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Metabolism of the sub-Antarctic caterpillar *Pringleophaga marioni* during cooling, freezing and thawing

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Summary

Although general models of the processes involved in insect survival of freezing exist, there have been few studies directly investigating physiological processes during cooling, freezing and thawing, without which these models remain hypothetical. Here, we use open-flow respirometry to investigate the metabolism of the freeze-tolerant sub-Antarctic caterpillar *Pringleophaga marioni* Viette (Lepidoptera: Tineidae) during cooling, freezing and thawing and to compare animals exposed to non-lethal (–5.8°C) and lethal (–6.0°C, after which caterpillars are moribund for several days, and –18°C, after which caterpillars are completely unresponsive) freezing stress. We found a large decrease in metabolic rate (that is not associated with freezing) at –0.6±0.1°C and calculated a Q_{10} of 2.14×10^3 at this breakpoint. This breakpoint is coincident with the critical thermal minimum (CT_{min}) and is hypothesised to be a metabolic manifestation of the latter, possibly a failure of the Na^+/K^+-ATPase pump. This provides a plausible link between processes at the cellular level and observations of the action of the CT_{min} at tissue and whole-organism levels. Caterpillars froze at –4.6±0.1°C and had detectable metabolism when frozen. Post-thaw, metabolic rates were lower than pre-freezing measurements. Post-thaw metabolic rates did not differ between temperatures that did and did not kill the caterpillars, which suggests that mortality may be a result of a breakdown in processes at the organismal, rather than cellular, level of organisation.

Key words: freeze tolerance, critical thermal minimum, chill-coma, metabolic rate, *Pringleophaga marioni*.

Introduction

Although the majority of insects are killed by freezing and must avoid internal ice formation to survive low temperatures, many insect species from a variety of habitats and taxa survive sub-zero temperatures by withstanding the formation of ice in their bodies and are termed ‘freeze tolerant’ (Lee, 1991; Ramløv, 2000; Sinclair et al., 2003a). Although the biochemical correlates of this freezing survival are reasonably well known (Lee, 1991; Sømme, 1999; Ramløv, 2000; Sinclair et al., 2003b), the actual processes that occur during freezing and thawing have not been investigated to the same extent (but see Sinclair and Wharton, 1997; Joanisse and Storey, 1998; Neufeld and Leader, 1998; Kristiansen and Zachariassen, 2001 for studies that do examine the freezing or thawing process explicitly). The general model of the response of freeze-tolerant insects to low temperatures is that as the temperature decreases, the insect will pass through the critical thermal minimum (CT_{min} – resulting in chill-coma) and eventually reach the temperature of crystallisation (T_c – the temperature at which the body fluids freeze). If the insect continues to cool after freezing, it will eventually reach a lower lethal temperature, after which it will not survive when thawed (Lee, 1991; Ramløv, 2000).

Survival of internal ice formation is generally attributed to a process of osmotic dehydration. Ice forming in the haemolymph creates concentrated fluid pockets that dehydrate cells and restrict ice to the extracellular spaces (Zachariassen, 1985; Sinclair and Wharton, 1997; Ramløv, 2000), although intracellular ice formation probably occurs in some species (Salt, 1962; Lee et al., 1993; Wharton and Ferns, 1995; Davis and Lee, 2001). During thawing, therefore, water must be redistributed and ion gradients re-established. In the woodfly *Xylophaga cincta*, re-establishing the ion balance takes several hours (Kristiansen and Zachariassen, 2001). Nonetheless, there are currently no explicit data about the cause of lower lethal temperatures in freeze-tolerant insects. Although mechanical stress (for example, ice crystals piercing cell membranes or lethal recrystallisation of ice) has been proposed as a cause of mortality in frozen insects (Salt, 1961), the relationship between water-to-ice conversion and temperature means that the overall proportion of water converted to ice will increase continuously with decreasing temperatures (Zachariassen, 1985), and most hypotheses of causes of mortality tend to hinge on an osmotic stress threshold being crossed. These hypotheses include the inability of individuals to re-establish ion gradients in the nervous system and transgression of a minimum cell volume

resulting in lethal Ca^{2+} concentrations (Meryman, 1971; Kristiansen and Zachariassen, 2001).

