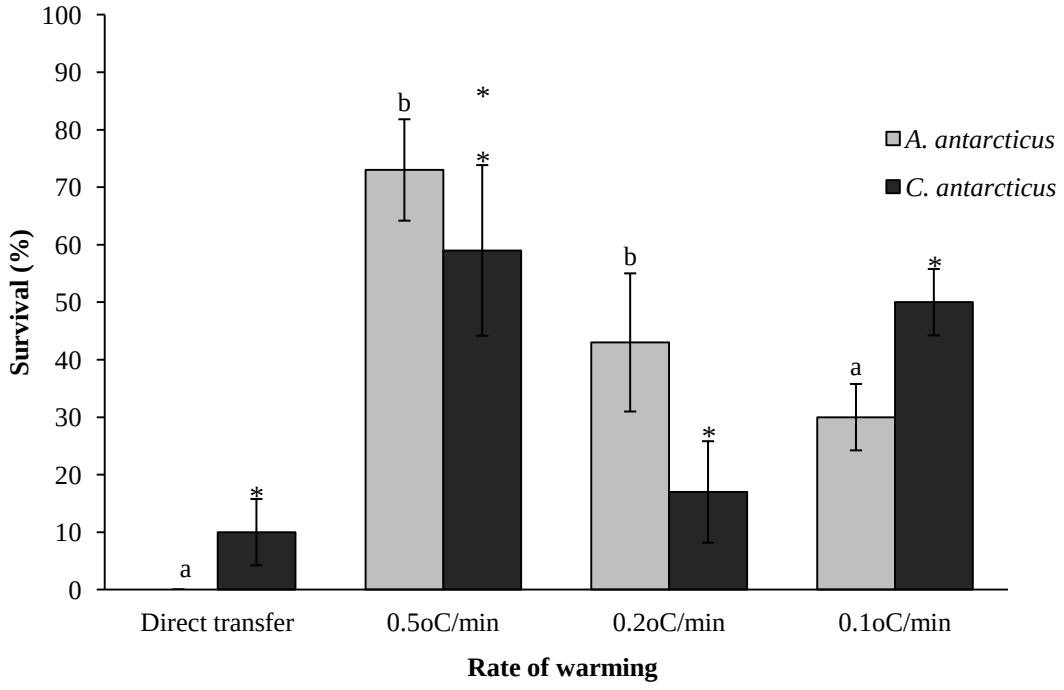

Fig. 7.5. *Mean percentage survival of C. antarcticus and A. antarcticus, following exposure to the discriminating temperature (35°C – C. antarcticus, 39.5°C – A. antarcticus) for 1 h, after being warmed to the discriminating temperature at one of three rates (0.5, 0.2 or 0.1°C min^{-1}). Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter (A. antarcticus) and same number of * symbols (C. antarcticus) are not significantly different within each species group at P < 0.05 (Tukey's multiple range test).*

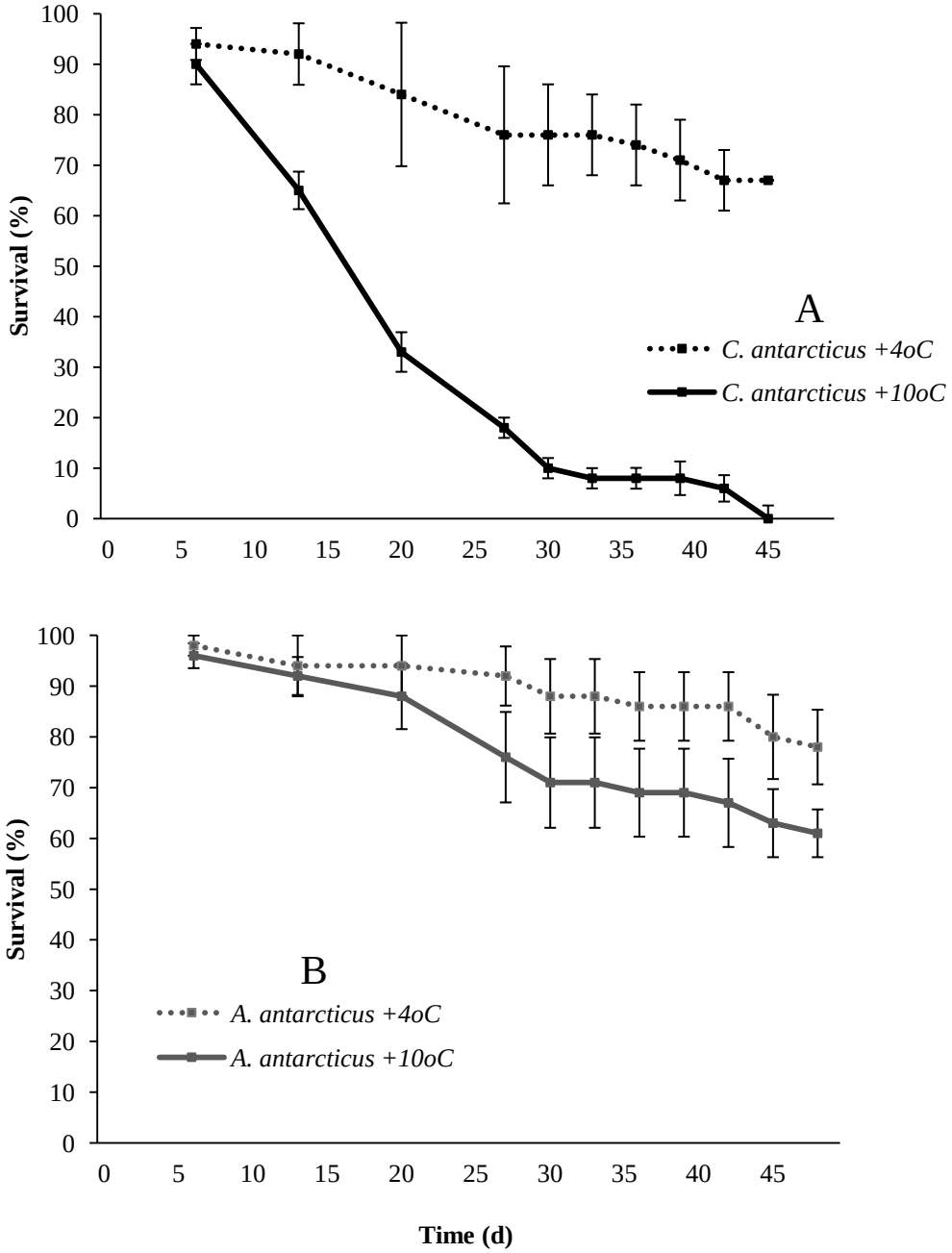

Fig. 7.6. *Mean percentage survival of C. antarcticus (A) and A. antarcticus (B) at +4 and +10°C over a period of 46 (C. antarcticus) and 49 d (A. antarcticus). Means ± S.E.M. are presented for five replicates of 10 individuals.*

7.4.5. Acute heat exposure

At 40 °C, *A. antarcticus* significantly outperformed *C. antarcticus* in all treatments (5, 10 and 20 min, Fig. 7.7) ($P<0.05$ two-way ANOVA, variances not equal). At 45 and 50 °C, both *C. antarcticus* and *A. antarcticus* survived poorly (Fig. 7.7).

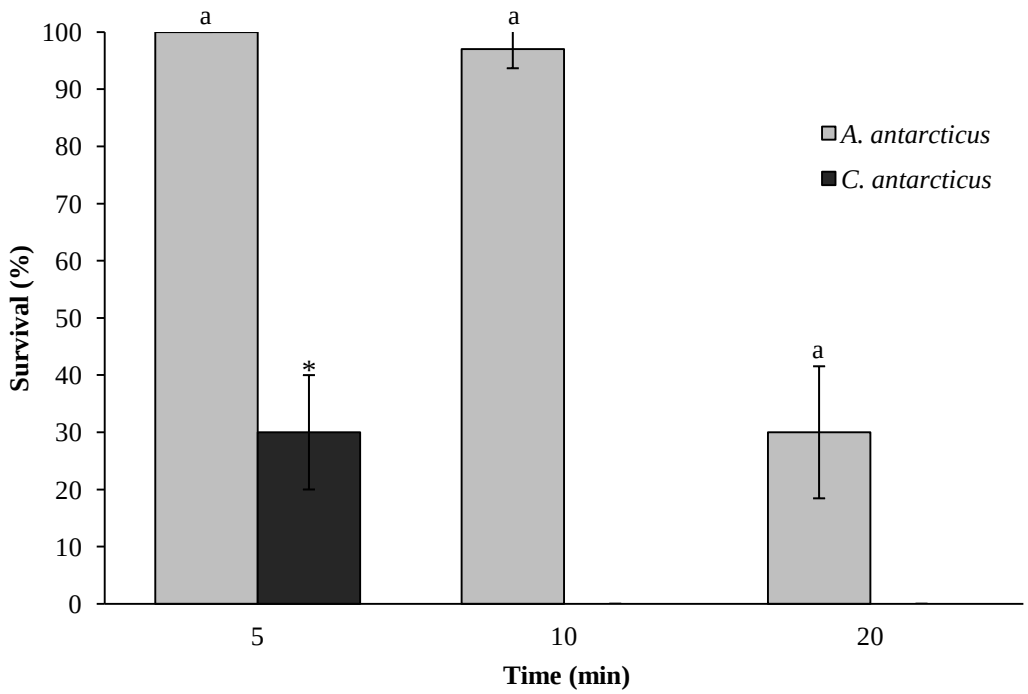

Fig. 7.7. *Mean percentage survival of C. antarcticus and A. antarcticus following exposure to 40°C for 5, 10 or 20 min. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter (A. antarcticus) and same number of * symbols (C. antarcticus) are not significantly different within each species group at P < 0.05 (Kruskal-Wallis test).*

7.4.6. Effect of acclimation on heat tolerance

Acclimation at 10 °C did not significantly enhance the heat tolerance of *C. antarcticus* or *A. antarcticus* at any of the temperatures tested ($P>0.05$ Mann–Whitney U test; one-way ANOVA, Fig. 7.8).

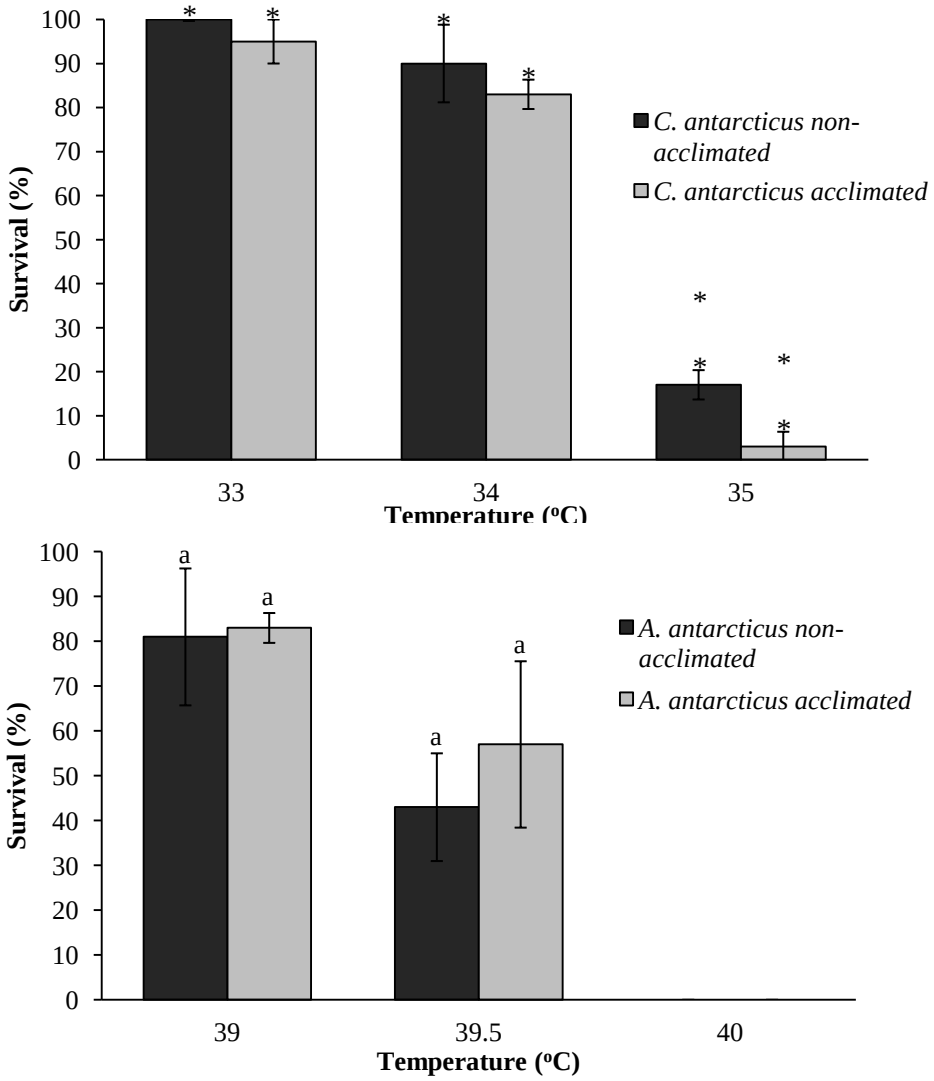

Fig. 7.8. *Mean percentage survival, following exposure to 33, 34 and 35°C – C. antarcticus, and 39, 39.5 and 40°C – A. antarcticus, for 1 h, before cooling at 0.2°C min^{-1} to 4°C. Both species were held at 10°C for one week prior to experimentation. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter (A. antarcticus) and same number of * symbols (C. antarcticus) are not significantly different within each species group at P < 0.05 (Tukey's multiple range test).*

