Pre-adapted to the maritime Antarctic? – Rapid cold hardening of the midge, *Eretmoptera murphyi*

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ABSTRACT

During the 1960s, the midge, *Eretmoptera murphyi*, was transferred from sub-Antarctic South Georgia (55ºS 37ºW) where it is endemic to a single location on maritime Antarctic Signy Island (60ºS 45ºW). 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.

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1. 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, 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 (60ºS 45ºW) from the sub-Antarctic island of South Georgia (55ºS 37ºW) (Block et al., 1984; Convey and Block, 1996). The species has since spread widely and now covers an area >2000 m², 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

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tory ability of larvae. Prior to acclimation, larvae exhibited moderate freeze-tolerance, with an $LTemp_{50}$ of $-13.19\,°C$, $\sim7\,°C$ 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,b; 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).

2. Materials and methods

2.1. 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 – 2.4 (ii), 2.5 and 2.7.

2.2. 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.

2.3. 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 $0.2\,°C\,min^{-1}$.

2.4. 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 $0.2\,°C\,min^{-1}$.

2.5. 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. 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 $0.2\,°C\,min^{-1}$.

2.6. 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
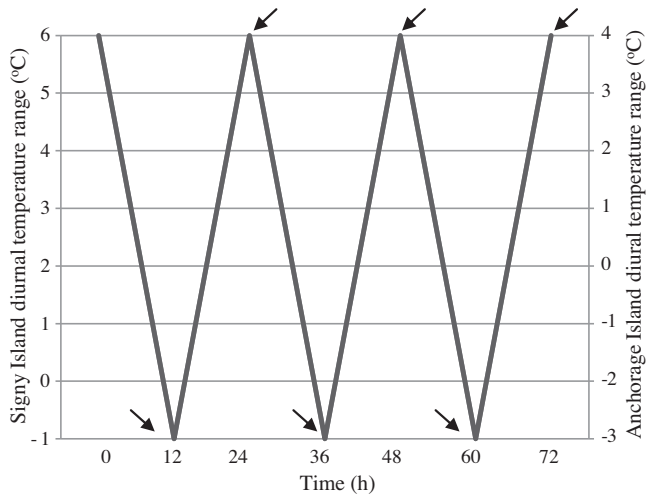
Fig. 1. Three day simulated thermoperiodic cycle for Signy (between 6 and −1 °C) and Anchorage (4 and −3 °C) Island. Arrows indicate the points at which 3 replicates of 10 mature larvae were removed from each thermoperiodic cycle and transferred directly to the DTemp (−12.5 °C).

(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).

2.7. 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.

2.8. 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. Results

3.1. 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. 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.
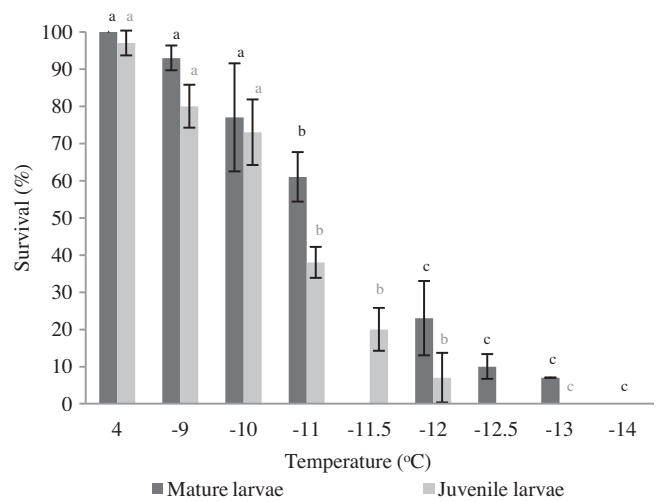

Fig. 2. Survival of juvenile and mature larvae after exposure to progressively lower sub-zero temperatures (−9 to −14 °C) for 8 h, before re-warming at 0.2 °C min⁻¹ to the rearing temperature (+4 °C). Temperatures of −11.5 and −12.5 °C were assessed for only juvenile and mature larvae, respectively, 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).