Although the hypothesised causes of lower lethal temperature act at the sub-organismal level, it is reasonable to assume that the results are manifest at higher levels of organisation. However, aside from the progression of ice formation (see Ramløv, 2000 for a summary), there is little information about processes at the organismal level during freezing and thawing of insects. Kozhantsikov (1938) suggested that cold-hardy insects are those that are able to maintain a detectable level of metabolism below 0°. This idea was challenged on methodological grounds by Scholander et al. (1953), who pointed out that Kozhantsikov heated his animals to above 0°C to perform measurements. Scholander et al. (1953) and Salt (1958) both showed that the rates of change of metabolic rates with temperature (i.e. Q_{10}) in frozen and supercooled insects were extremely high at sub-zero temperatures and that the rate of change in metabolic rate increases logarithmically as the 0° threshold is crossed. In later work, Zachariassen et al. (1979) directly addressed the manifestation of freezing injuries by investigating the post-thaw metabolism of freeze-tolerant *Eleodes blanchardi* beetles exposed to lethal and sub-lethal temperatures. They concluded that metabolic rate was elevated after freezing. Moreover, whole-organism metabolic rate did not differ between those beetles that survived and those that were killed by freezing, suggesting that (at least initially) mortality does not occur at the cellular level. Most recently, Block et al. (1998) investigated the metabolic rates of freeze-tolerant larvae and adults of the perimylopid beetles *Hydromedion sparsutum* and *Perimylops antarcticus* on sub-Antarctic South Georgia. They found that, while metabolic rates of both species did not differ pre- and post-chilling (exposure to non-freezing sub-zero temperatures), metabolic rates of both larvae and adults of *P. antarcticus* were significantly higher after a brief freezing event but did not change after freezing in *H. sparsutum*. They concluded that the elevated metabolic rate of the former species is indicative of a rapid repair of slight freeze-induced injuries that explains its greater cold tolerance but did not investigate the effects of freezing injury or mortality within the frozen beetles.

All five of the preceding studies were conducted using closed-system respirometry, which integrates instantaneous rate values over time periods that are often prolonged. Therefore, this method generally does not reveal activity on the part of the study animal nor does it provide the fine resolution required to investigate short-term temporal variation in metabolic rate (Lighton, 1991; Addo-Bediako et al., 2002). By contrast, open-flow respirometry has the advantage that it enables the investigation of rapid, real-time metabolic responses to thermal and other stressors. To date, only Irwin and Lee (2002) have used open-flow respirometry to compare the metabolic rate of frozen and supercooled insects. They examined the larvae of the goldenrod gallfly *Eurosta solidaginis*, concluding that the metabolic rate of frozen larvae was lower than that of supercooled larvae, although they did not present metabolic data from during the freezing, thawing or post-thawing processes. Here, we use open-flow respirometry to explore the metabolic changes that occur during cooling, freezing and thawing, using the sub-Antarctic caterpillar *Pringleophaga marioni* as a model freeze-tolerant organism, and compare metabolic patterns in individuals exposed to lethal and non-lethal freezing stress.

Materials and methods

Study animals

Pringleophaga marioni Viette (Lepidoptera: Tineidae) is a key decomposer on Marion Island (46°54′ S 37°45′ E; Crafford et al., 1986). The long-lived (several years) larvae occur in most of the habitats on the island, from sea level to ~1000 m, where they are likely to experience multiple freeze–thaw events during their lifetime (Crafford, 1990; Crafford and Chown, 1992). Klok and Chown (1997) studied the environmental physiology of *P. marioni* larvae and determined that caterpillars have a critical thermal minimum (CT_{min}) of –0.6±0.2°C and that they are freeze tolerant, with a mean temperature of crystallisation (T_c) of –5.0±0.2°C and mortality at temperatures below approximately –6°C to –10°C. Recently, Sinclair and Chown (2003) have shown that *P. marioni* larvae do not exhibit a rapid cold-hardening response but that cold hardiness may be enhanced by prior desiccation or heat stress, suggesting that heat shock proteins may play some role in the freezing survival of this species.

Pringleophaga marioni larvae were collected from abandoned albatross nests in the vicinity of the Research Station on Marion Island during the April 2002 relief voyage. Caterpillars were placed in groups of five in 250 ml plastic jars filled with nest material and were returned under refrigeration to the laboratory in Stellenbosch within 10 days, where they were housed in the dark in a refrigerator (6.0±0.4°C) until they were used for experiments. Animals housed in this manner fed and moulted normally, and several individuals pupated.

