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Deleterious effects of repeated cold exposure in a freeze-tolerant sub-Antarctic caterpillar

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Summary

Multiple freeze–thaw cycles are common in alpine, polar and temperate habitats. We investigated the effects of five consecutive cycles of approx. –5°C on the freeze-tolerant larvae of *Pringleophaga marioni* Viette (Lepidoptera: Tineidae) on sub-Antarctic Marion Island. The likelihood of freezing was positively correlated with body mass, and decreased from 70% of caterpillars that froze on initial exposure to 55% of caterpillars that froze on subsequent exposures; however, caterpillars retained their freeze tolerance and did not appear to switch to a freeze-avoiding strategy. Apart from an increase in gut water, there was no difference in body composition of caterpillars frozen 0 to 5 times, suggesting that the observed effects were not due to freezing, but rather to exposure to cold *per se*. Repeated cold exposure did not result in mortality, but led to decreased mass, largely accounted for by a decreased gut mass caused by cessation of feeding by caterpillars. Treatment caterpillars had fragile guts with increased lipid content, suggesting damage to the gut epithelium. These effects persisted for 5 days after the final exposure to cold, and after 30 days, treatment caterpillars had regained their pre-exposure mass, whereas their control counterparts had significantly gained mass. We show that repeated cold exposure does occur in the field, and suggest that this may be responsible for the long life cycle in *P. marioni*. Although mean temperatures are increasing on Marion Island, several climate change scenarios predict an increase in exposures to sub-zero temperatures, which would result in an increased generation time for *P. marioni*. Coupled with increased predation from introduced house mice on Marion Island, this could have severe consequences for the *P. marioni* population.

Supplementary material available online at
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Key words: climate change, fitness consequence, freeze–thaw cycle, Lepidoptera, *Pringleophaga marioni.*

Introduction

Cyclic changes in temperature are a daily feature of almost every terrestrial habitat on earth (Bonan, 2003), but this variation becomes of especial ecological importance when it is about a threshold important to the biology of living organisms, such as the freezing point of water (Sømme, 1995). Repeated freeze–thaw events are common daily, seasonally or annually in alpine (e.g. Krog et al., 1979; Mark, 1994), polar (e.g. Coulson et al., 1995a,b; Bockheim and Hall, 2002; Sinclair et al., 2003b), sub-polar (e.g. Davey et al., 1992; Blake, 1996; Boelhouwers et al., 2003; Grogan et al., 2004) and many temperate habitats (e.g. Inouye, 2000; Boelhouwers and Meiklejohn, 2002). Furthermore, the frequency and intensity (i.e. minimum temperature reached) of freeze–thaw is often greatly modified by interactions between precipitation, snow cover and ambient winter temperatures (Danks, 1991; Leather et al., 1993; Sømme, 1995), which may have fitness implications for overwintering insects. For example, midwinter thaw and freezing rain events can result in the loss of insulating snowpack and the development of a thick ice layer, which was responsible for killing 50% of collembolan populations in a high arctic field experiment (Coulson et al., 2000). On the Rock and Pillar Range, in alpine New Zealand, strong El Niño years lead to a decrease in insulating snow cover in the alpine zone, consequently increasing the frequency and intensity of freeze–thaw cycles (Sinclair, 2001b). Conversely, insulating snow cover results in an overall increase in winter energy consumption by the gall fly *Eurosta solidaginis* (Diptera: Tephritidae), leading to a decrease in fecundity for flies that experienced warmer winter conditions (Irwin and Lee, 2000, 2003).

The consequences of repeated cold exposure for cold-hardy insects are poorly known. In the absence of other evidence, Sinclair (2001b) made the assumption that cold hardiness parameters (for example, temperature of crystallisation, T_c, the

temperature at which an insect freezes) remain static across repeated frost cycles. However, Bale et al. (2001) showed that supercooling points (SCP, equivalent to the T_c) of larvae of *Hydromedion sparsutum* (Coleoptera: Perimylopidae) on South Georgia are plastic. The SCP of some individuals of *H. sparsutum* decreases after an initial freezing event, leading to the segregation of the population into individuals that continue to utilise freeze tolerance, and those that avoid freezing (Bale et al., 2001). Furthermore, Brown et al. (2004) demonstrated that SCP decreases and pupation and emergence success rates decline (but 72 h survival remains unaffected) in *Syrphus ribesii* (Diptera: Syrphidae) larvae that are repeatedly frozen and thawed. These effects do not differ markedly between groups of insects frozen at weekly, daily and hourly intervals (Brown et al., 2004). Mechanical and physiological stress during freezing and thawing (Zachariassen, 1985; Sinclair and Wharton, 1997; Kristiansen and Zachariassen, 2001), coupled with observations of increased post-thaw metabolic rate (Zachariassen et al., 1979; Block et al., 1998), suggest that repeated freeze–thaw will be accompanied by energetic and other costs, even in freeze-tolerant insects. However, several groups of insects may be well-adapted to repeated cold exposure cycles, especially those from temperate habitats in the southern hemisphere (Sinclair et al., 2003a). Sinclair et al. (2004) found a post-thaw decrease, rather than an increase, in metabolic rate of *Pringleophaga marioni* (Lepidoptera: Tineidae) larvae relative to pre-exposure, suggesting that the metabolic cost of a single freeze–thaw event might not be high in this species and others that share its response to low temperatures (Sinclair et al., 2003a). Thus, repeated freezing and thawing might not be necessarily associated with alterations in strategy and increases in metabolic and other costs.

Here we investigate the consequences of repeated cold exposure cycles for *Pringelophaga marioni* caterpillars, a freeze-tolerant species from sub-Antarctic Marion Island. We do so for several reasons. First, the responses of the larvae of *P. marioni* to freezing and to low temperatures are well understood (Klok and Chown, 1997; Sinclair and Chown, 2003; Sinclair et al., 2004). Second, caterpillars of *P. marioni* are ecologically important on sub-Antarctic Marion Island (46°54′S, 37°45′E) because of their wide distribution, large size (0.1–0.3 g) and role as keystone decomposers (Crafford et al., 1986; Smith and Steenkamp, 1992). Therefore, these freeze-tolerant larvae are a useful model for exploring the impact of the island's climate on the physiological responses of the fauna. Marion Island is a cold, wet island lying just north of the Antarctic convergence (Hänel and Chown, 1998), which has undergone rapid climatic change over the past 50 years (Smith, 2002). Among the likely continuing climatic changes on Marion Island are a decrease in precipitation, and at least one scenario envisages an increase in clear-sky days (Smith and Steenkamp, 1990). While the effects on the flora and fauna of the changes to the average moisture and thermal environments are currently under experimental investigation (M. A. McGeoch, manuscript submitted for publication), the increased insolation scenario suggests that an increase in daytime microclimate temperatures will be accompanied by a decline in night-time temperatures, resulting from a decrease in sky emissivity (Campbell and Norman, 1998). Thus, the third reason for this study is that the frequency of microhabitat sub-zero temperature events may increase, particularly at the high elevation sites on the island where snow cover is likely to decrease as a consequence of decreased precipitation and higher temperatures (cf. Sinclair, 2001b).