7.5. Discussion

The Antarctic environment is unable to support large biological communities and, in extreme cases, may only support a food web of less than five animal species (Convey and McInnes, 2005; Hodgson *et al.*, 2010). The few terrestrial invertebrates that inhabit

these communities play an important role in processes such as soil conditioning and nutrient cycling. In contrast to the temperate and tropical regions, which have greater species diversity and subsequently greater functional redundancy, polar communities will struggle to compensate for the loss of species and their associated services. Changing environmental conditions as a result of climate warming may put pressure on polar species. However, the thermal sensitivity of polar invertebrates to temperature increase has been suggested to be low, and warming may even result in more optimal conditions and a reduction in environmental constraints on invertebrate physiology (Addo-Bediako *et al.*, 2000; Convey, 2011; Deutsch *et al.*, 2008). The acute and chronic tolerances, as well as the physiological plasticity, of the collembolan, *C. antarcticus*, and the mite, *A. antarcticus*, are discussed here in the context of their ability to respond to climate warming.

7.5.1. Basal heat tolerance

The collembolan, *C. antarcticus*, and the mite, *A. antarcticus*, demonstrated considerable heat tolerance, with each having a ULT of over 35 °C (Fig. 3). In two sub-Antarctic studies on Marion Island (Deere *et al.*, 2006; Slabber *et al.*, 2007) and one study at Cape Hallet, North Victoria Land (Sinclair *et al.*, 2006), several mites and Collembola, including *C. antarcticus* on Marion Island, were also shown to possess ULTs above 30 °C. While this level of tolerance is somewhat lower than found in temperate or tropical species, such as the Asian brown planthopper, *Nilaparvata lugens*, which has a ULT_{50} of 41.8 °C to 42.5 °C (Piyaphongkul *et al.*, 2012), this nevertheless demonstrates a considerable capacity to cope with current conditions (Convey, 1996). Indeed, ULTs above 35 °C are high when considering the temperatures these Antarctic

species typically experience during the summer. Tinytag measurements on Léonie Island through February and March did not show surface temperatures exceeding 15 °C (Fig. 7.1). Likewise, temperatures recorded between 2002 and 2008 on nearby Anchorage Island did not rise higher than 20 °C. However, it should be noted that diurnal fluctuations in some microhabitats and years can exceed 30 °C for short periods of minutes to hours (Fig. 7.3; Smith, 1988; Convey, 1996). Both *C. antarcticus* and *A. antarcticus* were also able to survive for over 43 d at 10 °C (Fig. 7.6) and showed survival at 40 °C over periods of 5–20 min (Fig. 7.7). These two species are therefore well adapted to survive the summer on Léonie Island and have some capacity to tolerate higher temperatures than those that are currently experienced (Day *et al.*, 2009; Convey *et al.*, 2009).

Survival alone is not an accurate measure of fitness. Success is also influenced by the sub-lethal characteristics of a species, such as the effects of heat stress on reproduction and development. In many species, survival is possible at extremes of temperature, but they are then unable to fully develop and reproduce once usual temperatures are restored (Shreve *et al.*, 2004). Invertebrates are also hampered during temperature extremes (Piyaphongkul *et al.*, 2012; Powell and Bale, 2006; Shreve *et al.*, 2004; Wang and Kang, 2003). Uncoordinated movement 72 h after high temperature treatment in the current study (>30 °C, data not shown) indicates that permanent damage might have been incurred as a result of high temperature exposure, which could subsequently result in impaired development and reproduction. Thus, whilst *C. antarcticus* and *A. antarcticus* can survive above 35 °C, negative effects on them and their communities might be seen at much lower temperatures.

7.5.2. Interspecific comparisons

A. antarcticus showed significantly greater heat tolerance than *C. antarcticus*. This capacity was demonstrated across all experimental procedures; *A. antarcticus* had a higher ULT (Fig. 7.3), exhibited higher survival of acute heat exposure (Fig. 7.7) and survived for longer at 10 °C (Fig. 7.6). Previous studies also show that mite species tend to have higher heat tolerance than Collembola (Deere *et al.*, 2006; Sinclair *et al.*, 2006). It was initially hypothesised that higher desiccation resistance accounted for the greater heat tolerance in *A. antarcticus*. This is because *C. antarcticus* is a hygric species, with little or no control of water loss (Convey *et al.*, 2003; Worland and Block, 1986; Worland and Block, 2003), whereas *A. antarcticus* is a mesic species and has good control over its water content (Benoit *et al.*, 2007b; Worland and Block, 1986). However, there was little difference in water loss with temperature and no significant difference in the water lost between the two species over the experimental durations under all temperature treatments (Table 7.1). It seems, therefore, that *A. antarcticus* possesses a more adaptive heat tolerance physiology than *C. antarcticus*. Possible physiological adaptations capable of operating over these experimental timescales include the activation of heat shock proteins (Schill *et al.*, 2004; Rinehart *et al.*, 2006; Michaud *et al.*, 2008) and membrane remodelling (Hazel, 1995).

The results of this study suggest that, in a rapidly warming Antarctic, *A. antarcticus* would have some advantage over *C. antarcticus*. Climate manipulation studies also suggest that mites will be favoured over Collembola under warming. In both the Arctic (Coulson *et al.*, 1996) and the Antarctic (Bokhorst *et al.*, 2008; Convey *et al.*, 2002), Collembola numbers decreased significantly under artificially warmed conditions over

three years, while mite numbers remained largely unchanged. However, Webb *et al.* (1998) proposed that oribatid mite populations are slow to show a response to short-term environmental changes and that manipulations longer than those used in the aforementioned studies are required to identify any effect. A further consideration is how the heat tolerance of these species relates to their behaviour. Collembola are more mobile than oribatid mites, and so may be better able to relocate to habitats in their preferred temperature range. Consequently, the more rapid movement of *C. antarcticus* could compensate for reduced heat tolerance in this species. It is therefore only in a uniform thermal environment where *A. antarcticus* would be favoured (see also Hayward *et al.*, 2003).

7.5.3. Physiological plasticity

The Antarctic hosts a diversity of microclimates. In some of these, the daily temperature can fluctuate by as much as 50 °C (Convey, 1996). In the current study, measurements on a rock surface showed temperature variation approaching or exceeding 30 °C on a diurnal timescale (Fig. 7.2). Similar patterns have been reported in other microhabitats; temperatures within the moss cushion, *Schistidium antarctici*, were shown to cycle between −9.2 °C and 42.8 °C over 24 h (Smith, 1988). It could, therefore, be to an invertebrate's advantage to adapt quickly to changes in temperature. One means of tracking temperature changes is via a process termed Rapid Heat Hardening (RHH), which is the rapid induction of heat tolerance over minutes to hours (Benoit *et al.*, 2009b). Both *C. antarcticus* and *A. antarcticus* showed evidence of RHH, with enhanced survival at their discriminating temperatures following warming at the three rates of 0.1, 0.2 and 0.5 °C min^{-1} (Fig. 7.5). The rate of 0.5 °C min^{-1} gave the greatest

increase in survival for both species, and was likely due to the reduced time spent at harmful temperatures. Overall, the RHH response was small, however, giving an average rise in survivorship of only 38% across all treatments. It is possible that RHH has more of an influence on the sub-lethal characteristics of *C. antarcticus* and *A. antarcticus*. Although there is as yet little support for this occurring in other species, there is ample evidence of a sub-lethal influence during Rapid Cold Hardening (RCH) (Denlinger and Lee, 2010). For example, courting, reproduction, and the Critical Thermal minimum (CTmin–loss of coordination at low temperatures) were all improved in *D. melanogaster* following RCH (Shreve *et al.*, 2004; Kelty and Lee, 1999).

Physiological plasticity can also be seen over longer timescales in the form of experimental acclimation (Lachenicht *et al.*, 2010). The nematodes, *Steinernema carpocapsae* and *Steinernema feltiae*, for instance, showed enhanced heat tolerance, and higher virulence under heat stress, when reared at higher, and thus acclimatory, temperatures (Jagdale and Grewal, 2002). Similarly, heightened heat tolerance following time at higher rearing temperatures was exhibited in both marine and terrestrial mites found on Marion Island (Deere *et al.*, 2006). In the current study, a one week acclimation at 10°C had no significant impact on survivorship in either *C. antarcticus* or *A. antarcticus* (Fig. 7.8). A null response in the sub-Antarctic collembolan *Tullbergia bisetosa*, and a decline in heat tolerance in *C. antarcticus*, was also shown following acclimation at 15 °C (Slabber *et al.*, 2007).

Physiological plasticity across generations may also be important; species with sufficient genetic variation that produce progeny with higher physiological thermal optima may end up as the 'winners' in scenarios of climate warming (Somero, 2010). In a number of species, life at low temperatures has resulted in the loss of physiology

suited to warming conditions (Somero, 2010). The polar marine ectotherms of the Southern Ocean provide a particularly good illustration. These species are stenothermal and have experienced a narrow range of low temperatures for millions of years (at present −1.9 to +1.8 °C or much less) (Somero, 2010). As a result, many have lost their ability to initiate a heat shock response (Clark *et al.*, 2009a). The same might be true of polar terrestrial invertebrates with regard to their physiological plasticity, and if so these will therefore become less successful as climate change intensifies. However, it has also been suggested that the greater thermal variability typical of polar terrestrial environments will preserve heat tolerance adaptation (Peck *et al.*, 2006). Indeed, the climatic variability hypothesis (Stevens, 1989) suggests that the greater thermal variability at higher latitudes means that invertebrates must have a greater physiological range and subsequently retain physiological plasticity at higher temperatures. Also of note are the long generation times of these animals, which frequently extend to five years or more, and therefore limit their ability to adapt across generations (Convey, 1994; Convey, 1996).

7.6. Conclusion

It has been suggested that the thermal sensitivity of invertebrates to temperature change decreases from the tropics to the poles (Deutsch *et al.*, 2008). This statement is supported by the current study, which shows that both *C. antarcticus* and *A. antarcticus* have scope with which to tolerate current and future conditions. Warming may even alleviate the stresses experienced by these invertebrates and provide an opportunity for population growth. If these species are assumed to be characteristic of other Collembola

and Acari in the maritime Antarctic, a positive impact on the community and on ecosystem functions such as nutrient cycling, may also be seen.

Chapter transition

As mentioned previously, the following chapter also explores heat tolerance and physiological plasticity, but this time in the Arctic collembolan, *M. arctica*, and the Antarctic midge, *E. murphyi*.

CHAPTER 8: ARE THE ANTARCTIC DIPTERAN, *ERETMOPTERA MURPHYI*, AND ARCTIC COLLEMBOLAN, *MEGAPHORURA ARCTICA*, VULNERABLE TO RISING TEMPERATURES?

The work of this chapter has been accepted for publication by the Bulletin of Entomological Research.