Freezing and respirometry experiments

Caterpillars (0.202±0.016 g, mean mass ± S.E.M., N=29) were frozen individually in a 3 cm^3 plastic respirometry cuvette. A 44-SWG type-T thermocouple was affixed to the bottom of the cuvette's interior. The cuvette was then placed inside a plastic bag in the bath of a Grant LTD-6 (Grant Instruments, Cambridge, UK) cooling bath controlled by a Grant PZ-1 temperature programmer. Synthetic air (21% O_2, balance N_2) was scrubbed of CO_2 with soda lime and of water with silica gel and Drierite. The air flowed at 75 ml min^{-1} (Sidetrack mass flow controller) through a narrow-diameter copper coil (~1.5 m total length) in the cooling bath before passing through the cuvette to a LiCOR Li6262 CO_2/H_2O infra-red gas analyser (IRGA). The respirometry system was controlled by DATACAN V software (Sable Systems, Henderson, NV, USA), which also provided automatic baselining and recorded the temperature inside the cuvette *via* a Sable Systems TC1000 thermocouple thermometer. All

initial analyses of CO_2, water and temperature traces were also performed with DATACAN V software. All measurements were corrected to standard temperature and pressure and expressed in ml CO_2 h^{-1}.

At the start of each experiment, the caterpillars were removed from the moist nest material, surface dried, weighed and quickly transferred to the cuvette and placed in the water bath at 1.6°C to equilibrate for one hour. Caterpillars were then cooled at 0.1 deg. min^{-1} to the test temperature, where they were held for 15 h (5 h for –18°C treatment, which still resulted in ~10 h frozen and always resulted in mortality) before being rewarmed at 0.1 deg. min^{-1} to 1.6°C, where they were held for a further 2–5 h to enable the recovery from freezing to be recorded by the respirometer. Preliminary experiments allowed us to choose test temperatures that, under the experimental conditions, would be survived by all the caterpillars (–5.8°C, *N*=9), would result in mortality of all caterpillars with recovery to a 'moribund' state (some uncoordinated movement; –6.0°C, *N*=8) and would kill all caterpillars outright without any apparent recovery of movement (–18°C, *N*=5). After freezing, the caterpillars were weighed again and removed to individual Petri dishes filled with nest material. Recovery (coordinated movement and a righting response) was monitored for seven days, after which all caterpillars were preserved in ethanol.

Data analyses

In the CO_2 traces, a clear breakpoint in metabolism (*b*) was visible (Fig. 1), and the temperature at the start of this breakpoint was recorded. Freezing was evident in the temperature traces (owing to the sensitivity of the fine thermocouple), and the T_c – the temperature immediately before the exotherm – was recorded. Freezing was also clearly discernible in the CO_2 (Fig. 1) and water traces. Where CO_2 production was averaged for metabolic rate determination, temperature was averaged over the same period of recording. Temperatures of crystallisation and breakpoint distributions were not significantly different from normal, and means ± S.E.M. are presented. All caterpillars whose temperature dataset could yield breakpoint and T_c data were included in these analyses, including those from preliminary experiments to determine methods and test temperatures.

After baselining, mean CO_2 production (ml CO_2 h^{-1}) was calculated for a subset of data for each of four stages during the freeze–thaw cycle: (1) the equilibration period before freezing (24.6±1.2 min, *N*=16), (2) while the animal was frozen (382.3±43.2 min, *N*=4 per treatment), (3) during the immediate post-thaw period (the 34.5±2.9 min after cuvette temperature reached 1.6°, *N*=4 per treatment) and (4) >1 h post-thaw (105.4±20.2 min, *N*=4 per treatment) (Fig. 1). CO_2 production was converted to μW, assuming a respiratory quotient of 0.72 (Withers, 1992). Minute variations in the temperature of the IRGA chamber meant that some metabolic rate measurements (while the caterpillars were frozen) were characterised by regular (~30-min period) oscillations, in synchrony with small temperature changes in the room. After we had taken steps to better regulate the IRGA temperature, recordings where the range of metabolic rate (frozen) was greater than the mean metabolic rate (frozen) were not used for any metabolic rate comparisons, resulting in *N*=4 for each treatment for the majority of metabolic rate comparisons (although the data files were still useable for examination of *b* and T_c). Metabolic rate was log-transformed and compared between treatments and stages of the freeze–thaw cycle by a repeated-measures analysis of covariance (ANCOVA) using the general linear model (GLM) procedure on Statistica 6.1 (Statsoft Inc., Tulsa, OK, USA), using starting body mass of the caterpillar as the covariate. For ease of display, metabolic rate data are presented graphically as untransformed mass-specific data, although conclusions were drawn from the procedures outlined above.