3.2. 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). 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 ($F_{1,4} = 26.156$, $P < 0.05$; $F_{1,4} = 48.400$, $P < 0.05$, respectively). Under all treatments, the strength of the RCH response
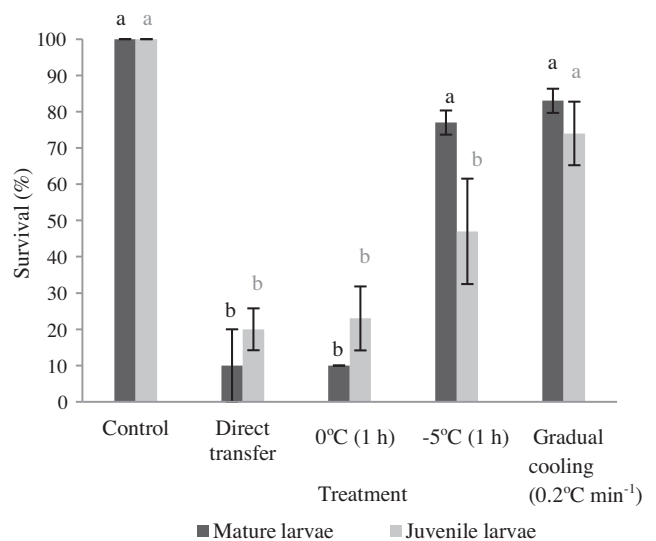

Fig. 3. Survival of juvenile and mature larvae after exposure to the DTemp for 8 h (−11.5 and −12.5 °C, respectively), following either direct transfer to the DTemp or 3 pre-treatments: 1 h at 0 °C, 1 h at −5 °C and gradual cooling (0.2 °C min⁻¹) from +4 °C to the DTemp. Mean ± S.E.M. are presented for 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).

was not significantly different between juvenile and mature larvae ($P > 0.05$ Tukey's multiple range test).

3.3. Limits of RCH

RCH lowered the lower lethal temperature (LLT) by 2.5 and 6.5 °C in mature and juvenile larvae, respectively (Fig. 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. 5).

3.4. 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. 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. 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 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 ($F_{1,4} = 11.560$, $P < 0.05$).

3.5. 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 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).

3.6. 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.

4. 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 (Greens-
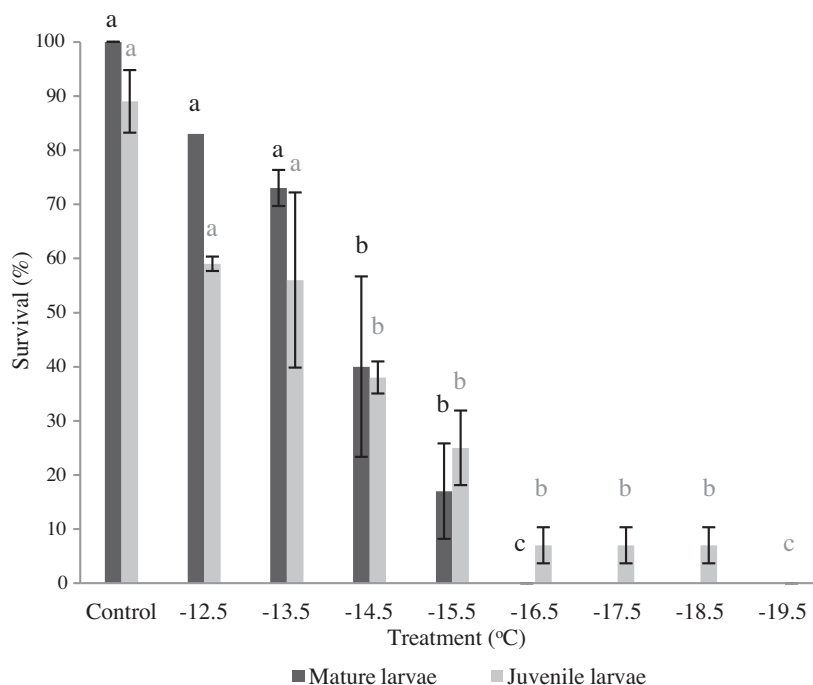

Fig. 4. Survival of juvenile and mature larvae following gradual cooling (0.2 °C min⁻¹) from the rearing temperature (+4 °C) to progressively lower temperatures below the DTemp (−11.5 to −19.5 °C) for 8 h at these temperatures. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival 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).