8.1. Abstract

Polar terrestrial invertebrates are suggested as being vulnerable to temperature change relative to lower latitude species, and hence possibly also to climate warming. Previous studies have shown Antarctic and Arctic Collembola and Acari to possess good heat tolerance and survive temperature exposures above 30^{o}C. To test this feature further, the heat tolerance and physiological plasticity of heat stress were explored in the Arctic collembolan, *Megaphorura arctica*, from Svalbard and the Antarctic midge, *Eretmoptera murphyi*, from Signy Island. The data obtained demonstrate considerable heat tolerance in both species, with upper lethal temperatures ≥ 35^{o}C (1 h exposures), and tolerance of exposure to 10 and 15^{o}C exceeding 56 d. This tolerance is far beyond that required in their current environment. Average microhabitat temperatures in August 2011 ranged between 5.1 and 8.1^{o}C, and rarely rose above 10^{o}C, in Ny-Ålesund, Svalbard. Summer soil microhabitat temperatures on Signy Island have previously been shown to range between 0 and 10^{o}C. There was also evidence to suggest that *E. murphyi*

can recover from high temperature exposure and that *M. arctica* is capable of rapid heat hardening. *Megaphorura arctica* and *E. murphyi* therefore have the physiological capacity to tolerate current environmental conditions, as well as future warming. If the features they express are characteristic, such polar terrestrial invertebrates will likely fare well under a climate warming scenario.

8.2. Introduction

It is becoming increasingly clear that many terrestrial invertebrates resident in the Antarctic and Arctic are remarkably heat tolerant. Block *et al.* (1994), Hodkinson *et al.* (1996), Deere *et al.* (2006), Everatt *et al.* (2013a), Sinclair *et al.* (2006) and Slabber *et al.* (2007) have shown survival above 30^{o}C in a number of Antarctic Collembola and Acari, including "polar model organisms", such as *Cryptopygus antarcticus*, *Megaphorura arctica* and *Alaskozetes antarcticus*. In the Antarctic, typical summer microhabitat temperatures range between 0 and 10^{o}C, whereas in the Arctic, the temperature range is slightly higher (Davey *et al*., 1992; Coulson *et al.*, 1996; Hodkinson *et al.*, 1996; Block *et al.*, 2009). Temperatures above 30^{o}C are rare, occurring only in certain microhabitats for brief periods of minutes to hours and not consistently between years (Smith, 1988; Convey, 1996; Hodkinson *et al.*, 1996; Everatt *et al.*, 2013a). It is generally assumed that invertebrates respond behaviourally to such temperatures, and rapidly relocate if/when they become stressful (Hayward *et al.*, 2003). Polar Collembola and Acari of the Antarctic therefore have ample capacity to tolerate current conditions. Annual mean temperatures have risen by over 2^{o}C in parts of the polar regions in the last 50 years and similar, possibly more extreme, increases are predicted to occur over the next half century (Convey *et al.*, 2009; Turner *et al.*, 2009).

Such warming is within the physiological thresholds of the resident Collembola and Acari (Deere *et al.*, 2006; Sinclair *et al.*, 2006; Slabber *et al.*, 2007; Everatt *et al.*, 2013a).

The capacity of polar invertebrates to tolerate future warming is in line with Deutsch *et al.* (2008), who suggested that the sensitivity of terrestrial invertebrates to a temperature change decreases with increasing latitude (see also Addo-Bediako *et al.*, 2000). It has even been suggested that climate warming might alleviate the stresses of living in a low temperature environment and actually benefit some polar species (Convey, 2006, 2011; Bale and Hayward, 2010). This proposal is consistent with the results of some climate manipulation studies (e.g. using cloches) which have shown warming to increase populations of invertebrates in Antarctic communities (Convey *et al.*, 2002; Convey and Wynn-Williams, 2002; Day *et al.*, 2009). Convey *et al.* (2002) and Day *et al.* (2009), however, highlighted that continued water availability during warming is crucial, and some Arctic studies have shown declines or no change following artificial increases in temperature alone (Coulson *et al.*, 1996; Webb *et al.*, 1998). Manipulation studies should therefore be treated with care for they are complex in their effects and often inconsistent in the consequences identified, emphasising that the changes observed are strongly influenced by the specific microhabitat characteristics and invertebrate populations investigated, as well as the seasonal timing and duration of the study (Convey *et al.*, 2002, 2003; Bokhorst *et al.*, 2011, 2013). Climate manipulation studies also lack an assessment of the potential impact of possible new colonists as a result of climate change.

The first studies investigating heat tolerance in polar terrestrial invertebrates concentrated on Arctic species, including three species of Collembola (*M. arctica*,

Onychiurus groenlandicus and *Hypogastrura tullbergi*) and four species of mite (*Camisia anomia*, *Diapterobates notatus*, *Hermannia reticulata* and *Ceratoppia hoeli*) (Block *et al.*, 1994; Hodkinson *et al.*, 1996). The current study also uses *M. arctica* and, although it revisits this collembolan's short-term and long-term tolerance to heat, the methods used here take into account more ecologically relevant rates of warming and cooling. The ability of the collembolan to acclimate using rapid heat hardening (RHH) is also investigated for the first time. *Megaphorura arctica* (formerly *Onychiurus arcticus*) is a pale yellow collembolan found predominantly in the palaearctic regions of Iceland, Norway and Svalbard (Fjellberg, 1994). This collembolan is common under rocks and within moss beneath bird cliffs, where it commonly aggregates in groups of 100 or more individuals (Worland, 1996). Partly because of its ability to cryoprotectively dehydrate, *M. arctica* is considered a "model" in Arctic terrestrial invertebrate ecophysiological research (Worland *et al.*, 1998). However, to date, there has only been one study investigating physiological heat tolerance of *M. arctica* and other Arctic microarthropods (Hodkinson *et al.*, 1996). This study showed good survival in a number of species following 1 h durations at temperatures ≥ 30°C, and survival in *M. arctica* specifically for up to 68 d at 25°C.

Previous Antarctic studies have examined heat tolerance in Collembola and Acari (Deere *et al.*, 2006; Sinclair *et al.*, 2006; Slabber *et al.*, 2007; Everatt *et al.*, 2013), but have given little attention to Antarctic Diptera. In this study, the capacity of the midge, *Eretmoptera murphyi*, to respond to high temperature is investigated, including an assessment of its CTmax, and its ability to recover from heat stress. *Eretmoptera murphyi* is native and endemic to the sub-Antarctic island of South Georgia (55^{o}S 37^{o}W). Likely as a result of plant transplant experiments in the 1960s, this midge was

accidentally transferred to maritime Antarctic Signy Island (60°S 45°W) and is now established as a non-native species there (Block *et al.*, 1984; Convey and Block, 1996). The species has since spread to cover an area > 2000 m^2 and is now having a significant impact on the local environment (Hughes *et al.*, 2013). *Eretmoptera murphyi* is closely related to the endemic *Belgica antarctica* of the maritime Antarctic (Allegrucci *et al.*, 2012). While heat tolerance has received some attention in the latter species, the subject has not been explored in detail (Hayward *et al.*, 2007; Benoit *et al.*, 2009a).

8.3. Materials and methods

8.3.1. Invertebrate collection and storage conditions

Summer-acclimatised individuals of *M. arctica* were collected from moss-covered slopes at Krykkefjellet and Stuphallet, near Ny-Ålesund, Spitsbergen, Svalbard (78°55'N, 11°56'E) between 14th and 24th August 2011. Summer acclimatised larvae of *E. murphyi* were collected from soil and moss on Signy Island (60°S, 45°W) near to the British Antarctic Survey Signy Research Station between January and March 2012. Samples of *M. arctica* and *E. murphyi* were subsequently transported to the University of Birmingham under refrigerated conditions and held in plastic boxes containing substratum from the site of collection at 4-5°C (0:24 L:D). The duration of travel was approximately 2 d from the Arctic and two months from the Antarctic. Numbers of *M. arctica* were limited, and as a result this species was not assessed for the effect of recovery or heat coma (sub-sections 8.3.4 and 8.3.6).

8.3.2. Arctic site microhabitat temperatures

The thermal regime experienced by *M. arctica* during the summer was measured at four different sheltered sites (laid on surface, but covered by rocks), two at Krykkefjellet and two at Stuphallet, between 17th and 24th August 2011. Temperature was measured at each site using a Tinytag Transit 2 Datalogger, and data were uploaded using Tinytag Explorer Software (Gemini Data Loggers, Chichester, U.K.). Fieldwork was not conducted on Signy Island as part of this study and microhabitat temperature data for *E. murphyi* will therefore be inferred from previous studies.

8.3.3. Upper Lethal Temperatures (ULTs)

The upper temperature at which a species is no longer able to survive (ULT) was determined for *M. arctica* and *E. murphyi* by warming individuals at 0.2^{o}C min^{-1} from 4^{o}C (rearing temperature) to progressively higher temperatures (30 to 36^{o}C for *M. arctica*, 35 to 39^{o}C for *E. murphyi*). Individuals were subsequently held at the target temperature for 1 h, before being cooled back to 4^{o}C at the same rate. Three replicates of 10 individuals of each species were placed in Eppendorf tubes, inserted into glass test tubes that were then plugged with sponges, and placed inside an alcohol bath (Haake Phoenix II C50P, Fisher Scientific UK Ltd, Loughborough, U.K.), prior to each experimental treatment. Control groups were handled, and exposed, in the same way at 4^{o}C. The temperature experienced by the invertebrates was measured by placing a thermocouple within an identical Eppendorf tube into one of the glass test tubes. Humidity typically remains high within this experimental set-up, and is assumed not to impact survival based on previous findings (Everatt *et al.*, 2013a). At the end of experimental treatments, individuals were rapidly transferred (over ice) from the

Eppendorf tubes into plastic universal tubes containing substratum, and returned to the rearing conditions (see also Everatt *et al.* 2013a). Survival, defined by individuals moving either spontaneously or in response to gentle contact stimulus, was assessed 72 h after treatment.

8.3.4. Activity thresholds

Activity thresholds were assessed for *E. murphyi* only within an aluminium block arena. The temperature within the arena was regulated using an alcohol bath, and activity monitored using a digital video camera with a macro lens (see Hazell *et al.*, 2008). Thirty larvae in groups of 10 were transferred into the arena and allowed to settle before video recording (Studio Capture DT, Studio86Designs, Lutterworth, UK) and the alcohol bath programme began. The temperature of the arena was raised from 4 to 40^{o}C at two different rates, 0.2 and 0.1^{o}C min^{-1}. The temperature at which each individual larva last moved its body was recorded.

8.3.5. Long-term heat tolerance

Five replicates of 10 individuals of *M. arctica* and *E. murphyi* were transferred to either 4, 9 or 15^{o}C for up to 210 d. Individuals were held in universal tubes with a base of moist Plaster of Paris and a small amount of substratum within an incubator or temperature controlled room (9^{o}C). The temperature inside the incubators and room was checked using a Tinytag Transit 2 Datalogger. Survival was assessed every 7 d (see also Everatt *et al.*, 2013a).

8.3.6. Effect of recovery on heat tolerance

To test the effect of recovery at cooler temperatures on heat tolerance, three replicates of 10 individuals were exposed to one of three treatments: i) 25^{o}C for 10 d, ii) ten 24 h exposure periods at 25^{o}C, each separated by 1 h recovery at 4^{o}C and iii) ten 24 h exposure periods at 25^{o}C, each separated by 2 h recovery at 4^{o}C. Larvae were kept in plastic universal tubes with a base of moist Plaster of Paris and substratum. Transfer from and to 25^{o}C was followed and preceded by 1 h at 15^{o}C to avoid cold and heat shock. Survival was assessed after each day (treatment i) or 24 h exposure period (treatment ii and iii).

8.3.7. Rapid Heat Hardening (RHH)

8.3.7.1. Determination of the discriminating temperature

The discriminating temperature is defined as the temperature at which there is 10-20% survival (Lee *et al.*, 1987). Three replicates of 10 individuals of *M. arctica* were exposed directly (without ramping at 4^{o}C) to progressively higher temperatures (30 – 36^{o}C) for 1 h, before cooling to 4^{o}C at 0.2^{o}C min^{-1}. Invertebrate collection and handling, controls, thermocouple use, recovery and survival assessment were as described in sub-section 8.3.3. Preliminary trials on *E. murphyi* suggested that the midge did not show RHH (data not shown) and so RHH was only assessed in *M. arctica*.

8.3.7.2. Induction of RHH

To test for the RHH response, three replicates of 10 individuals were warmed to the discriminating temperature at three different rates, 0.5, 0.2 and 0.1^{o}C min^{-1}. As before, samples were held for 1 h at the discriminating temperature and then cooled back to 4^{o}C

at 0.2°C min^{-1}. Invertebrate collection and handling, controls, thermocouple use, recovery and survival assessment were the same as in sub-section 8.3.3.