Q_{10} was calculated from the slope of a regression of $\log_{10}$metabolic rate on temperature using the equation $Q_{10}=10^{(10\times slope)}$ (Cossins and Bowler, 1987). Q_{10} was calculated at five points during the freeze–thaw cycle: (1) during cooling before the breakpoint, (2) across the breakpoint, (3) during cooling after the caterpillar had frozen (–18°C treatment only), (4) during rewarming between –15°C and –10°C (–18°C treatment only) and (5) during thawing (–5°C to –2°C). Prior to cooling, metabolic rate was highly variable, and relationships where the slope was non-significant (*F*-test, $P>0.05$) were excluded from the analyses. Q_{10} was compared between treatments using Kruskal–Wallis Rank analysis of variance (ANOVA) and between cooling and thawing using Wilcoxon's paired test (Sokal and Rohlf, 1995).

Water loss

Water loss rate measured by the IRGA was baselined and then integrated for the 30-min period immediately prior to temperature decrease and for 240 min (or as long as the trace allowed if less than this) to give total mg H_2O lost during these periods. For the post-thaw water loss, time was standardised to 200 min to allow comparison between treatments. Water loss was not normally distributed, despite transformation, and was compared between treatments using a generalized linear model (GLZ) in Statistica 6.1 with starting body mass as a covariate.

Mass loss during the course of the experiment was assumed to be entirely water (although several caterpillars did produce faecal pellets and/or vomit in the cuvette, the non-water mass of these was assumed to be negligible compared with the water lost). An ANCOVA with starting body mass as the covariate was used to compare total water lost between treatments.

Results

Metabolic rate declined as the temperature decreased from 1.6°C to <0°C (Fig. 1). Almost all (16 of 17) animals showed a clear breakpoint in CO_2 production where the slightly irregular CO_2 trace (indicative of asynchronous spiracular control; Wasserthal, 1996) decreased precipitously and disproportionately to the rate of temperature decline (point *b* in Fig. 1). The mean breakpoint temperature (± S.E.M.) was –0.6±0.1°C (*N*=16, range –1.2°C to 0.4°C), and the decline in