An important aspect of investigating population-level effects on insect cold tolerance is the selection of measures of performance. While survival is adequate for studies of tolerance mechanisms, if questions are being addressed on an evolutionary scale, and particularly if the effects are likely to be sublethal, the correct response variable is the fitness (*sensu* Endler, 1986) of the individuals in question (Baust and Rojas, 1985; Bale, 1987; Chown and Nicolson, 2004). Although following individuals through to measure (for example) fecundity is not always feasible, other measures relevant to reproduction (e.g. locomotor performance in frogs; Irwin et al., 2003) or longer-term survival (e.g. emergence as adults in syrphids; Brown et al., 2004) can be suitable surrogates. Pupal mass in Lepidoptera is strongly correlated with both potential and realised fecundity (Tammaru et al., 2002; Thurston and MacGregor, 2003; but see Leather, 1988). Thus, factors that affect the ability of larvae to feed and obtain a high pupal mass are likely to be related to fitness, particularly in flightless species (see Thurston and MacGregor, 2003). We assess critical thermal minimum, below which the caterpillars cannot feed (Lee, 1991), and several measures of larval size and body composition (larval mass, gut mass and water and lipid stores) as measures of larval performance, and therefore as fitness proxies, in our assessment of the sublethal effects of repeated exposure to low temperatures. In particular, we ask (1) do repeated exposures to cold have fitness consequences for *P. marioni*, and (2) does *P. marioni* alter its cold tolerance strategy in response to repeated exposure to cold?

Materials and methods

Animals studied

Pringleophaga marioni Viette larvae are found in most habitats on the island from sea level up to heights of approx. 1000 m, and are postulated to live several years before pupation (Crafford, 1990). Klok and Chown (1997) found that *P. marioni* larvae are freeze tolerant, with a mean T_c of –5.0°C and mortality beginning at temperatures below –6°C. The mean critical thermal minimum (CT_{min}) was –0.6°C. *Pringleophaga marioni* larvae do not exhibit a rapid cold-hardening response (Sinclair and Chown, 2003), but cold hardiness is enhanced by prior exposure to desiccation or heat stress. Death of caterpillars after acute exposure to low temperatures seems to be associated with nervous system failure leading to water loss at the organismal level, as metabolic rate does not differ between thawed dead and living caterpillars (Sinclair et al., 2004).

Caterpillars (⩾ third instar) were collected in during the April (Autumn) 2004 relief voyage from abandoned wandering albatross nests within a 3–4 h walk of the meteorological station, and returned to the laboratory in bulk in bags of nest material (which doubles as food) within 5 h of collection. Larvae were then transferred into individual plastic Petri dishes with nest material and held in darkness at 5°C for 5 days. Nest material was moistened with island stream water every 2 days. Larvae were weighed (start mass) and assigned to treatment groups (control N=200, or treatment N=150) before being kept overnight in the incubator prior to the commencement of experiments the following day. With the exception of dissection of the 30-day recovery animals (see below), all experiments and dissections were conducted in the laboratory on Marion Island.

Effects of repeated cold exposure

Treatment caterpillars were exposed to five cold exposure cycles on consecutive days [repeated cold exposure (RCE) caterpillars]. Both control and treatment caterpillars were transferred individually into numbered 7 ml plastic tubes which were treated in groups of 50. Viability of caterpillars was monitored at this stage, and any caterpillars that did not show a righting response, or were turgid and immobile, were removed from the experiment and noted as dead. Tubes containing control caterpillars were then kept in the incubator in the dark at 5°C for the duration of the day's cold exposure experiment. Tubes containing treatment caterpillars were placed in plastic bags in the bath of one of three Grant LTC-12 refrigerated circulators (Grant Instruments Ltd, Cambridge, UK) and allowed to equilibrate for 30 min at 5°C. The caterpillars were then cooled at 0.1°C min^{-1} to –5.5°C, where they were held for 2 h. –5.5°C was chosen as a temperature at which most caterpillars were expected to freeze, but no mortality was expected. Dummy tubes containing a thermocouple indicated that in practice the caterpillars were exposed to a temperature of approx. –5°C. After 2 h, the caterpillars were re-warmed at 0.5°C min^{-1} to 5°C, and both control and treatment animals were transferred back to their Petri dishes and returned to the incubator at 5°C.

Before commencement of the experiment, and after each cold exposure cycle, 40 caterpillars were permanently removed from the experiment for determination of CT_{min} (N=10 each for control and treatment) and body composition (N=10 each for control and treatment). To examine the effects of recovery after five cold exposure cycles, a total of 25 treatment and 30 control caterpillars were kept in the incubator at 5°C. Five days after the final exposure, CT_{min} was again determined (N=10 for both control and treatment) and caterpillars were dissected (N=10 for both control and treatment). The remaining caterpillars were returned alive to the laboratory in Stellenbosch, South Africa and dissected 30 days after the final cold exposure cycle (control N=10, treatment, N=5). Thus, we have measures of body composition and CT_{min} for caterpillars exposed to 0, 1, 2, 3, 4 and 5 cold cycles, for caterpillars exposed to 5 cold cycles and given 5 days recovery, and matching measurements of animals subjected to the handling control at each stage of exposure. Body composition only was measured for control and treatment caterpillars (allowed 30 days recovery after the final exposure).