8.3.8. Statistical analyses

The Kolmogorov-Smirnov test was used to confirm whether survival and heat coma data were normally distributed. Normally distributed data were analysed using analysis of variance (ANOVA) and Tukey's multiple range test, and non-normally distributed data were analysed using either the Mann-Whitney U test or the Kruskal-Wallis test.

8.4. Results

8.4.1. Arctic site microhabitat temperatures

Temperatures remained above 3°C throughout the period 17th - 24th August 2011 (Fig. 8.1) at both locations. At Stuphallet, temperatures averaged 6.6°C when combining data from both Tinytag sites, and at Krykkefjellet, 7.8°C. Temperatures deviated considerably from these averages, rising as high as 16°C at Krykkefjellet. The first 3 d were noticeably warmer, averaging 0.8 and 1.3°C higher than over the whole period in Stuphallet and Krykkefjellet, respectively. The time at which these temperatures were recorded also coincided with the warmest period on Svalbard to date (Coulson, S. J. pers. Comm.).

8.4.2. Upper Lethal Temperatures (ULTs)

Individuals of *M. arctica* survived up to 35°C, while larvae of *E. murphyi* survived up to 39°C (Fig. 8.2). The difference in survival between the two species at 35°C was

significant ($P < 0.05$ one-way ANOVA, variances not equal). Survival in both species declined rapidly, falling by > 80%, within 2-3°C as they approached the ULT.

8.4.3. Heat coma

The point at which *E. murphyi* larvae no longer showed signs of electrophysiological activity or movement (heat coma) occurred above 31°C under two different rates of warming, 0.1 (31.4 ± 0.14°C) and 0.2°C min^{-1} (32.3 ± 0.18°C). The heat coma temperature was significantly higher under faster warming ($P < 0.05$ one-way ANOVA).

8.4.4. Long-term heat tolerance

Survival of both species was greatest at 4°C (Fig. 8.3). *Megaphorura arctica* tolerated 9°C for 91 d, while survival of *E. murphyi* was still above 75% following 56 d, when the experiment finished. Both species tolerated a 15°C exposure for at least 56 d (Fig. 8.3), at which point survival was greater in *E. murphyi* (32%) than in *M. arctica* (13%). Survival of *E. murphyi* larvae at all temperatures was not significantly different after 35 d ($P > 0.05$ Tukey's multiple range test, variances not equal in some cases). However, survival after 56 d was significantly lower for larvae exposed to 15°C compared to 4 or 9°C ($P < 0.05$ Tukey's multiple range test). Survival of *E. murphyi* at 9 or 4°C did not differ significantly for any of the durations tested ($P > 0.05$ Tukey's multiple range test).

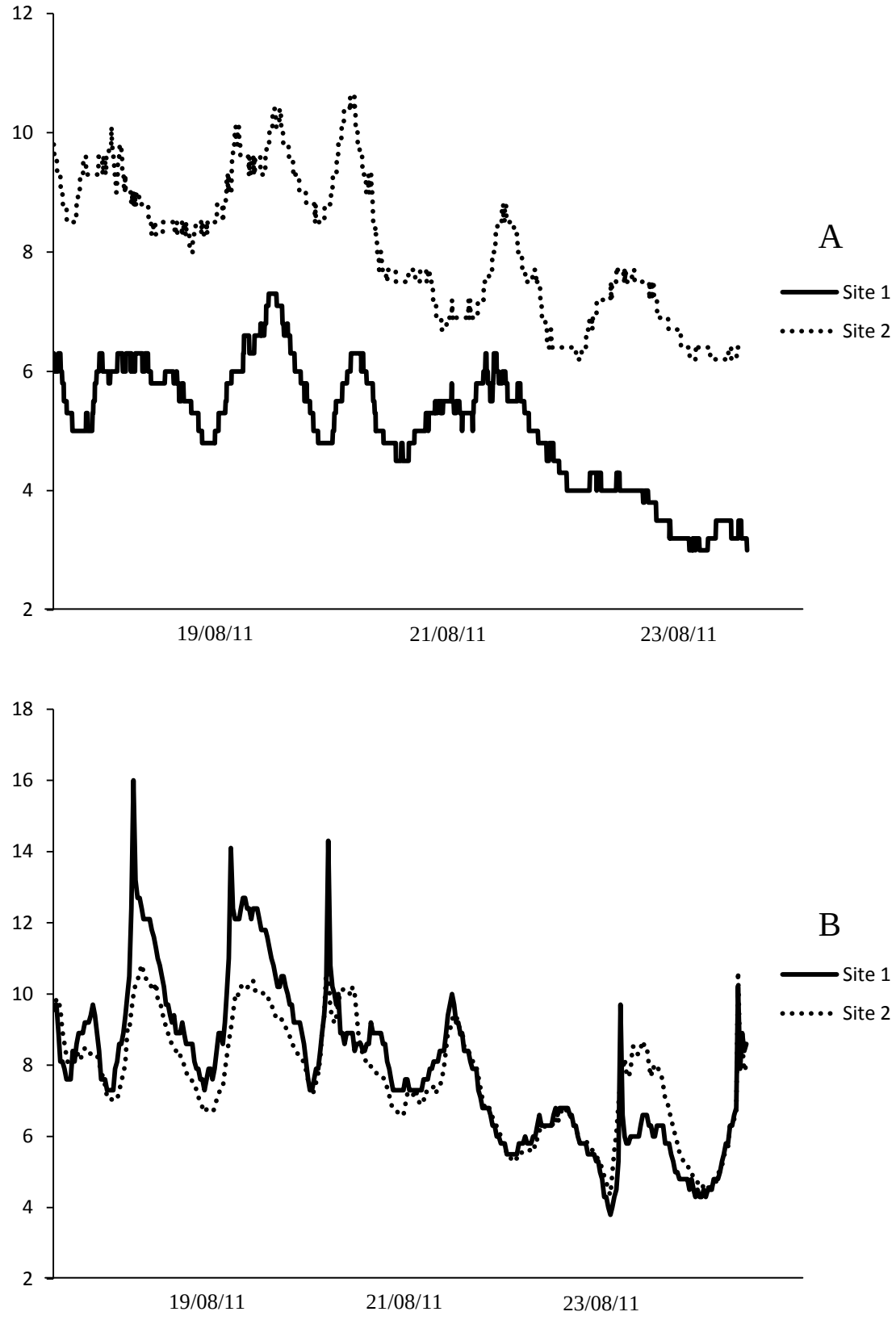

Fig. 8.1. *Surface temperature at four sites, two at Stuphallet (A) and two at Krykkefjellet (B), near Ny-Ålesund, Svalbard, between 17th and 24th August 2011.*

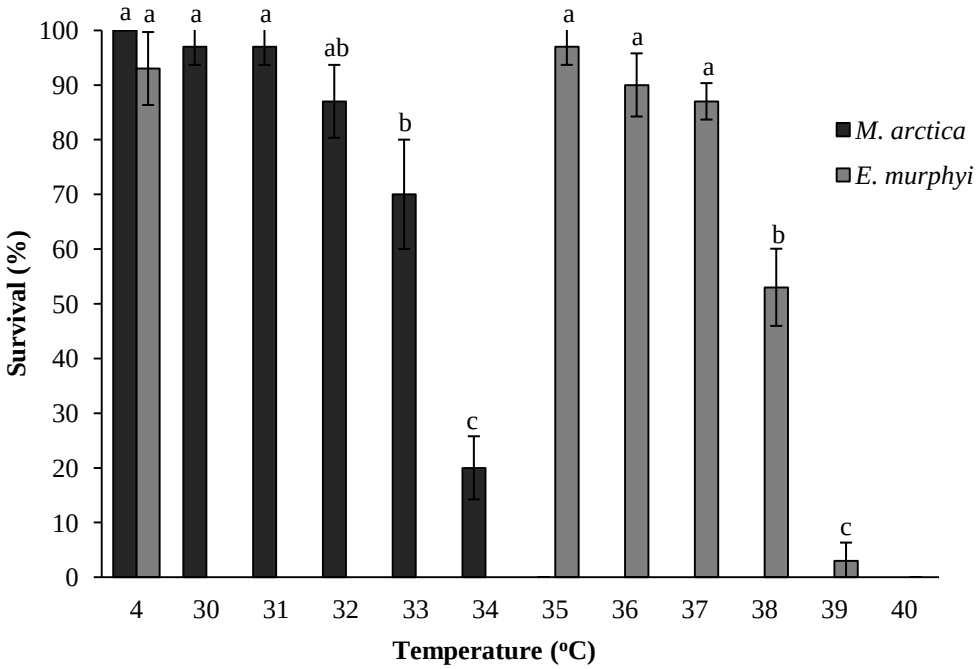

Fig. 8.2. *Survival (%) of M. arctica and E. murphyi following exposure to progressively higher temperatures (30-35°C for M. arctica, 35-40°C for E. murphyi) for 1 h. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different within each species group at P < 0.05 (Tukey's multiple range test, variance not equal for M. arctica).*

8.4.5. Effect of recovery on heat tolerance

Constant exposure to 25°C was lethal after 8 days, but survival increased with the introduction of daily recovery periods of 1 or 2 h at 4°C (Fig. 8.4). This was significant overall ($F_2 = 9.064$, $P < 0.05$ two-way ANOVA), but the interaction between time and recovery was not significant ($F_{14} = 1.849$, $P > 0.05$ two-way ANOVA). Survival following a daily 2 h recovery period at 4°C was greater than survival without recovery over the course of the entire experiment (day 2 to day 8), though the difference in survival was only significant after 6 d ($P < 0.05$ Tukey's multiple range test). A 1 h recovery period also gave greater survival for days 3-5 and day 8, but none of these differences were significant.

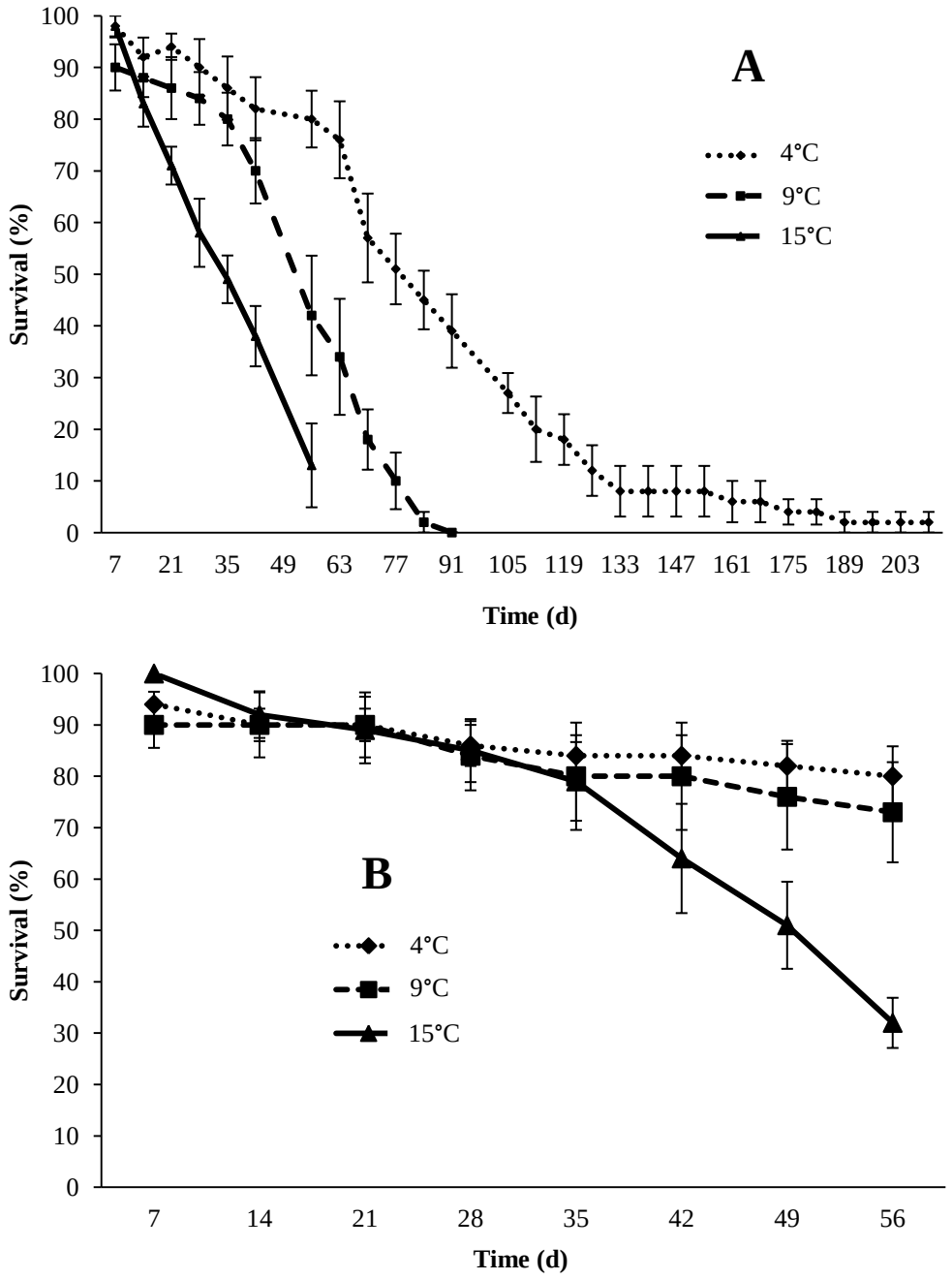

Fig. 8.3. *Survival (%) of M. arctica (A) and E. murphyi (B) at 4, 9 and 15°C over a period of up to 210 d. Means ± S.E.M. are presented for five replicates of 10 individuals.*