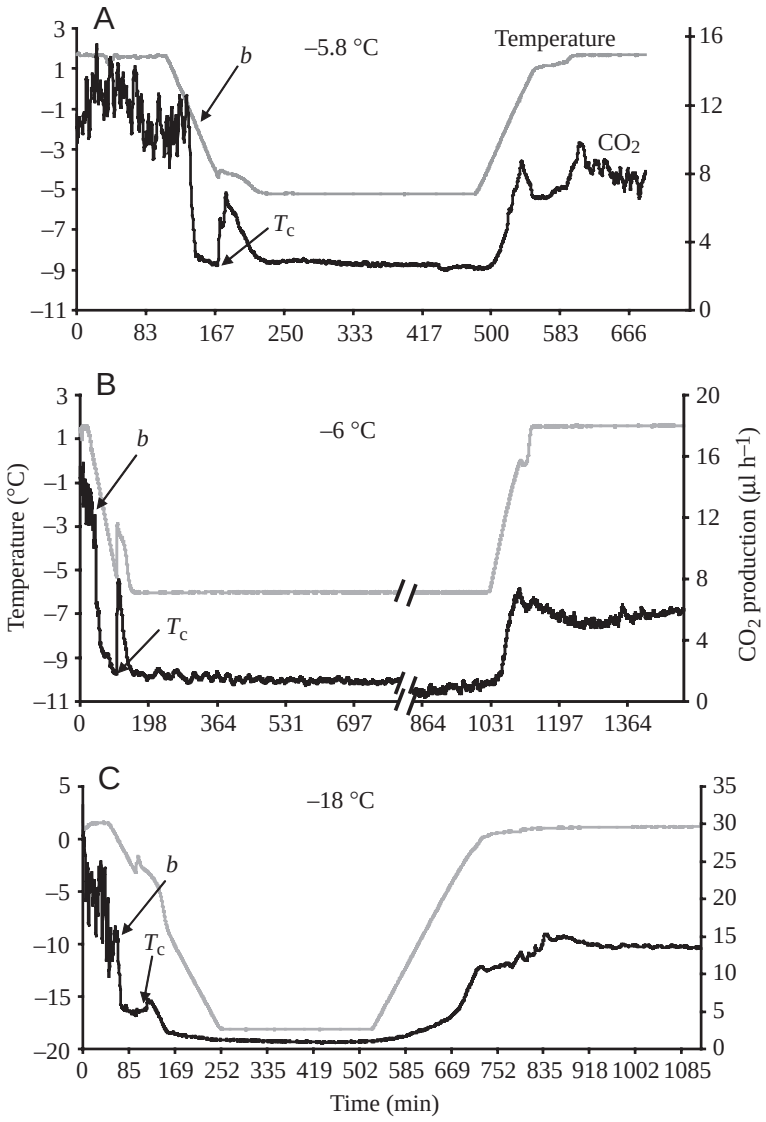

Fig. 1. Representative data plots of temperature (grey, upper lines) and CO_2 production (black, lower lines) from *Pringleophaga marioni* caterpillars exposed to freezing treatments at (A) –5.8°C (in which all caterpillars survive), (B) –6°C (which results in caterpillars being left moribund and then dead) and (C) –18°C (after which no caterpillars show muscle tone or response to stimuli). Breakpoint (*b*) and temperature of crystallisation (T_c) are marked. Small cycles in the CO_2 trace shown in B are a result of minor temperature fluctuations at the IRGA. Whenever the range of these fluctuations exceeded the mean, the data were discarded. The break in the middle graph represents a change in data files (we were restricted by the memory that the acquisition software was able to allot to data gathering), and the slight difference in trace afterwards (equivalent to a maximum of 2.1 µW) is a result of re-baselining mid-data-acquisition. The masses of the caterpillars used in the presented data were 0.2342 g (A), 0.1977 g (B) and 0.3826 g (C), accounting for the variation in the scale of the right-hand *y*-axes.

metabolic rate occurred over a period of 14.4±1.1 min (N=16). The freezing event was recorded simultaneously by the thermocouple (the latent heat of crystallisation) and by the water and CO_2 channels. The latter was presumably a result of expulsion of water vapour and gases from the tracheal system with the expansion of water in the haemocoel [assuming a tracheal CO_2 partial pressure (P_{CO_2}) of 5 kPa (Levy and Schneiderman, 1966), the volume of gas released is 9.5±1.2% (N=15) of the volume of the caterpillars, which is close to estimates of tracheal volume of insects, which usually vary between 3% and 10% (Bridges et al., 1980; Schmitz and Perry, 1999)]. The mean T_c was –4.6±0.1°C (N=29, range –5.7°C to –3.2°C). Upon rewarming, metabolic rate increased with temperature and remained steady after thawing in all three temperature treatments (Fig. 1). All animals in the –5.8°C treatment survived and were active and coordinated upon removal from the cuvette. Three of eight animals exposed to –6°C showed limited response to stimulus upon removal from the cuvette but died within 5 days (of the remainder, one survived and was excluded from analysis, while the rest never responded to stimulus). There was no consistent qualitative or quantitative difference in any of the variables measured between moribund and dead caterpillars exposed to –6°C, so the results were considered together. None of the –18°C animals showed any response and all completely lacked muscle tone when removed from the cuvette.

In all treatments, metabolic rates before freezing were significantly higher than after thawing and, when frozen, caterpillars had significantly lower metabolic rates ($F_{3,21}$=18.364, P<0.0001; Fig. 2). Metabolic rates measured in the first 30 min post-thaw did not differ significantly from those measured longer after thawing and did not differ between treatments ($F_{2,7}$=3.624, P=0.08; Fig. 2).

There was no significant difference (Kruskal–Wallis Rank ANOVA, P>0.1) between treatments in pre-freezing Q_{10} (comparison only possible between –6.0°C and –18°C groups due to noisy data; median test, χ^2=0.667, d.f.=2, P=0.72), so these results were pooled. Q_{10} before the breakpoint was highly variable (Table 1) but had a median of 2.20 (range 1.18–2.98, N=10) whereas across the metabolic breakpoint it ranged from 4×10^2 to 1×10^5 (N=15). During cooling after the animal had frozen, Q_{10} was low, as it was when the caterpillars were being warmed between –15°C and –10°C. During thawing, Q_{10} was significantly higher in the –6.0°C group (Kruskal–Wallis H=7.05, d.f.=2, N=11, P=0.0294).

Flow-through gas analysis indicated that water loss rate did not differ between groups before freezing (Wald χ^2=1.42, d.f.=2, P=0.49; Fig. 3A). The –18°C group lost considerably more body mass than the other two groups (Wald χ^2=44.35, d.f.=2, P<0.0001; Fig. 3B), and the IRGA results suggest that this difference is attributable to the post-thaw period ($F_{2,18}$=11.313, P<0.001; Fig. 3A).