Fig. 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).
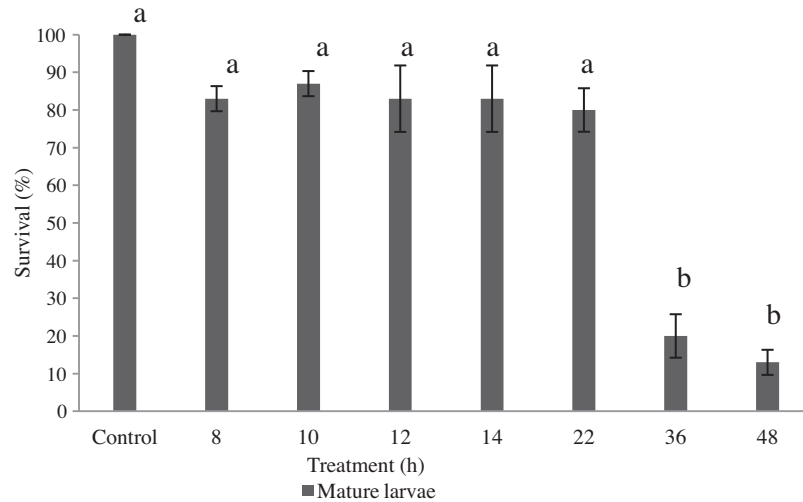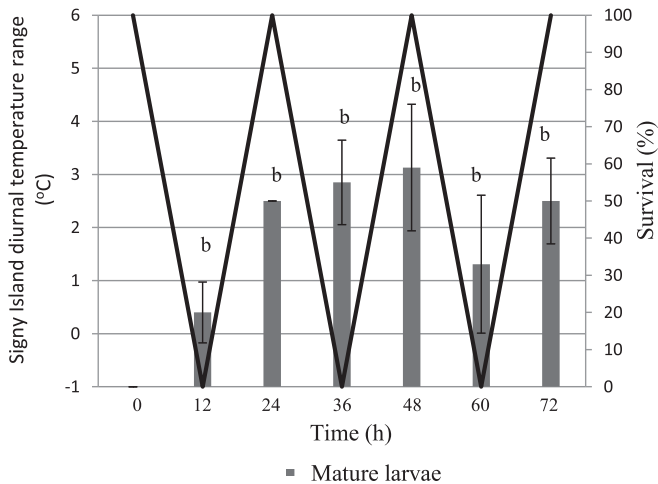

Fig. 6. Survival of mature larvae (bars) after exposure to the DTemp at specific points (−1 and 6 °C after 1, 2 and 3 d) during a thermoperiodic cycle. Mean ± S.E.M. are presented for three replicates of 10 individuals. Survival was assessed 72 h after treatment. Means with the same letter are not significantly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

Fig. 7. Survival of mature larvae (bars) after 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.
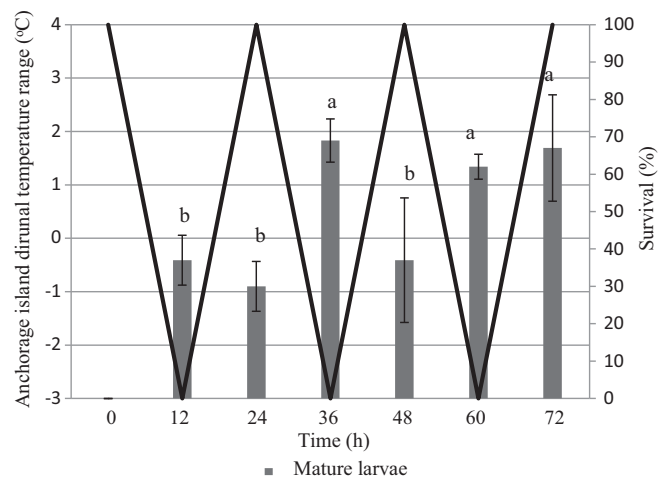
lade et al., in review). As with the non-native species (>200) known from the sub-Antarctic islands, these organisms may have significant impacts on the native ecosystems (Frenot et al., 2005). *H. viatica* is described as an aggressive invader on South Georgia and Macquarie Islands (Frenot et al., 2005; Tin et al., 2009). Likewise, *E. murphyi* has been shown by Hughes et al. (in review) 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.

4.1. Basal cold tolerance

Although this study centres on the RCH response of *E. murphyi*, the data obtained also confirm that both juvenile and mature lar-

Table 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 24 replicates of single individuals. Survival was assessed 72 h after treatment.

	SCP (°C) ± S.E.M.
0.2 °C min^{-1} (mature larvae)	−5.42 ± 0.18
0.2 °C min^{-1} (juvenile larvae)	−7.29 ± 0.54
1 °C min^{-1} (mature larvae)	−5.00 ± 0.18
Direct transfer (−12.5 °C, mature larvae)	−5.60 ± 0.23
Direct transfer (−11.5 °C, juvenile larvae)	−5.86 ± 0.43

vae 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-adapta-

tion, 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 46ºW) (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).

4.2. 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, 2005, 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). 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. 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. 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).

4.3. 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), and only slightly at −1 °C (Fig. 6), while a much stronger response was induced at −3 (Fig. 7) and −5 °C (Fig. 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). 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. 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.

4.4. 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 sur-

vive 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 sublethal 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).

4.5. 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,b; 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.

5. 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., 2006a,b). 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° 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.

Acknowledgements

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