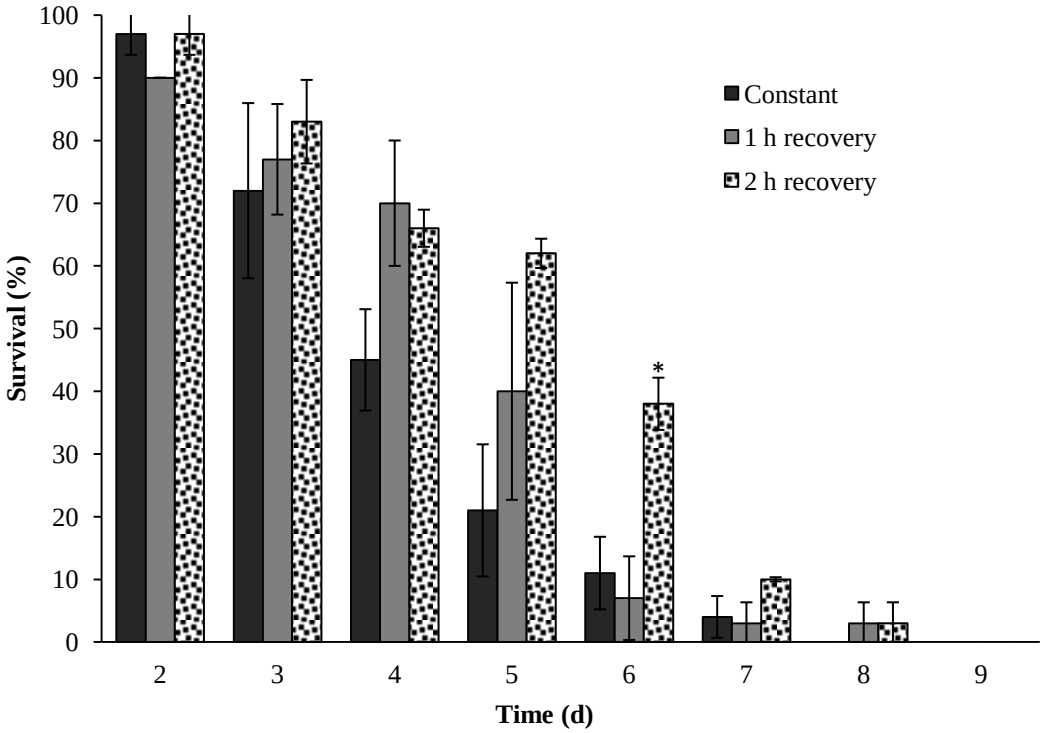

Fig. 8.4. *Survival (%) of E. murphyi at 25°C over a period of 9 d. Larvae were either given no recovery period, 1 h at 4°C after each 24 h period or 2 h at 4°C after each 24 h period. Means ± S.E.M. are presented for three replicates of 10 individuals. Asterisks indicate a recovery treatment significantly different from the constant treatment at P < 0.05 (Tukey's multiple range test, variances not equal).*

8.4.6. Rapid Heat Hardening (RHH)

8.4.6.1. Determination of the discriminating temperature

The discriminating temperature was determined to be 34.5°C for *M. arctica* (17% survival, Fig. 8.5).

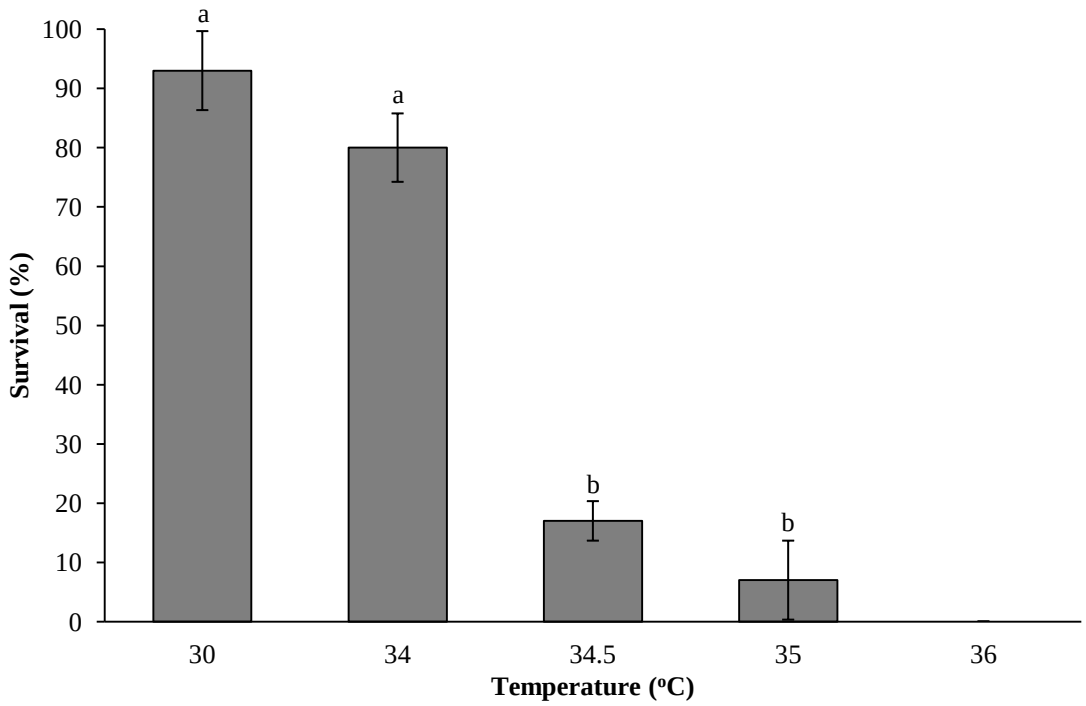

Fig. 8.5. *Survival (%) of M. arctica following direct exposure (without ramping) to progressively higher temperatures (30-35°C) for 1 h. Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different at P < 0.05 (Tukey's multiple range test).*

8.4.6.2. RHH induction

Mean survival was significantly higher following warming at a rate of 0.1°C min^{-1} (73%), compared with survival after direct transfer (17%) to the discriminating temperature ($P < 0.05$ Tukey's multiple range test, variances not equal, Fig. 8.6). Survival was also raised following warming at a rate of 0.2 and 0.5°C min^{-1}, but this was not significant ($P > 0.05$ Tukey's multiple range test, variances not equal).

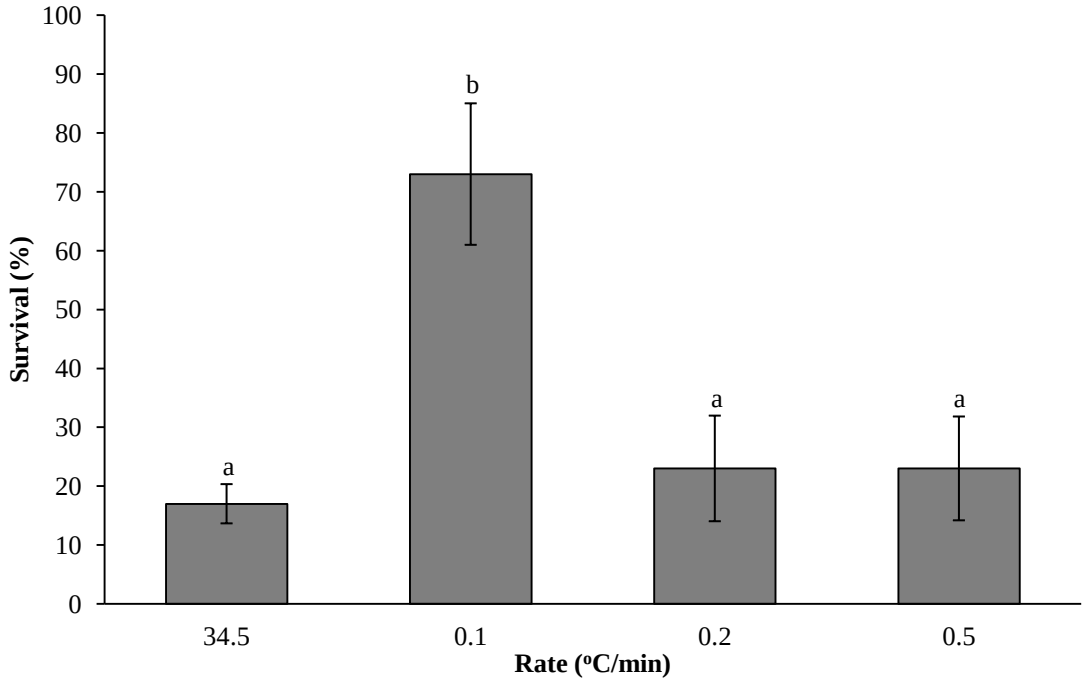

Fig. 8.6. *Survival (%) of M. arctica, following exposure to the discriminating temperature (34.5°C) for 1 h, after being warmed to the discriminating temperature at one of three rates (0.5, 0.2 or 0.1°C min^{-1}). Means ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly different at P < 0.05 (Tukey's multiple range test).*

8.5. Discussion

As poikilothermic ectotherms, invertebrate body temperatures are determined by, and vary with, the external environment (Speight *et al.*, 2008). Invertebrates are therefore susceptible to injuries, and impaired development and reproduction, resulting from exposure to temperature alterations, such as those that may result from climate change (Bale and Hayward, 2010). Changes in temperatures due to climate warming are already known to affect invertebrate population dynamics and distribution (Parmesan, 1996; Walther *et al.*, 2002). For example, climate warming has led to the occurrence of extreme heat events, which have resulted in the mass mortality of tropical species, such as corals (Walther *et al.*, 2002). Tropical species are particularly vulnerable to

temperature change as the upper temperatures they are able to tolerate lie very close to the upper temperatures experienced in their environment (Somero, 2010). Indeed, in some cases, tropical species live at temperatures which exceed their physiological optima (Somero, 2010). The current study considers whether polar species are also vulnerable to climate warming, by examining the heat tolerance and activity thresholds of the dipteran, *E. murphyi*, from the Antarctic, and further examining the heat tolerance capacity of the Arctic collembolan, *M. arctica*.

8.5.1. Basal tolerance

Both study species demonstrated considerable heat tolerance and showed survival above 34^{o}C for a period of 1 h (Fig. 8.2.). The heat coma temperature of *E. murphyi* was also very high, averaging above 31^{o}C following warming at 0.1 or 0.2^{o}C min^{-1}. Correspondingly, Everatt *et al.* (2013a) demonstrated survival up to 37^{o}C in the collembolan, *C. antarcticus*, and survival up to 40^{o}C in the mite, *A. antarcticus*, with similar results also being demonstrated in other Antarctic species (Deere *et al.*, 2006; Sinclair *et al.*, 2006; Slabber *et al.*, 2007). Block *et al.* (1994) and Hodkinson *et al.* (1996) likewise demonstrated high temperature survival in Arctic Acari and Collembola, including in *M. arctica*. The survival of *M. arctica* in this study was almost identical to that found by Block *et al.* (1994) and Hodkinson *et al.* (1996), with all three studies showing virtually 100% survival at 30^{o}C and an upper lethal temperature of 35^{o}C. Extending the exposure time to 3 h shifted survival downwards, but still gave survivorship above 30 ^{o}C (Block *et al.*, 1994; Hodkinson *et al.*, 1996). These temperatures are considerably higher than the temperatures experienced throughout the year in both the Antarctic and Arctic, including in summer and short duration extreme

maxima. Temperature conditions varied across small spatial scales at both the Stuphallet and Krykkefjellet sites (Fig. 8.1), and microhabitat buffering would further protect terrestrial invertebrates from temperature extremes. *Megaphorura arctica* and *E. murphyi* therefore have considerable capacity to tolerate current summer conditions, including conditions that are unusually warm. These species also have the capacity to tolerate the much higher temperatures that will likely occur as a result of climate warming (Arctic Council, 2005; Convey *et al.*, 2009; Turner *et al.*, 2009), further consolidating the hypothesis set out by Deutsch *et al.* (2008).