Measures of performance: critical thermal minimum and body composition

Critical thermal minimum (CT_{min}) of caterpillars was determined using a method similar to that of Klok and Chown (1997). Individual caterpillars were placed in one of ten water-jacketed chambers connected to a Grant LTC12 refrigerated circulator (Grant Instruments). A 40-gauge type-T thermocouple was inserted into a control chamber to measure chamber temperatures with an electronic thermometer. The caterpillars were allowed to equilibrate for 30 min at 10°C before CT_{min} assessment. After equilibration, the chambers' temperature was decreased at 0.25°C min^{-1}. The temperature of onset of critical thermal minimum ($CT_{min,onset}$) was recorded for each individual when coordinated muscle function was lost at decreasing temperatures. Subsequent to $CT_{min,onset}$, the chamber temperature was lowered 1°C below the lowest $CT_{min,onset}$ and held for 10 min before being rewarmed at 0.25°C min^{-1}. As the temperature increased, the recovery of the caterpillars from chill coma was recorded until they had regained a coordinated righting response ($CT_{min,recovery}$). Determination of CT_{min} was performed on two groups of five individuals for both control and treatment caterpillars. The position of control and treatment individuals within the array of chambers was randomised. If individual caterpillars froze during the CT_{min} run (N=10 of 126 individuals), their $CT_{min,recovery}$ was not used in analyses because it would be confounded by the time taken to melt internal ice.

To assess the condition of the RCE caterpillars, ten control and ten treatment caterpillars were dissected in random order before the commencement of the experiment, after each cold exposure, and after 5 and 30 days of recovery, as follows. Caterpillars were narcotised with CO_2 gas and weighed (mass at dissection). Their abdomens were then gently torn open and head capsule carefully pierced, and all haemolymph blotted onto filter paper. The haemolymph-free larva was then weighed to give an estimate of haemolymph mass (Kristiansen and Zachariassen, 2001). The gut was then dissected out, and the gut and body weighed separately (fresh gut and fresh body mass, respectively; mass at dissection – gut mass = gutless mass). Gut and body were then dried and reweighed (dry mass of each, or combined to give total dry mass of the caterpillar). Mass lost on drying was assumed to be water, and water content was therefore calculated as fresh mass – dry mass. The dry body and gut were then subjected to three changes of a 1:1 chloroform:methanol mixture and dried and reweighed to give lipid-free dry mass (and therefore an estimate of lipid content; method modified from Naidu and Hattingh, 1988). Whole and haemolymph-free caterpillar masses were obtained to 0.1 mg using a Mettler AE163 balance, while masses of fresh and dry gut and body were obtained to within 1 μg (Mettler UMT-2 (Marion Island) or

UMX-2 (Stellenbosch) balances; Mettler-Toledo, Columbus, OH, USA).

Effect of repeated cold exposure cycles on temperature of crystallisation

A different set of larvae (TC caterpillars) consisting of three groups of 16 caterpillars (total N=43 after loss of three animals during handling and loss of data from two more due to thermocouple failure during one or more runs) were weighed and placed individually in 1 ml plastic pipette tips in contact with a 40-gauge Copper–constantan (Type T) thermocouple held in place with cotton wool. The thermocouples were connected to a computer *via* two Pico Technology TC-08 thermocouple interfaces (Pico Technology Ltd, Cambridge, UK), whose proprietary software logged the 16 channels of input. The pipette tips and caterpillars were placed in a plastic bag in the bath of a Grant LTC-12 programmable refrigerated circulator (Grant Instruments Ltd), and allowed to equilibrate for 30 min at 5°C before being cooled at 0.1°C min^{-1} to –5.5°C, where they were held for 2 h before being rewarmed at 0.5°C min^{-1} to 5°C. Caterpillars were then removed from the pipette tips, and returned in their containers to the incubator at 5°C until the next day's freezing run. If the caterpillars had frozen, freezing exotherms were readily observable in the traces from the thermocouples (see Lee, 1991). Temperature of crystallisation T_c of animals that froze >0.2°C above the minimum temperature of the run were included in analyses. After five such cold exposure cycles, caterpillars were dissected as below. To determine the effects of handling on the T_c, ten handling control caterpillars removed from the repeated exposure experiment were also cooled in contact with thermocouples under control conditions each day, and their survival monitored. Thus, each measurement of T_c in the repeatedly exposed caterpillars is matched with a measurement of T_c in caterpillars that had received equal handling, but no cold exposure. The body composition of all the TC caterpillars was assessed 24 h after the final cold exposure cycle using the methods described above.

Data analysis

All mass analyses were conducted on $\log_{10}(x)$-transformed data and all data were checked for normality prior to all analyses. Pearson's correlation coefficients were used for correlations of body mass, CT_{min} and T_c. Body composition was compared between treatments and across exposures (RCE caterpillars) or freezing frequencies (TC caterpillars) using analyses of covariance (ANCOVA, with total dry mass, dry gut mass or dry body mass as covariates where appropriate). For the repeated cold exposure experiment, significant treatment or treatment × exposure interactions are reported, as these imply differences between treatment and control groups. Critical thermal minima and T_c values were also compared between treatment and control groups across exposures, using ANCOVA with fresh body mass as a covariate. Comparison of the proportion of caterpillars frozen was made between treatment and control groups using Generalized Linear Models (GLZ, using log-link and assuming a binomial distribution). Within-individual repeatability (± 95% confidence limits) of T_c was calculated using the ratio of between and within individual variances (Krebs, 1999) from an ANOVA calculated in NCSS 2000 (NCSS, Kaysville, Utah, USA). All other analyses were conducted on Statistica 6.1 (Statsoft, Tulsa, Oklahoma, USA).

Microclimate measurements

Soil temperatures (1 cm depth) were monitored at hourly intervals using iButton Thermochron data loggers (accurate to ± 0.5°C; Dallas Semiconductors, Dallas, TX, USA) along a transect from sea level to 800 m a.s.l. (above sea level) on the eastern side of Marion Island since April 2002, and will be subject to more detailed analysis elsewhere (S. Slabber and S. L. Chown, manuscript in preparation). In order to determine the likelihood of cycles of subzero temperatures occurring at a frequency greater than or equal to that used in our experiment, we chose a subset of data from the beginning of July to the end of September 2002 (the time of year when the coldest air temperatures are recorded; Hänel and Chown, 1998) at 200 m and 800 m a.s.l. We then used the macros described by Sinclair (2001a) to count the number of times the soil temperature cycled below 0°C, and used Microsoft Excel to calculate the mean ± standard deviation (S.D.) of duration of subzero temperatures, as well as the overall mean, maximum and minimum temperatures experienced in the habitat.