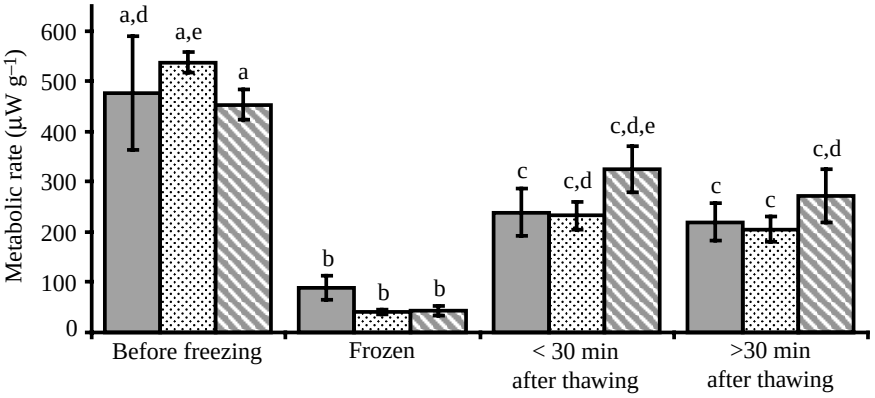

Fig. 2. Mass-corrected metabolic rate of *Pringleophaga marioni* larvae at different stages of freezing and thawing. Treatments: grey bars, –5.8°C; stippled bars, –6°C; hatched bars, –18°C. Means ± S.E.M. are presented. Although mass-specific data are presented here, analyses were performed as a repeated-measures analysis of covariance, with mass as a covariate. Bars with the same letter are not significantly different from one another (Tukey's HSD, $P<0.05$).

Discussion

The metabolic breakpoint and critical thermal minimum

There is a steep decline in metabolic rate as *Pringleophaga marioni* larvae are cooled (the breakpoint; Fig. 1), evidenced by an increase in Q_{10} of several orders of magnitude at the breakpoint (Table 1). A threshold in metabolism around 0°C that results in an exponential change in metabolic rate was reported by both Salt (1958) and Scholander et al. (1953), who interpreted this decline to be a consequence of freezing. One advantage of flow-through respirometry is that observations can be made in real time, so that Q_{10} can be calculated from the actual rate of change of metabolic rate over a temperature change. We have shown that there is a rapid change in metabolic rate at a threshold temperature that is clearly not associated with the freezing of the animal (Fig. 1). Rather, the start of this decline (–0.6±0.1°C) closely matches the CT_{min} measured previously for this species (–0.6±0.2°C; Klok and Chown, 1997). This strongly suggests that the observed breakpoint is a metabolic manifestation of the CT_{min}.

Hosler et al. (2000) found a steady decrease in the resting potential of flight muscle neurons of both honey bees and *Drosophila* with decreasing temperature. They proposed that the chill-coma temperature was the temperature at which the Na^+/K^+-ATPase pump could no longer maintain nerve cell polarisation to a level where action potential could be produced. Since the contribution of transmembrane ion pumps to total metabolism is estimated to be >55% of basal metabolic rate at normal temperatures (Zachariassen, 1996; Hulbert and Else, 2000), a threshold temperature for pump activity is a plausible explanation for the drop in metabolism we observed.

Because of its ecological significance, the CT_{min} (or the

Table 1. *Q_{10} measured at different points during the freezing and thawing of* Pringleophaga marioni *larvae frozen to different temperatures (–5.8°C, –6.0°C and –18°C)*

Point during freeze/thaw	Treatment	Median Q_{10}	Upper quartile	Lower quartile
Before breakpoint	All	2.20	2.79	1.60
Across breakpoint	All	2.14×10^3	4.24×10^3	1.26×10^3
Cooling when frozen	–18°C	1.49	1.69	1.33
Warming –15°C to –10°C	–18°C	1.83	2.18	1.70
Thawing –5°C to –2°C	–5.8°C	8.65	16.72	7.11
	–6°C	48.20*	75.14	23.00
	–18°C	7.37	8.16	5.87

A significant difference ($P<0.05$) in post-thaw Q_{10} between the –6.0°C treatment and the other treatments is indicated with an asterisk.