In addition to the well characterised cellular damage inflicted during acute exposure to temperature extremes, injury can also occur following long-term exposure to more moderate temperatures (e.g. Czajka and Lee, 1990). In the current study, *M. arctica* and *E. murphyi* were exposed to 9 and 15^{o}C for several weeks. Although mortality occurred at these temperatures, both species survived well for the first 4 weeks, particularly at 9^{o}C (Fig. 8.3). The collembolan survived until 91 d at 9^{o}C and 56 d at 15^{o}C and, while the experiment was only carried out over 56 d for *E. murphyi*, mean survival at 9°C was still above 70%. Hodkinson *et al.* (1996) showed similarly good survival in *M. arctica* at 10^{o}C, with the collembolan surviving up to 196 d, with less than 50% mortality after 140 d, in that instance. Some individuals were also able to survive up to 68 d at 25^{o}C. Such tolerance is notable when compared with their Arctic microhabitat temperatures. For only a few periods, of no more than 24 hours, did temperatures exceed 9^{o}C, and at only one point did temperatures exceed 15^{o}C (Fig. 8.1). Likewise, maximum temperatures 3 cm below the soil surface recorded between 1991 and 1993 did not exceed 14^{o}C (Hodkinson *et al.*, 1996). Temperatures above 9^{o}C are even more unusual

on Signy Island or more generally in the maritime Antarctic (Davey *et al.*, 1990; Bokhorst *et al.*, 2008).

8.5.2. Physiological plasticity

Polar terrestrial invertebrates are exposed to a highly variable climate. Temperatures can vary seasonally by up to 100^{o}C and daily by as much as 50^{o}C (Convey, 1996). Even in buffered microhabitats, there will be ample variation. Terrestrial invertebrates will therefore not be exposed to either constant low or high temperatures, and will also be exposed to milder transitional temperatures, giving them an opportunity to recover from thermal injuries. It has already been shown in a number of invertebrates, including the firebug, *Pyrrhocoris apterus* (Kostal *et al.*, 2007), the beetle, *Alphitobius diaperinus* (Renault *et al.*, 2004; Kostal *et al.*, 2007; Colinet, 2011; Lalouette *et al.*, 2011), the parasitic wasp, *Aphidius colemani* (Colinet *et al.*, 2007), and the flesh fly, *Sarcophaga crassipalpis* (Dollo *et al.*, 2010), that pulses at warmer temperatures allow recovery from chilling injury. However, few studies have looked at analogous recovery from higher temperatures. In the current study, larvae of *E. murphyi* exhibited improved survival following daily recovery of 1 h, but particularly following 2 h, at 4^{o}C (Fig. 8.4). Greater survival with increasing duration of recovery has also been demonstrated in *A. diaperinus* (Colinet *et al.*, 2011). The lethal time (Lt_{50}) of the beetle increased significantly from a 0.5 to 4 h recovery period. We speculate that longer recovery times than used in the current study would further enhance survival of *E. murphyi* larvae. Recovery from, and repair of, chilling injury has been shown to involve ion gradient homeostasis (Kostal *et al.*, 2007), induction of antioxidants (Lalouette *et al.*, 2011), and the up-regulation of key proteins (Colinet *et al.*, 2007). Analogous responses during

recovery from high temperature injury may also occur. The up-regulation of heat shock proteins (HSPs), for example, is a common response to stressful conditions and is known as the 'heat shock response' because of its role in repair of heat shock injuries (Clark and Worland, 2008). Heat shock proteins help refold and stabilise proteins and other macromolecules during stress (Clark and Worland, 2008), and may also be involved with the recovery of microfilament dynamics (Tammariello *et al.*, 1999) and the regulation of apoptosis (Yi *et al.*, 2007).

A further means by which terrestrial invertebrates show physiological plasticity to high temperatures is through acclimation. However, the benefits of long-term acclimation (weeks to months) have so far been shown to be slight in polar terrestrial invertebrates. Following long-term acclimation, the widespread collembolan, *C. antarcticus*, and mite, *A. antarcticus*, were shown to either exhibit no improvement in their survival, or reduced survival, at high temperatures (Slabber *et al.*, 2007; Everatt *et al.*, 2013a). Acclimation to higher temperatures can also occur over shorter timescales in the form of rapid heat hardening (RHH), which is defined as the rapid induction of heat tolerance over minutes to hours (Benoit *et al.*, 2009b). Unlike rapid cold hardening, which has now been demonstrated in an increasing number of species (e.g. Kelty and Lee, 1999; Powell and Bale, 2004; Lee *et al.*, 2006b; Owen *et al.*, 2013), including *E. murphyi* (Everatt *et al.*, 2012), RHH has been little explored. In polar terrestrial invertebrates, there is only evidence for the effect in *C. antarcticus* and *A. antarcticus* (Everatt *et al.*, 2013a). The current study also showed an RHH response in *M. arctica* (Fig. 8.6). Following a warming rate of 0.1^{o}C min^{-1}, survival of *M. arctica* at 34.5^{o}C was increased by 56%, compared with survival after a direct transfer to the same temperature. However, survival was not raised at 34.5^{o}C following a rate of 0.2 or 0.5^{o}C min^{-1}.

Greater survival at a rate of 0.1°C min^{-1} can be explained by an increased time being available for *M. arctica* to respond physiologically. Greater time at protection-inducing temperatures has also been shown to give greater survival at lower temperatures, including in the western flower thrips, *Frankliniella occidentalis* (McDonald *et al.*, 1997). While 0.1°C min^{-1} is a slow rate compared with other studies, rates will be slower still in nature (Convey and Worland, 2000, also see Fig. 8.1). It is therefore speculated that, with more time to acclimate, *M. arctica* will show an even greater RHH response and thereby possess an additional mechanism improving its tolerance of a temperature change.

8.5.3. Water availability and alien species in an era of climate warming

Although the direct impacts of high temperature are important, climate warming in the polar regions is also associated with changes in water availability and a heightened threat of alien species establishment. As climate warming intensifies, precipitation is predicted to increase at mid-high latitudes (Walther *et al.*, 2002; Ávila-Jiménez *et al.*, 2010). Under conditions of increased water availability, Antarctic invertebrates have been shown to thrive under warming, with increases in both Collembola and mite numbers (Convey *et al.*, 2002; Schulte *et al.*, 2008; Day *et al.*, 2009). However, rising temperatures are also expected to reduce snow cover and thaw ice earlier in the season, in turn resulting in the earlier evaporation of meltwater during the summer, which may instead leave invertebrates susceptible to desiccation (Callaghan *et al.*, 1992; Walther *et al.*, 2002; Ávila-Jiménez *et al.*, 2010). Under this scenario, polar terrestrial invertebrates have been shown to fare less well. Block *et al.* (1994) and Hodkinson *et al.* (1996) demonstrated the heat tolerance of collembola, including *M. arctica*, to be reduced

when desiccated, as compared to those which were hydrated, while Coulson *et al.* (1996), Convey *et al.* (2002) and Day *et al.* (2009) showed decreasing numbers of Collembola under field conditions. Even so, because the heat tolerance of polar terrestrial invertebrates far exceeds buffered microhabitat temperatures, as shown in the current study, and because their heat tolerance still remains high under desiccation (Block *et al.*, 1994; Hodkinson *et al.*, 1996), we speculate that changes associated with climate warming will result in a positive change to the invertebrate fauna.

The probability of alien species establishment is also predicted to increase with climate warming. As temperatures rise, areas which were previously too stressful for invading organisms are beginning to open up (Frenot *et al.*, 2005; Chwedorzewska, 2009). Increasing human activity, as a result of scientific research and, more recently, tourism is also aiding the transfer of alien species by allowing them to bypass geographical and environmental barriers, particularly in the Antarctic (Frenot *et al.*, 2005; Chown *et al.*, 2012). Events in the sub-Antarctic provide a glimpse into what might happen, with native flora and invertebrate fauna of many islands suffering in the presence of invasive alien species (Frenot *et al.*, 2005; Chwedorzewska, 2009).

8.6. Conclusion

As with the polar Collembola and Acari that have been studied to date, the Antarctic midge, *E. murphyi*, possesses considerable heat tolerance that equips it to survive current and predicted future environmental conditions. This species and the Arctic collembolan, *M. arctica*, also demonstrate physiological plasticity with respect to recovery from high temperature, and RHH, respectively. Polar terrestrial invertebrates may therefore be protected from the harmful consequences of a temperature rise that

may result from climate change, at least at a physiological level (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008). However, to identify likely consequences at community level, it is imperative that this is also balanced with other factors, including changes in water availability and competition from alien species, and that the sub-lethal characteristics of invertebrates, including development and reproduction, are also considered.

CHAPTER 9: GENERAL DISCUSSION

The Antarctic and Arctic contain some of the most extreme environments on Earth. Soil temperatures frequently fall below -10^{o}C in winter and rarely rise above 10^{o}C in summer (Block *et al.*, 2009; Strathdee and Bale, 1998). The availability of fresh water is similarly low and it is largely inaccessible during the winter and mid-summer (e.g. Treonis and Wall, 2005). There are also a number of other potential stressors, which include salinity (Elnitsky *et al.*, 2009), extremes of pH (Rinehart *et al.*, 2006), anoxia (Lopez-Martinez *et al.*, 2008), UV radiation (Strathdee and Bale, 1998), and pollution (Avila-Jimenez *et al.*, 2010; Bindesbol *et al.*, 2009). It is because of this multitude of stressors, and the overall severity of stress, that it is widely perceived that these regions could not possibly contain life, let alone terrestrial invertebrate life. Life does exist, however, and terrestrial invertebrates are a large part of it. Their roles in ecosystem services, such as decomposition, carbon mineralisation and nutrient cycling, are vitally important to the functioning of polar ecosystems (Ávila-Jimenez *et al.*, 2010; Barret *et al.*, 2008; Bokhorst *et al.*, 2007; Freckman, 1988; Hughes *et al.*, 2013). This is particularly clear in both the Antarctic and Arctic, where food webs are much simpler than at lower latitudes. Significantly, this also means that they can be used as models of ecosystem function.

The research presented within this thesis builds upon existing knowledge and further clarifies how certain species survive and even thrive under such extreme circumstances. Using whole organism experimental techniques, including the assessment of survival and activity under temperature and water manipulation, the preceding chapters have explored the capacity of polar terrestrial invertebrates to tolerate stress, the

physiological strategies they have evolved to minimise the impacts of stress, and their capability to tolerate changes associated with climate warming. Here, I further expand on these core concepts, integrating the various strands (chapters) of the thesis, and identify areas of future study.