Results

Effects of repeated cold exposure on performance of caterpillars

No control and four of 150 treatment caterpillars died during the repeated cold exposure experiment. Body mass was negatively correlated with $CT_{min,onset}$ (r=–0.207, P=0.020, N=125), and near-so with $CT_{min,recovery}$ (r=–0.171, P=0.067, N=116), and was used as a covariate in all CT_{min} analyses. Critical thermal minima did not differ between treatment and control caterpillars at any stage of the experiment ($CT_{min,onset}$: $F_{1,121}$=1.429, P=0.234; $CT_{min,recovery}$: $F_{1,111}$=0.734, P=0.393; Fig. 1A). Caterpillars that were repeatedly exposed to –5°C lost mass relative to their control counterparts (Fig. 1B). This mass was not regained with 5 days of recovery, and 30 days after the final exposure, the mass of treatment larvae had increased to initial levels compared to control larvae, which had significantly gained mass over the recovery period (Fig. 1B). Gutless mass declined relative to initial mass with repeated exposures, and this became more pronounced after 5 exposures and 5 days of recovery (Fig. 1C). Gutless mass was significantly lower in experimental than in control larvae 30 days after the final exposure (Fig. 1C). During dissections, treatment caterpillars were observed to have empty guts with thinner, more delicate walls than their control counterparts. Measurements of body composition showed a significant decrease in gut mass in treatment caterpillars as compared to controls (main effects: fresh mass: $F_{1,131}$=29.474, P<0.001; dry mass: $F_{1,131}$=38.113, P<0.001; total dry mass as covariate), and

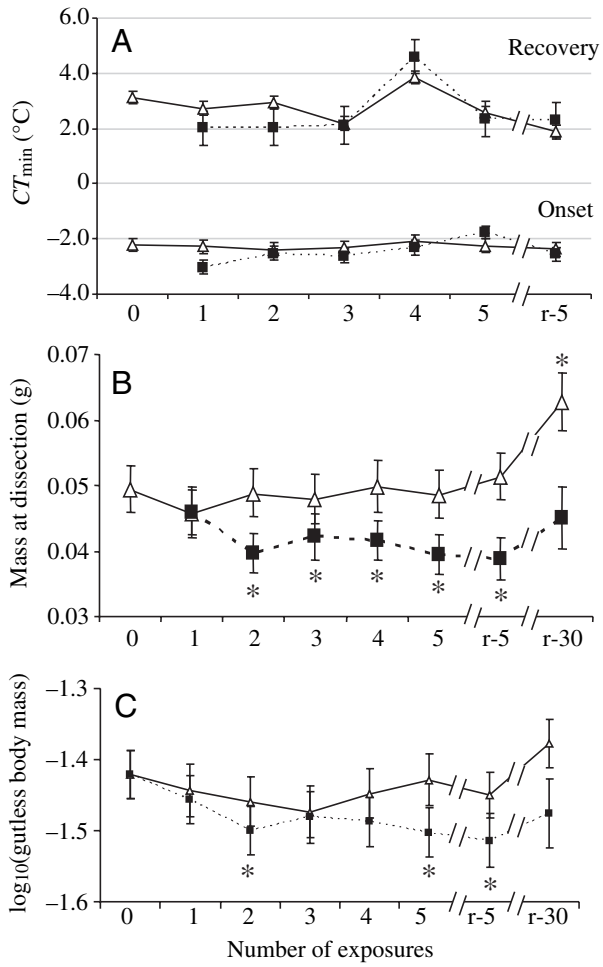

Fig. 1. (A) Mass-independent critical thermal minimum (CT_{min}) onset and recovery (least-square means ± 95% confidence intervals) of *P. marioni* larvae repeatedly exposed to cold (filled squares, broken lines) or handling controls (open triangles, solid lines). Number of exposures on *x* axis includes 5 days recovery after final exposure (r-5). (B) Mass at dissection (g) and (C) gutless mass (g; mass at dissection – gut + contents) of *Pringleophaga marioni* larvae exposed to –5°C on up to 5 occasions (filled squares, broken line) and handling controls (open triangles and solid lines). Number of exposures on *x* axis, includes 5 days (r-5) and 30 days (r-30) recovery after 5 exposures. Values are least-square means with initial mass as covariate ± 95% confidence intervals. All analyses were performed as ANCOVA on $\log_{10}$-transformed data. Asterisks indicate masses that are significantly different from initial mass (0 exposures), on the basis of non-overlapping confidence intervals. Control-treatment pairs whose confidence intervals do not overlap are significantly different ($P<0.05$). Mass at dissection: main effect $F_{1,134}=80.99$, $P<0.0001$; treatment × exposure interaction $F_{7,134}=4.721$, $P<0.0001$. Gutless mass: main effect $F_{1,133}=21.61$, $P<0.0001$; treatment × exposure interaction $F_{7,133}=1.71$, $P=0.112$.

higher gut lipid mass in treatment compared to control animals (treatment effect: $F_{1,129}=11.876$, $P<0.001$; treatment×exposure interaction: $F_{7,129}=3.477$, $P=0.002$; Fig. 2). There were no significant differences in total or body lipids, haemolymph mass or water content between control and treatment animals ($P>0.05$; Fig. 2; Table S1 in supplementary material).

Effect of repeated cold exposure cycles on temperature of crystallisation

Of the 43 caterpillars cooled in contact with thermocouples, 39 caterpillars (91%) froze at least once, and 11 (26%) froze on every exposure (Fig. 3). Because freezing did not necessarily occur upon exposure to cold, we make a distinction between the effects of freeze–thaw cycles (in which freezing events were confirmed in individual caterpillars attached to thermocouples), and the effects of repeated cold exposure (regardless of whether the larvae froze). A reduced proportion of treatment caterpillars froze after the initial exposure, and this was not observed in control animals (Wald statistic $\chi^2=6.04$, $P=0.014$, d.f.=1; Fig. 4A). The probability of freezing was significantly positively correlated with caterpillar mass (Fig. 5). In the absence of prior cold exposure, larger caterpillars froze at higher temperatures [T_c–($\log_{10}$mass) correlation at first exposure: control: $r=0.371$, $P=0.012$, $N=46$; treatment: $r=0.756$, $P<0.001$, $N=43$], and mass was therefore used as a covariate in all analyses of T_c. The profile of freezing across the exposures for each individual caterpillar is given in Table S2 of the supplementary material. Least-squares mean T_c (approx. –4.3°C) did not change with repeated exposures in control ($F_{3,40}=0.9079$, $P=0.45$) or treatment groups ($F_{4,119}=0.0655$, $P=0.992$; Fig. 4B). However, within-individual repeatability of T_c in treatment animals was not significantly different from zero ($r=0.114$, upper/lower 95% confidence limits 0.315/–0.007; $MS_{Between\ exposures}=0.335$, $MS_{Within\ exposures}=0.147$, $N=10$, $F_{5,10}=2.28$, $P=0.13$), suggesting that, while the mean T_c remains stable, it is highly variable within an individual between exposures.