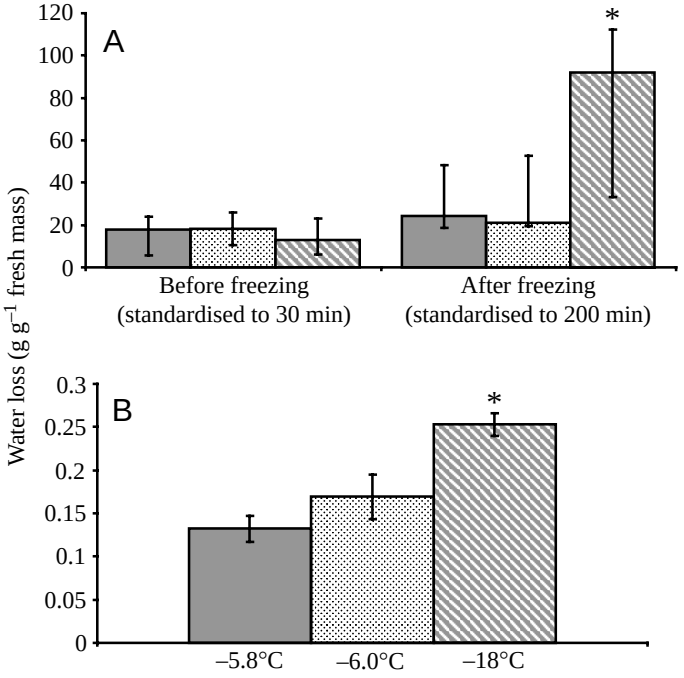

Fig. 3. Water lost by *Pringleophaga marioni* caterpillars after freezing, measured by (A) infra-red gas analyzer before freezing and after thawing during the same run (note that quantity of water lost is standardized to different time periods) and (B) by total mass lost during the run. Grey bars, –5.8°C; stippled bars, –6°C; hatched bars, –18°C. (A) IRGA data are medians ± minimum and maximum. Sample sizes: –5.8°C (N=4); –6°C (N=6); –18°C (N=4). (B) Mass loss data were analysed as an ANCOVA with starting body mass as a covariate, although mass-specific means ± S.E.M. are presented. Sample sizes: –5.8°C (N=9); –6°C (N=8); –18°C (N=5).

closely comparable chill-coma temperature) has received attention in both field and laboratory studies of several species of insects (Chown, 2001; Gibert and Huey, 2001; Kelty and Lee, 2001; Hoffmann et al., 2003). For example, Gibert and Huey (2001) found that the chill-coma temperature of *Drosophila* differed between species and populations from different latitudes (suggestive of natural selection) and could be modified within those populations by developmental temperature (indicative of developmental plasticity). Klok and Chown (2003) found that the CT_{min} of sub-Antarctic weevils (*Ectemnorhinus*) responded to acclimation, with higher acclimation temperatures resulting in significantly higher CT_{min} values. Activity of the Na^+/K^+-ATPase pump may be modulated to reduce energy expenditure in response to heat shock and anoxia (Hochachka et al., 1996; Wu et al., 2002), and there is a variety of isomeric sodium pumps produced by insects under different environmental conditions, stages of development and tissues (Emery et al., 1998). This variation could be adequate both as a substrate for natural selection and possibly as a mechanistic explanation of acclimation responses in the CT_{min}.

An alternative mechanism explaining variation in CT_{min} has been proposed by Pörtner (2001, 2002), who suggested that the CT_{min} is a consequence of a mismatch between oxygen demand and delivery at low temperatures. However, because of the very large capacity for oxygen delivery in tracheated insects (Chapman, 1998), it seems unlikely that delivery failure sets limits to low temperature activity in these animals (Chapman, 1998; C. J. Klok, B. J. Sinclair and S. L. Chown, manuscript submitted for publication). Nevertheless, an oxygen-limited process (for example, ATP production by mitochondria), rather than a failure in the kinetics of the Na^+/K^+-ATPase pump at the membrane, would result in the same depolarisation, so the working hypothesis presented here is not inconsistent with Pörtner's (2001, 2002) assertions. Further investigation at the subcellular level is necessary to understand the processes leading to the CT_{min} in insects.

Metabolic processes during freezing and thawing

The metabolic rate of frozen caterpillars ($89.2\pm24.9\ \mu W\ g^{-1}$) was low but not zero (Fig. 2) and, during cooling after freezing, showed a Q_{10} comparable with that measured pre-freezing (pre-breakpoint) (Table 1). This suggests that the osmotically dehydrated cells are still functional and that, although the tracheal volume is presumably greatly reduced by the expansion of ice in the haemocoel, the tracheal system is still open and continues to provide an atmospheric interface for the cells. Assuming the animal's habitat allows access to atmospheric oxygen [see Scholander et al. (1953) for examples of chironomids frozen into lake ice; see Conradi-Larsen and Sømme (1973) for an example of terrestrial beetles locked in ice; see Lighton (1998) for a discussion of the hypoxic nature of below-ground habitats], this potentially allows the caterpillars to respire aerobically when frozen. An alternative explanation for this phenomenon is that the conversion of water into ice in freeze-tolerant insects is a slow process, continuing towards an asymptote over a period of hours (Lee and Lewis, 1985; Ramløv and Westh, 1993) and continuing to expand and expel CO_2 from the tracheal system. While this is probably a partial explanation, the rate of CO_2 does not attenuate to zero with increasing time frozen (Fig. 1), suggesting that there is a metabolic contribution to CO_2 production.