9.1. Acclimation to low temperatures

Invertebrates adapt to gradual changes in their environment via the process of acclimatisation (or, in the laboratory, acclimation) (Deere *et al.*, 2006). This process is almost universal and is found amongst invertebrates inhabiting each of the Earth's biomes, including those of the tropics (e.g. Piyaphongkul *et al.*, 2012), temperate regions (e.g. Powell and Bale, 2005), and the poles. In the Antarctic and Arctic, invertebrates commonly acclimatise physiologically for the low temperatures of winter by enhancing their cold tolerance, thereby increasing survivorship as exemplified in the midge, *Eretmoptera murphyi* (Worland, 2010), the mite, *Halozetes belgicae* (Hawes *et al.*, 2007), and the Collembola, *Cryptopygus antarcticus* and *Megaphorura arctica* (Šustr and Block, 1998; Worland *et al.*, 1998, 2007). This form of acclimatisation is referred to as beneficial acclimation or the improvement of performance at temperatures close to those which invertebrates have previously experienced (Leroi *et al.*, 1994). In Chapter 2, beneficial acclimation to cooler conditions was also shown at the sub-lethal level. The two Collembola, *C. antarcticus* and *M. arctica*, and the mite, *Alaskozetes antarcticus*, all exhibited a depression of their lower thermal thresholds of movement following 1-2 months at -2 or 0^{o}C. Previously, only two other polar species, the Arctic aphid, *Myzus polaris*, and the continental Antarctic collembolan, *Isotoma klovstadi*, have been demonstrated to show beneficial acclimation of their thermal activity

thresholds (Hazell *et al.*, 2010; Sinclair *et al.*, 2006). Acclimatisation of the lower thermal thresholds of activity in the aforementioned species would, hypothetically, allow them more time to forage and reproduce during the growing season. Currently, active seasons in the Antarctic and Arctic are short, lasting for at most 4-6 months of the year and often much less (Convey, 1996; Coulson *et al.*, 1995b; Somme, 1986). Extending the time during which invertebrates are active may have important consequences at the organismal and population level.

To illustrate this further, consider a hypothetical scenario (Fig. 9.1.). Prior to acclimation, all three study species can remain active on Signy Island (maritime Antarctic) for the whole of summer and the beginning of autumn, as well as for much of mid-late autumn in the case of the two Collembola. The low temperature activity they already show gives them a large window of opportunity that would not be open to their temperate and tropical counterparts. However, this window is extended even further following low temperature acclimation. After two months at -2°C, *A. antarcticus* and *C. antarcticus* show activity for several more days than they would have previously. Although this may not seem much, small changes at the level of the organism can have large effects when scaled up to the level of a population or community. *Megaphorura arctica* is the exception in that it shows a rise in its CTmin following acclimation, though it should be noted that this collembolan lives in the Arctic (Strathdee and Bale, 1998) and shows a different overwintering strategy than both the Antarctic mite and collembolan (Worland *et al.*, 1998), which may have a bearing on the acclimation of its thermal activity thresholds. Further investigation into the plasticity of polar terrestrial invertebrates' activity in relation to more realistic microhabitat temperatures would

better reveal the true activity windows of these animals. Discerning the temperatures at which they are no longer able to forage and reproduce would also be of great value.

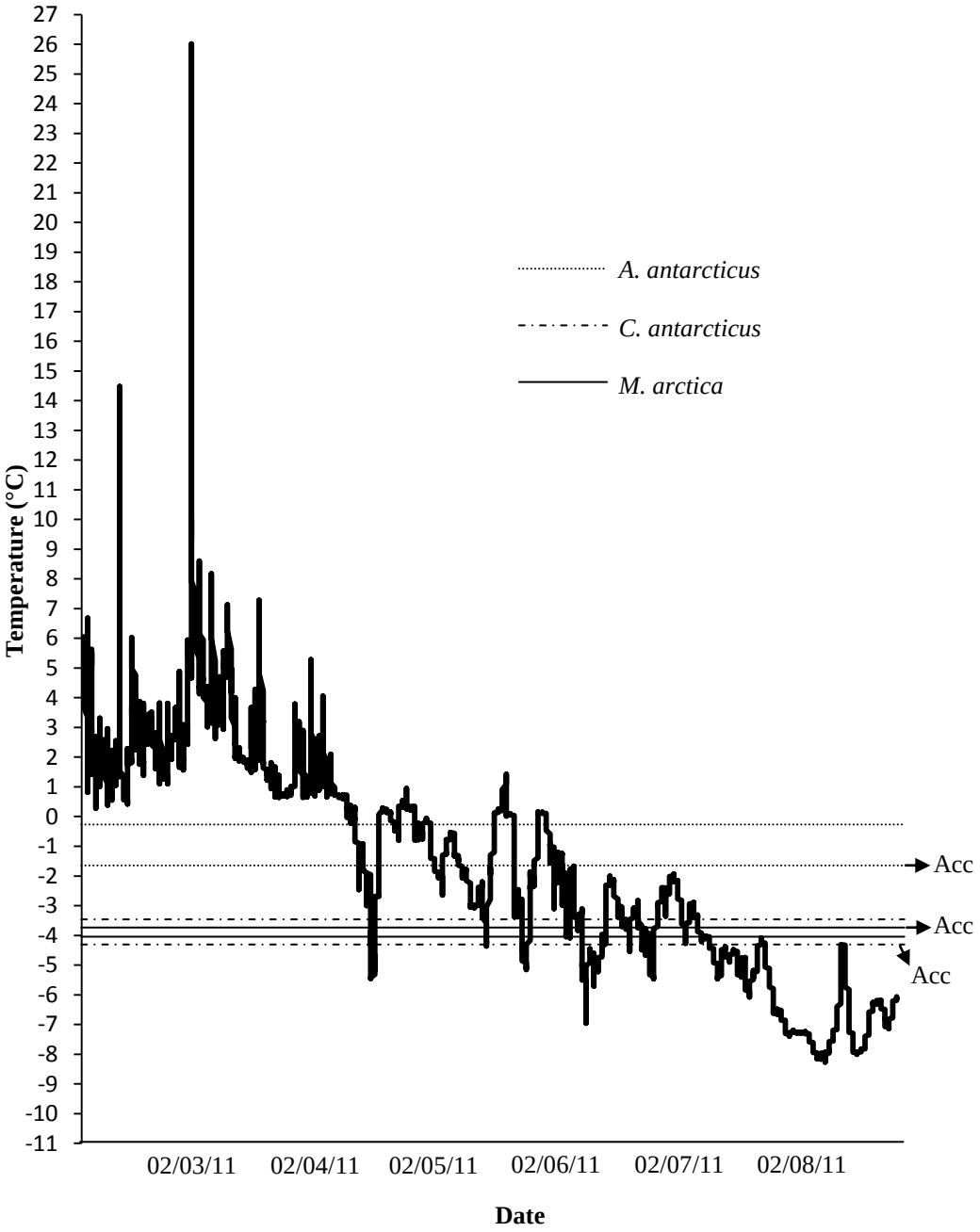

Fig. 9.1. *Soil temperature on Signy Island between February and September 2011. Horizontal lines denote the CTmin of A. antarcticus, C. antarcticus and M. arctica before and after acclimation at -2°C. The acclimated condition has been labelled (Acc), while the non-acclimated condition has been left blank.*

Acclimatisation to low temperatures can also occur over much shorter timescales, within minutes to hours, through rapid cold hardening (RCH). Rapid cold hardening was first described in the flesh fly, *Sarcophaga crassipalpis*, by Lee *et al.* (1987) and has since been observed in a wide range of organisms, including polar invertebrates such as the collembolan, *Cryptopygus antarcticus*, the mites, *Alaskozetes antarcticus* and *Halozetes belgicae* (Worland and Convey, 2001; Hawes *et al.*, 2007), and the midge, *Belgica antarctica* (Lee *et al.*, 2006b). The response has now also been confirmed in the Antarctic midge, *E. murphyi* (Chapter 2), only the second time the response has been observed in a freeze-tolerant invertebrate. Rapid cold hardening allows an organism to adjust rapidly to sudden changes in temperature that may occur on a temporal or spatial scale (Powell and Bale, 2005; Sinclair and Chown, 2006). In the polar regions, RCH may therefore afford protection during a brief cold exposure. However, as demonstrated in Chapter 3, larvae of *E. murphyi* are able to survive all temperatures to which they are likely to be exposed on Signy Island without the need for the induction of RCH. It was therefore hypothesised that RCH may serve a greater purpose at sub-lethal temperatures, via the promotion of sub-lethal characteristics, such as reproduction and development. Previous studies have already shown the role RCH can play on sub-lethal characteristics (Kelty and Lee, 1999; Powell and Bale, 2006; Shreve *et al.*, 2004) and further support to this hypothesis is provided by the data presented in Chapter 2, which indicate that sub-lethal characteristics, in this case activity, are amenable to acclimatory change.

9.2. Desiccation and cross-tolerance

While low temperature is often assumed to be the principal stressor in the polar regions, the availability of liquid water may be as important, if not more important, in regulating population and species dynamics (Block, 1996; Kennedy, 1993). During the winter, water is locked up as ice and is inaccessible, while in summer, melt water evaporates, sometimes resulting in drought (Block *et al.*, 2009; Strathdee and Bale, 1998). Entrapment in, or exposure to, sea water during the summer also provides an environment in which access to fresh water is low (Hawes *et al.*, 2008). When these conditions are placed in combination with the small body size, and subsequently high mass-specific metabolic rate, of polar terrestrial invertebrates, the threat of desiccation and its associated injuries is great (Schmidt-Nielsen, 1997). To avoid or ameliorate these injuries, invertebrates generally adopt one of two strategies: desiccation resistance, in which water loss from the body is minimised, or desiccation tolerance, in which extensive water loss is tolerated through the induction of various physiological mechanisms. In Chapter 5, examples of the use of both of these strategies were identified. The Arctic dipteran, *Heleomyza borealis*, exhibited just a 6.9% loss of body water over 12 d at 98.2% RH (= desiccation resistance), whereas the Antarctic midge, *E. murphyi*, exhibited high survival in spite of losing nearly half of its body water (= desiccation tolerance). A further example of desiccation tolerance was demonstrated in Chapter 4, in the collembolan, *C. antarcticus*. *Cryptopygus antarcticus* and other springtails are frequently observed floating or 'rafting' on sea water (Coulson *et al.*, 2002; Hawes *et al.*, 2008), either on the sea itself or in puddles of sea water on land. As a result of evaporation, these can become concentrated. The data presented in Chapter 4 suggest that *C. antarcticus* is capable of tolerating water loss up to 30% following high

salinity exposure and only begins to suffer mortality following > 40% water loss, as can occur in saturated sea water.

Cross-tolerance, which is the enhancement of tolerance to one stress following exposure to another, is becoming increasingly apparent among invertebrates. It has been shown between low temperature and anoxia in the flesh fly, *Sarcophaga bullata* (Yoder *et al.*, 2006), between desiccation and high temperature in tardigrades (Hengherr *et al.*, 2009), between desiccation and radiation in the midge, *Polypedilum vanderplanki* (Gusev *et al.*, 2010), and between desiccation and low temperature in *B. antarctica* (Benoit *et al.*, 2009a). These invertebrates take advantage of the interrelationship that exists between stresses. Whether an invertebrate is experiencing low temperature, desiccation or radiation, the injuries and physiological symptoms that result are often similar. The physiological mechanisms and strategies that an invertebrate responds with may therefore also be analogous. Consequently, cross-tolerance serves as a distinct form of acclimation.

In polar studies, cross-tolerance has to date only been shown in the Antarctic midge, *B. antarctica* (Benoit *et al.*, 2009a; Hayward *et al.*, 2007). In Chapters 4 and 5, cross-tolerance was demonstrated in two further organisms, the Antarctic collembolan, *C. antarcticus* and the Antarctic midge, *E. murphyi*. In these species, additional protection to low temperatures was afforded following a desiccation (or salinity) pre-treatment. Low temperature and desiccation are the two most prevalent stressors in the polar regions and often occur simultaneously (Block *et al.*, 2009; Strathdee and Bale, 1998). The confluence of protection between these two stresses is therefore likely to be highly beneficial. It should be noted, however, that the sub-lethal benefits of cross-tolerance have received less attention in previous studies, with the exception of Sinclair *et al.*

(2007), who demonstrated that *D. melanogaster* previously selected for greater desiccation resistance/tolerance had a shorter, and thus improved, chill coma recovery time as compared with controls. Yet, in Chapter 4 of this thesis, the upper and lower thermal activity thresholds were shown to be negatively affected following salinity exposure. Cross-tolerance may thus come at a cost in some species and be subject to an evolutionary trade-off.

9.3. Climate warming and alien species invasion

Since 1880, global temperatures have risen by 0.85^{o}C (IPCC, 2013). The rise in temperature has been particularly acute at higher latitudes, averaging 2^{o}C over the last 50 years in parts of the polar regions (Arctic Council, 2005; Convey *et al.*, 2009; Turner *et al.*, 2009). Less or equivalent warming has led to species extinctions in non-polar regions and has been predicted to reduce endemic species by a further 15-52% by 2050, if current general circulation models of warming are accurate (Millennium Ecosystem Assessment, 2005).