Because the TC caterpillars froze a variable number of times during their five exposures to –5.5°C, we were able to partition out the effect of the number of times a caterpillar froze on its body composition. Increasing numbers of freeze–thaw events were associated with increased gut water content ($F_{5,35}=4.000$, $P=0.006$), but no other significant changes were detected (Fig. 6; Table S3 in supplementary material).

Microclimatic conditions

During the period for which microclimate data were analysed, soil temperatures at both 200 and 800 m a.s.l. dropped below 0°C repeatedly (Table 1, Fig. 7). At 800 m, there were 13 frost cycles in the 13 day period from 11 to 23 August, and at 200 m there were 9 frost cycles in the 13 day period from 1 to 13 September (Fig. 7). The minimum temperature at 200 m was –2.5°C, higher than the highest *P. marioni* T_c recorded in our experiments (–2.6°C), but at 800 m, the temperature dropped below –5°C on eight occasions over the winter (Table 1), although these never occurred on consecutive days.

Discussion

Arthropods that ordinarily experience freeze–thaw cycles in their habitat (for example, sub-Antarctic and alpine habitats)

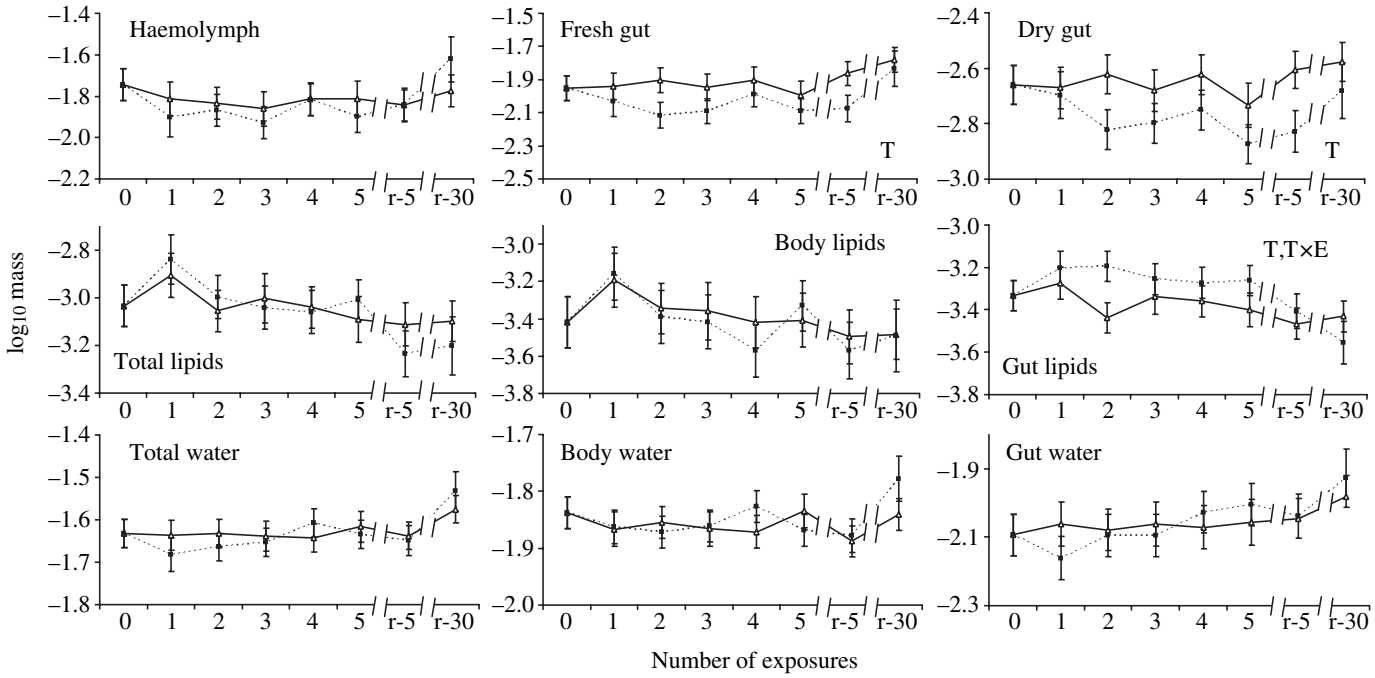

Fig. 2. Body composition in *Pringleophaga marioni* larvae exposed to increasing numbers of freeze–thaw cycles (filled squares, dotted lines) or handling controls (open triangles, solid lines), and 5 (r-5) and 30 (r-30) days after the final exposure. Values are least-square means ± 95% confidence intervals of nine measurements. T indicates a significant difference between treatment and control, T×E indicates a significant treatment × exposure interaction. See Table S1 in supplementary material for statistics and covariates used for each analysis.

might be expected to be well-adapted to repeated cold exposure. Indeed, many species of insects display a rapid cold-hardening response, which is an increase in tolerance to low temperature after a previous exposure (Lee et al., 1987; Sinclair et al., 2003c). Although hourly cycles (as used by Brown et al., 2004) are probably unrealistic, daily cold exposure cycles are frequent events in tropical high mountain (Krog et al., 1979), temperate alpine (Sinclair, 2001b) and Antarctic (Sinclair et al., 2003b) habitats. Recordings of soil temperature during the winter of 2002 show that five consecutive frost events is, in fact, a conservative set of exposures for habitats at 200 and 800 m a.s.l. occupied by *P. marioni* on Marion Island (Fig. 7). Although the minimum soil temperature at 800 m was –12°C, in neither habitat did the temperature drop to –5°C on consecutive days, so the frequency of exposure in our experiment was realistic, even though the temperature chosen may have been somewhat extreme.