The generally accepted model of the processes of freezing and thawing in freeze-tolerant insects requires that ice formation begins in the haemocoel and is confined to the extracellular spaces, resulting in osmotic dehydration of cells (Zachariassen, 1985; see Sinclair and Wharton (1997) for a demonstration of this). This osmotic dehydration, and subsequent passive movement of ions, means that water and ions must be redistributed and gradients re-established upon thawing (Kristiansen and Zachariassen, 2001). It stands to reason from this model that the freezing process is passive, but the apparent expulsion of CO_2 from the tracheal system during freezing did not allow us to test this part of the model.

Redistribution of ions between intra- and extracellular compartments after thawing is probably an active process taking a few hours (Kristiansen and Zachariassen, 2001). There is a decrease in metabolic rate in *P. marioni* in the four hours after thawing relative to before freezing (Fig. 2), suggesting that the initial redistribution of ions is not energetically expensive and that any necessary tissue repair does not begin immediately. Joanisse and Storey (1998) suggest that it would be advantageous for freeze-tolerant animals to depress post-freezing metabolism to reduce oxidative damage caused by reactive species accumulated while the animal is in the frozen state. The metabolic rate of *Eleodes blanchardi* beetles is elevated 20 h after thawing (Zachariassen et al., 1979), as is the metabolic rate of frozen *Perimylops antarcticus* beetles from South Georgia (Block et al., 1998), and it seems that this elevation may be associated with the repair of cellular or tissue damage sustained during the freeze–thaw process. Assuming that some cellular or tissue damage is sustained by *P. marioni*, metabolic rate might be expected to be elevated longer after thawing. Measurements of free radicals and antioxidants (along with longer-term monitoring of post-thaw metabolic rate) will be necessary to test this hypothesis.

Causes of low temperature mortality in frozen P. marioni

Low temperature mortality in frozen cells that would otherwise survive freezing has been hypothesised to be due to a threshold ice content resulting in a lethal minimum cell volume (Meryman, 1971; Zachariassen et al., 1979; Zachariassen, 1985). However, Zachariassen et al. (1979) did not find any metabolic differences between beetles that were exposed to lethal or sub-lethal freezing and concluded that mortality due to freezing might occur at the organismal, rather than the cellular, level as a result of neuronal membrane impermeability to sodium ions. Recent work by Yi and Lee (2003) on the gallfly *Eurosta solidaginis* examined the levels of injury sustained by different tissues at the lethal temperature of –80°C. Although nerve tissue was not tested, there is differential mortality between tissues, with gut tissue most

tolerant to freezing and several tissues (intertegumentary muscle, the distal segment of Malpighian tubules and haemocytes) least tolerant, suggesting that damage to a specific tissue group such as nervous tissue could account for mortality associated with freezing. We compared metabolic rate and water loss in caterpillars that survived exposure to –5.8°C (which results in no mortality) to caterpillars exposed to –6.0°C (which results in moribund larvae that exhibit uncoordinated movement but die after a few days) and –18°C (after which larvae never show any response to stimuli). In common with Zachariassen et al. (1979), there was no difference in post-freezing metabolism between the three groups (Fig. 2), suggesting that, even in the dead caterpillars, most cells remained intact and capable of metabolism. The major difference between the three groups was that the –18°C group lost substantially more water than the other groups (Fig. 3), and this water loss is concentrated in the post-thaw period. This post-thaw water loss is likely to be a direct consequence of a lack of muscle tone due to nervous system disruption: thawed, dead caterpillars often vomit, and it is also likely that the water from this, as well as from the open anus and spiracles, provides an outlet for water loss from the caterpillar's body (Wharton, 1985). Immediate water loss does not, however, provide an explanation for mortality in the –6.0°C group. The notable feature of this group is their elevated Q_{10} during thawing (Table1), which may suggest that the cellular processes associated with recovery from freezing have been disrupted.

Conclusions

In this paper, we have used open-flow respirometry to explicitly examine the metabolic processes associated with cooling, freezing and thawing in a freeze-tolerant insect. We have demonstrated a pronounced decline in metabolic rate that is not associated with freezing but is coincident with the CT_{min}. This finding provides a plausible link between the CT_{min} and mechanistic changes at the cellular and whole-organism levels. It also indicates ways in which natural selection might alter mechanisms at the cellular level, so giving rise to environmental variation in the CT_{min}. We have also shown that frozen caterpillars continue to respire when frozen, but we found no evidence that either freezing or thawing were active metabolic events. Moreover, there were no significant differences in metabolic rate between caterpillars that were and were not killed by freezing, suggesting that mortality from freezing occurs at an organismal, rather than cellular, level.

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