In Chapters 7 and 8, the tolerance of heat, and thus also of climate warming, was investigated in four polar invertebrates; *C. antarcticus*, *A. antarcticus*, *E. murphyi* and *M. arctica*. All of these species demonstrated considerable heat tolerance over both short and long time scales, and the heat tolerance shown far exceeds what is currently required during Antarctic and Arctic summers. This level of tolerance was also shown in Chapter 2, in which both Collembola and the mite exhibited CTmax and heat coma temperatures above 30°C. These results support the hypothesis presented by Deutsch *et al.* (2008), which proposes that the sensitivity of terrestrial invertebrates to a temperature change decreases with increasing latitude. However, the physiological

plasticity these invertebrates show in relation to heat stress is comparatively limited. Survival of *C. antarcticus* and *A. antarcticus* following high temperature exposure was negatively affected by an acclimation pre-treatment at 9^{o}C. Acclimation over shorter time scales in the form of rapid heat hardening (RHH) similarly provided only minimal benefit for these two species, as well as for *E. murphyi*, with a more substantial role of RHH indicated only for *M. arctica*.

Regardless of whether these species have the capacity to respond plastically to high temperatures or not, their basal heat tolerance is more than sufficient to tolerate current conditions. These species will also more than likely be able to tolerate future warming, as based on general circulation models (Ávila-Jimenez *et al.*, 2010; Convey *et al.*, 2009). It should be noted that certain microhabitats can rise above 30°C on occasion, and these extreme events are expected to increase as climate change intensifies. However, it is expected that the invertebrates will be able to quickly relocate to more thermally buffered microhabitats if necessary. If the species studied in this thesis are representative of other polar terrestrial invertebrates, the survival of polar terrestrial communities during an era of climate warming is anticipated. Polar terrestrial invertebrates may therefore not provide an early warning system for climate warming as has been described previously for the polar regions in general and for ice extent in particular (e.g. Spielhagen, 2012). Instead, they are likely to respond positively and may show enhanced growth, both with respect to their abundance and their distribution, as has also been concluded by Convey (2011).

However, climate warming is not just about the direct impacts of temperature, but also indirect impacts on ice melt/flooding, as well as precipitation (e.g. Convey *et al.*, 2003), pollution (e.g. Callaghan *et al.*, 1992; Convey *et al.*, 2002) and alien species invasion,

all of which could have a marked impact on native invertebrate communities. Alien species invasion increases in likelihood as climate warming intensifies. Areas that were previously too stressful for invading organisms are beginning to open up (Chwedorzewska, 2009; Frenot *et al.*, 2005). Increasing human activity, as a result of scientific research and, more recently, tourism is also aiding the establishment of alien species by allowing such species to bypass geographical and environmental barriers and colonise the polar regions at an ever increasing rate (Chown *et al.*, 2012; Frenot *et al.*, 2005). The sub-Antarctic islands have now witnessed numerous species introductions and there are some islands, such as South Georgia, which host more alien species than native species in some of the major taxonomic groups (Chown and Convey, 2007, Chown *et al.*, 2008; Frenot *et al.*, 2005). The result has not been entirely positive, with competition, predation and disease becoming ever more prevalent as more alien species establish (Bale, 2000; Chwedorzewska, 2009). Further, there is little opportunity for dispersal in the remote and isolated regions of the Antarctic and Arctic, making any kind of escape from alien invaders a slim possibility (Block *et al.*, 2009; Strathdee and Bale, 1998).

In contrast to the sub-Antarctic islands, alien species introductions to the maritime and continental Antarctic have been more limited, with only eight known establishment events in the maritime Antarctic to date (Hughes and Convey, 2012). These establishment events are by no means any less important due to their limited number, however, with a substantial impact already being shown by some species (e.g. Hughes *et al.*, 2013). One of these species is *E. murphyi*, a midge accidentally introduced to maritime Antarctic Signy Island probably in the 1960s. By investigating the physiology of *E. murphyi* and other invasive species in the Antarctic, we can better understand how

they are able to establish, and we may even be able to produce a template of traits that are characteristic of a successful alien species in the maritime Antarctic, as well as elsewhere. Exploring the traits of unsuccessful invertebrates, which have been reported to colonise the polar regions, but have failed to establish, or those invertebrates which have been less successful, may also be useful. For example, much may be gained from exploring the biology and physiology of the enchytraeid worm, *Christensenidrilus blocki*, which though established on Signy Island, has not spread as widely as the midge, *E. murphyi* (Hughes and Worland, 2010).

Very much like the checklist required to pass a biocontrol agent for use (Bale and Walters, 2001; Lenteren *et al.*, 2003), a similar checklist could be created for potential alien species of the maritime and continental Antarctic, and High Arctic. Such a checklist has already been developed for Collembola likely to colonise and establish on the sub-Antarctic islands, Heard Island and South Georgia (Greenslade, 2002; Greenslade and Convey, 2012), and this may allow for the better prevention and/or management of these invertebrates in future. Here, a schematic representing a risk assessment of alien species establishment in the maritime and continental Antarctic, and High Arctic, is presented (Fig. 9.2). The following paragraphs expand on the different steps of the assessment and put them into the context of studies conducted for this thesis.

The risk assessment begins by assessing the opportunity for colonisation (step 1). In much of the Arctic, where the land is generally connected and approachable, colonisation can occur through natural step-wise dispersal. To do so, the invertebrate requires a certain level of physiological tolerance while exposed to climate variations during travel. In contrast, if an invertebrate is to be transferred to the remote Antarctic,

and also to High Arctic archipelagoes such as Svalbard, it is largely reliant on humans (e.g. Block *et al.*, 1984; Hughes *et al.*, 2010). Consequently, the stresses during transfer become less important, but are replaced by the timing of transfer. If it is immediately exposed to conditions in winter, it is unlikely to survive. Conversely, if it is transferred in summer, there is potential for acclimatisation. Even the native invertebrates would struggle if transferred directly to winter conditions when in their summer state (e.g. Worland and Convey, 2008).

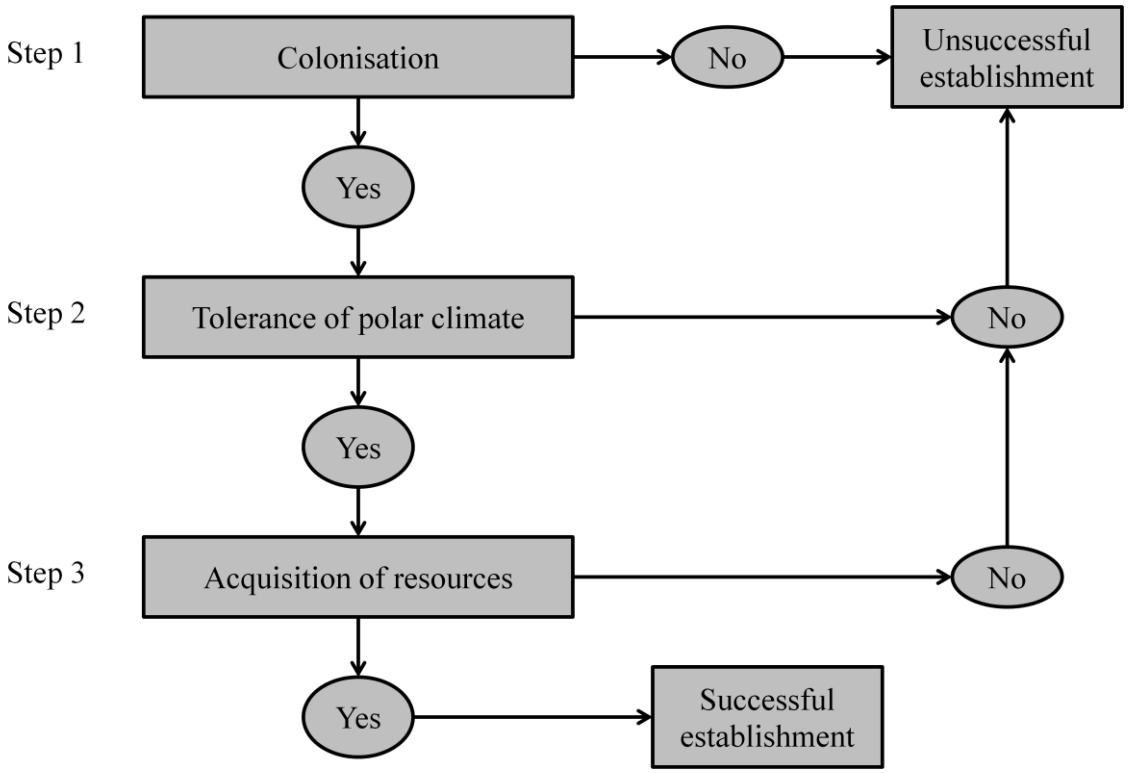

Fig. 9.2. Schematic representation of an alien species risk assessment.

Once an alien species has colonised a new region, its continued persistence is determined by its tolerance of the climate (step 2). This will first depend on its ability to locate an adequately buffered microhabitat, and so it must show some capacity for activity at low temperatures. Experiments, as performed in Chapter 2, provide a useful indicator of an invertebrate's lower thermal activity thresholds, and these can be

subsequently mapped against the temperatures of the local climate. To persist over more than just the short term and survive winter, the alien invader must also possess extensive tolerance of low temperatures and water availability. Chapters 3 and 5 offer examples of protocols that could be used to test these traits. Lower lethal temperatures and humidity manipulation demonstrate the baseline tolerance of the animal. While investigation into their acclimatory ability, both over long and short periods with respect to rapid cold hardening, provides a picture of their physiological plasticity - their ability to shift from their baseline. Snow melt and subsequent flooding can also occur during the summer, and the capacity to either tolerate anoxia or respire in water may therefore be necessary. In Chapter 6, *E. murphyi* was shown to perform the latter, providing the first documented example of an amphibious terrestrial midge. Clearly there are other stresses in both the polar regions and more widely, which would also have to be taken into account in designing protocols to be applied to specific species or regions.

The final barrier to the alien invader is the sufficient availability of food resources and mates (step 3). Food webs supported by the polar regions are small, and in the case of the maritime Antarctic and continental Antarctic, limited to just a few cryptogams and lower invertebrate groups (Block *et al.*, 2009; Convey, 2013). Being a food generalist may therefore be of benefit. Although invertebrates are known to survive starvation for several months (e.g. Hawes *et al.*, 2008), they will eventually perish if they are too selective in their diet. They will also eventually fail if they are unable to reproduce. It is therefore imperative that they are transferred alongside others of their own species, though an ability to produce young from unfertilised eggs through parthenogenesis can circumvent this, as is true of *E. murphyi* (Convey, 1992). It should be noted that like for

step 2, specifically the location of an adequate microhabitat, the attainment of food and mates will also require activity at low temperatures.

9.4. Conclusion

Polar terrestrial invertebrates are ancient and have likely spent at least the last few million years experiencing selective pressure to adapt their physiology to the extreme environments in which they live (Convey and Stevens, 2007; McGaughran *et al.*, 2010). Investigating polar terrestrial invertebrates is therefore of great value to the field of invertebrate ecophysiology and may also provide applications in, for instance, cryopreservation and pest management (Bale, 2002; Katkov, 2006). Cells and tissues are susceptible to deterioration and damage during storage (Katkov, 2006), and studies exploring the molecular mechanisms that such invertebrates use may shed light on possible cryopreservation solutions. In two recent studies, insect-derived AFPs improved cell viability during cryopreservation (Campbell *et al.*, 2011; Halwani *et al.*, 2011). Likewise, exploration of low temperature physiology in polar invertebrates may unearth alternative, or complementary, forms of pest control, such as the external application of INAs (Bale, 2002).

The work of this thesis informs the field of invertebrate ecophysiology, having shown only the second example of RCH in a freeze-tolerant insect, beneficial acclimation of thermal activity thresholds, desiccation resistance and tolerance, cross-tolerance between desiccation and low temperature, and the first example of aquatic respiration in a terrestrial midge.

Climate warming is currently of great significance and, with warming occurring at an accelerated rate in the polar regions, its impact on their contained terrestrial

communities is of great interest. The research presented within this thesis indicates that these animals have extremely large thermal tolerance windows compared with temperate or tropical species, which will not only allow them to endure climate warming, but also to profit from it (see Gaston, 2009).

Perhaps what this thesis has shown most of all is that invertebrates have great flexibility and adaptability in their physiology at the level of the individual, species and population. Such is their diversity and adaptability that they have been able to thrive in all habitats, even those as extreme and inhospitable as the Arctic and Antarctic.

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