We experimentally exposed larvae of *P. marioni* to five sequential low temperature (–5°C) events. This repeated exposure did not result in a change in either $CT_{min,onset}$ or $CT_{min,recovery}$, suggesting that repeated cold exposure does not affect threshold temperatures for activity and feeding by the caterpillars. Nevertheless, repeated exposure led to a decrease in total body mass, largely accounted for by a decrease in gut mass (although accompanied by a decrease in gutless mass), which suggests that the caterpillars stopped feeding after the second cold exposure. This cessation of feeding, however, is not simply a short-term effect: after 30 days of recovery, treatment caterpillars had merely regained their initial (pre-exposure) body mass, and gutless body mass was significantly lower than controls, which had substantially gained mass by both measures. After 30 days, relative gut mass did not differ between the groups, suggesting that feeding had recommenced in treatment caterpillars. Thus, repeated freeze–thaw cycles, which resulted in very little mortality, had substantial and long-lasting sublethal effects on growth rate of the larvae, and hence their fitness. Brown et al. (2004) exposed syrphid larvae to repeated low temperatures, and also found sublethal effects, manifested in a substantial reduction in successful pupation and emergence.

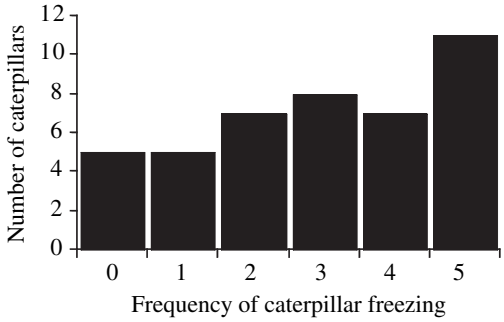

Fig. 3. Frequency distribution of freezing events of *Pringleophaga marioni* larvae exposed five times to –5.5°C. Note that some caterpillars froze on every exposure, while others did not freeze at all; *N*=43. See Table S2 in supplementary material for individual freezing profiles.

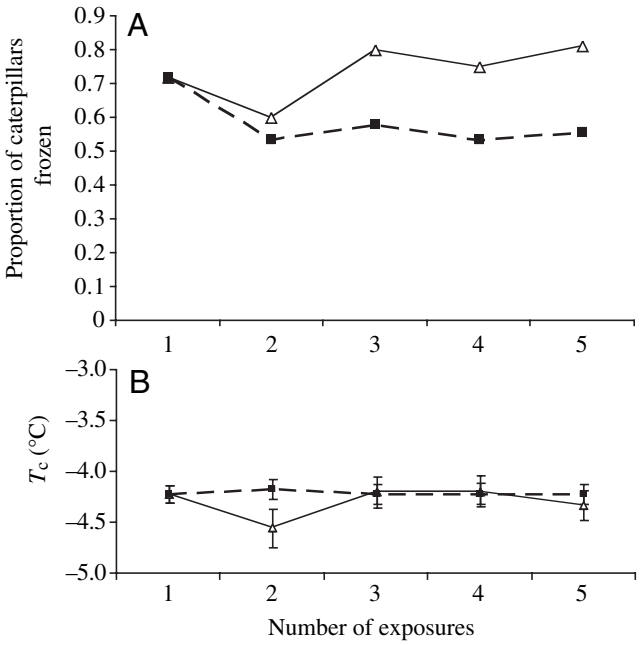

Fig. 4. (A) Proportion of caterpillars that froze at each exposure to –5.5°C. Filled squares, broken line: treatment animals, exposed to –5.5°C each time; N=43. Open triangles, solid line: handling controls, exposed to –5.5°C for the first time at each exposure; N=16. (B) Least-squares mean temperature of crystallisation (T_c) ± 95% confidence intervals (after ANCOVA with body mass as covariate) of control (open triangles, solid line) and treatment (filled squares, broken line) caterpillars exposed to repeated cold events. Only individuals that froze every time are included in the treatment data. Control animals were exposed to the same handling as the treatments, but each exposure was their first exposure to cold for those individuals.

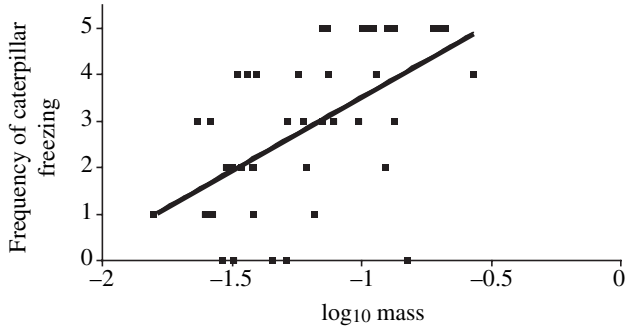

Fig. 5. Positive relationship between caterpillar mass (g) and the number of times an individual caterpillar froze during five exposures to –5.5°C. Pearson's correlation coefficient r=0.552, P<0.001, N=43.

During dissection, we observed that the guts of treatment caterpillars were very fragile. In combination with a cessation of feeding, this suggests that repeated cold exposure resulted in damage to the gut. Feeding had clearly not resumed after 5 days of recovery, which suggests that this damage to the gut was not transient, but persisted, and possibly required extensive repair when animals were no longer exposed to cold. Temperature and other perturbations can have a marked effect on the insect gut, leading to a decrease or cessation in feeding, and sometimes death (Chown and Nicolson, 2004). The larval gut of *Drosophila melanogaster* is particularly susceptible to heat shock (which may be mitigated with increased Hsp70 expression; Feder and Krebs, 1998), and non-lethal virus infection can result in increased sloughing of gut cells and consequent loss of gut function in Lepidoptera (Brooks et al., 2002). The deleterious effects of repeated cold exposure in *P. marioni* probably result from damage to the gut. This suggests that the gut cells of *P. marioni* may not be as robust to low temperature damage as has been observed in some other freeze-tolerant taxa (Yi and Lee, 2003; Worland et al., 2004).

The increase in gut lipids in treatment larvae could be confounded with a decrease in gut contents in those animals, as *P. marioni* diet has a low lipid content, and full dry guts are likely to contain little lipid. Given the fragile nature of the gut in treatment animals, removing and weighing gut contents was not practical. However, the increase in gut lipids in treatment caterpillars remained if dry body mass (rather than dry gut mass) was used as a covariate in the model (main effect $F_{1,128}$=11.796, P<0.001), suggesting that there is a genuine increase in gut lipid mass. The source of this increased lipid mass is not easily explicable, as there was no concomitant decrease in body lipids, and a significant proportion of the fat body is associated with the gut in lepidopteran larvae (Gullan and Cranston, 2005) making redistribution of lipid among tissues unlikely. However, membranes are particularly sensitive to temperature, and as well as changes to lipid composition, fluidity can be maintained in the face of temperature stress by incorporation of additional cholesterol or polyunsaturated fatty acids into the membranes (Logue et al., 2000; Hochachka and Somero, 2002; Hulbert, 2003). It is likely that our chloroform-methanol extraction would also remove non-polar cholesterol, possibly causing the increase in measured 'lipids'. However, this extraction method is a somewhat blunt instrument, and changes in gut lipid associated with repeated cold exposure require further qualitative, as well as quantitative, investigation.

A switch between the dichotomous cold tolerance strategies of freeze avoidance and freeze tolerance has been observed in the wild in *Dendroides canadensis* (Coleoptera: Pyrochroidae) and *Cucujus clavipes* (Coleoptera: Cucujidae) larvae (Horwath and Duman, 1984). In both cases, this switch was from freeze tolerance to freeze avoidance, perhaps as a result of a series of mild winters that might have repeatedly exposed the insects to freeze–thaw cycles (Duman, 1984). Similarly, Bale et al. (2001) and Brown et al. (2004) assert that *Hydromedion sparsutum* and *Syrphus ribesii* larvae (respectively) actually change their cold tolerance strategy in response to repeated freezing. However, repeated cooling of *P. marioni* did not result in a change in the mean T_c, and although there was a reduction in the proportion of individuals that froze after the first exposure (possibly a consequence of a decrease in mass, Figs 1B, 4A), all individuals that froze still survived freezing. This suggests that there was no systematic change in the cold-tolerance strategy of *P. marioni* individuals from freeze tolerance to freeze avoidance during the course of the experiment. Nucleation *via* gut material is expected to be

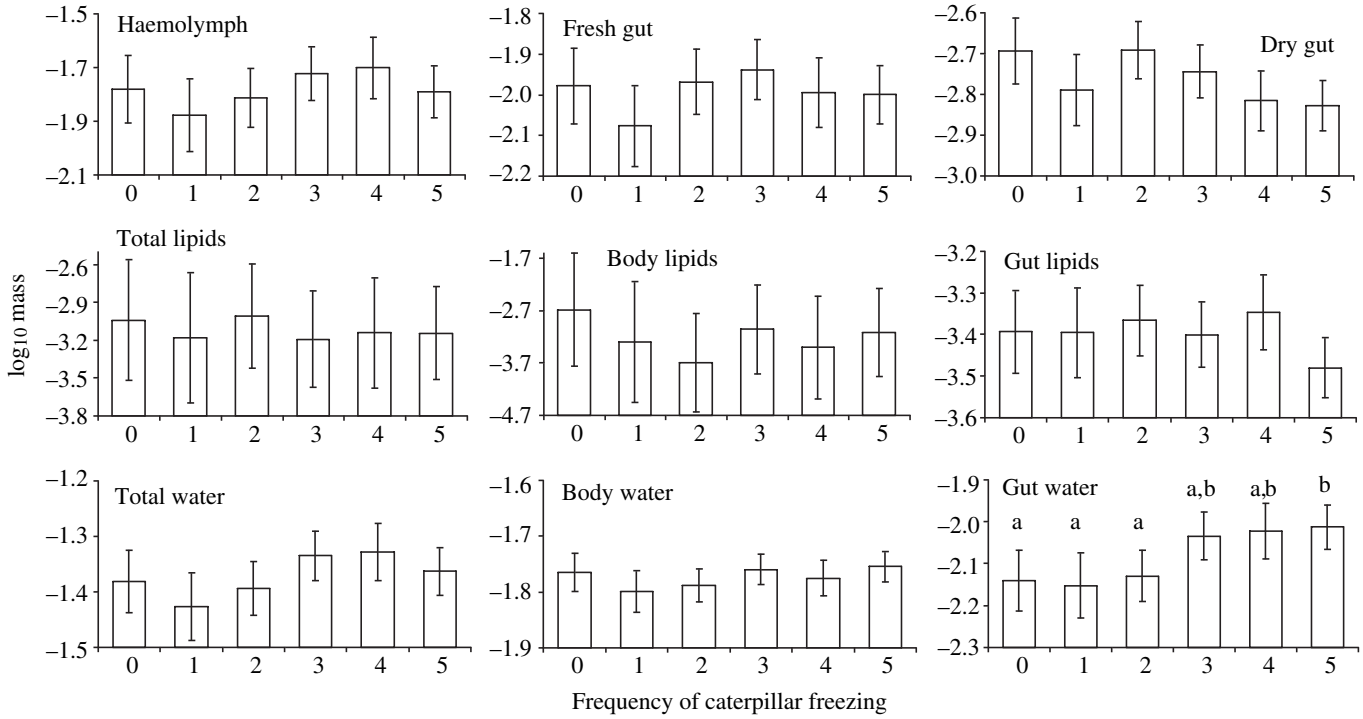

Fig. 6. Body composition of larvae of *P. marioni* exposed five times to –5.5°C, and that froze between 0 and 5 times during those exposures. Values are least-square means ± 95% confidence intervals of mass (g) measurements. Gut water differed significantly with frequency of freezing; significant differences are indicated by different letters above the bars. See Table S2 in supplementary material for statistics and covariates used in ANCOVA.

prevalent in winter-active insects (Sinclair et al., 2003a; see Palmer et al., 2004, for a graphic demonstration), which means that we would expect T_c to decline as the gut is emptied. Mean T_c in treatment *P. marioni* remained unchanged despite a decline in gut mass, suggesting that other factors besides gut contents, such as resident gut flora or ice-nucleating proteins, may be as important as food content in determining the temperature at which caterpillars freeze; however, our experimental design did not allow us to measure a T_c below –5.5°C, and the eleven individuals which froze above that temperature on every occasion may not provide an accurate representation of ice nucleation in the population as a whole. The reduced proportion of caterpillars that froze after the initial exposure (Fig. 4A) may be due to a decrease in overall mass, to a reduction in gut contents and thus susceptibility to nucleation by gut material, or to changes in the efficacy of gut nucleators as a result of (for example) increased sloughing of the gut epithelium. However, because we only measured gut mass at the conclusion of the five exposures of the TC caterpillars, we are unable to tease these explanations apart here.

Of interest here is that the effects of repeated freezing events were relatively minor (only gut water content increased with increasing numbers of freezing events in an individual). By contrast, repeated exposure to cold had a significant effect on feeding, regardless of whether the individual caterpillars froze or not. This means that the deleterious effects we demonstrate cannot be ascribed solely to the obvious mechanical and physiological effects of internal ice formation (Ramløv, 2000). Non-freezing low-temperature injuries are not well understood, but among the candidate causes are oxidative stress, structural changes to proteins and phase transitions in membranes (Ramløv, 2000; Chown and Nicolson, 2004). In a

Table 1. *Summary of microclimate temperatures, number and duration of freeze–thaw cycles at two threshold temperatures on Marion Island*

	Altitude (m)	
	200	800
Microclimate temperature (°C)		
Mean	3.3±0.1	0.7±0.1
Maximum	18.5	23.0
Minimum	–2.5	–12.0
0°C threshold		
Number of events	18	49
Mean duration (h)	5.9±1.1	15.5±1.9
–5°C threshold		
Number of events	0	8
Mean duration (h)		4.5±1.3

Mean values are ± S.E.M.

Measurements were taken at 1 cm depth in soil at two altitudes on the eastern side of Marion Island in July, August and September 2002.

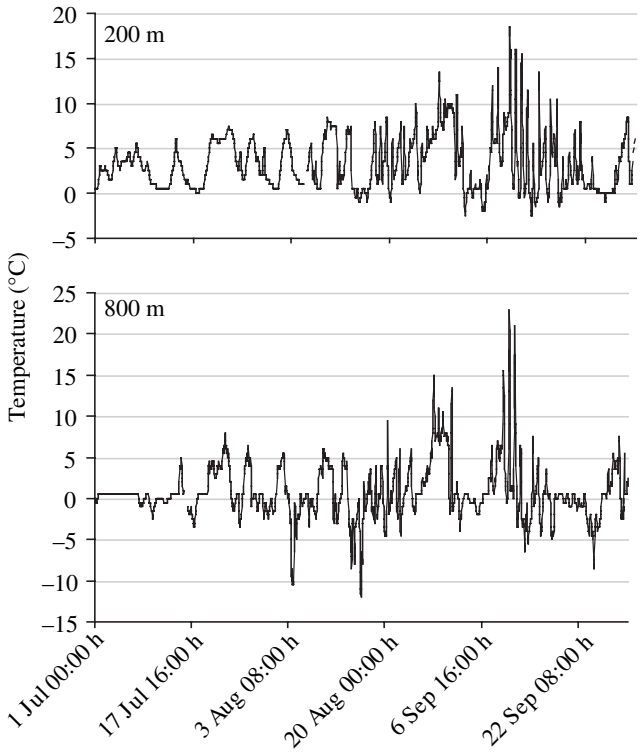

Fig. 7. Hourly microclimate temperatures measured at 1 cm depth in soil at 200 and 800 m above sea level on the eastern side of Marion Island from the beginning of July to the end of October 2002.

situation where low temperature *per se* is a source of deleterious effects, there is limited benefit to a switch in cold-tolerance strategy compared to avoiding the low-temperature stress entirely. Indeed, freezing and thawing in *P. marioni* do not seem to be associated with increased energy expenditure (Sinclair et al., 2004), and there is some evidence of the preferential selection of beneficial thermal microenvironments by *P. marioni* on Marion Island (B. J. Sinclair and S. L. Chown, manuscript submitted).

It is clear that insects respond to low temperatures at temporal scales ranging from minutes (in the case of rapid cold-hardening), through diurnal changes in cold tolerance to seasonal acclimatization (Bale, 2002; Sinclair et al., 2003c). However, as Bale and colleagues (Bale et al., 2001; Brown et al., 2004) have pointed out, investigations of these responses have generally focussed on the effects of a single exposure. Nevertheless, this study, and those of Bale et al. (2001) and Brown et al. (2004), demonstrate significant sublethal effects for larvae repeatedly exposed to low temperatures. This raises something of a paradox, as cold-exposure cycles of various frequencies are common in many terrestrial habitats, and are thought to select for freeze tolerance in those habitats where freeze–thaw may be experienced year-round (Sinclair et al., 2003a). Although we observed a cessation of feeding and apparent damage to the gut in caterpillars repeatedly exposed to cold, mortality of treatment caterpillars was less than 3%, and after 30 days recovery caterpillars had resumed feeding. Thus, we make a distinction between selection associated with survival (e.g. the selection of a cold tolerance strategy; Sinclair et al., 2003a), and selection associated with life-history traits like growth and development (in this case, feeding performance).

The microclimate data shown in Fig. 7 demonstrate that sub-zero temperatures do not occur every day during the winter, even at high altitude on Marion Island, which means that there is ample opportunity for recovery and resumption of feeding by caterpillars in the field. However, a temporary cessation in feeding as a result of cold exposure means that the feeding and growth performance of caterpillars in the field will be substantially decreased. This will result in lengthened life cycles, for example, limitations to growth rate imposed by freezing stress and aestivation to avoid parasitoids results in a 7 year life cycle in *G. groenlandica* (Morewood and Ring, 1998). Indeed, slow rates of growth and long life cycles are typical for many sub-Antarctic terrestrial arthropods (Crafford, 1990; Convey, 1996, 2000), in spite of apparently normal Q_{10} and preferred temperatures (Ottesen, 1990; Crafford and Chown, 1993; Todd, 1997), and this particular paradox is resolved if responses to low temperature are viewed not simply as a linear relationship, but as a step function, with a considerable decrease in growth and development beyond threshold microclimatic conditions (in this case, repeated cold exposure). The exact nature of the threshold in terms of frequency and intensity of cold exposure remains to be elucidated for *P. marioni*, but it is clear that knowledge of these parameters would be necessary to model this species' responses to climate change.

Under the clear-skies scenario for climate change on Marion Island (Smith and Steenkamp, 1990), an increase in daytime temperature would result in a decrease, rather than an increase, in developmental rate if balanced with increased frequency of sub-zero night-time temperatures. Thus, the biological consequences of climate change on Marion Island cannot be predicted simply from mean temperatures, but must encompass changes to the patterns of variation about that mean. Moreover, *P. marioni* is the primary prey of introduced house mice on Marion Island (Smith et al., 2002), and an increase in predation is expected if mean temperatures continue to rise (Smith and Steenkamp, 1990). Coupled to an increased development time, such increased predation could lead to severe impacts on the Marion Island population of *P. marioni*.

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