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RESEARCH ARTICLE

Metabolism and energy supply below the critical thermal minimum of a chill-susceptible insect

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SUMMARY

When exposed to temperatures below their critical thermal minimum (CT_{min}), insects enter chill-coma and accumulate chilling injuries. While the critical thermal limits of water-breathing marine animals may be caused by oxygen- and capacity-limitation of thermal tolerance (OCLT), the mechanisms are poorly understood in air-breathing terrestrial insects. We used thermolimit respirometry to characterize entry into chill-coma in a laboratory population of fall field crickets (*Gryllus pennsylvanicus*). To detect potential oxygen limitation, we quantified muscle ATP, lactate and alanine concentrations in crickets following prolonged exposure to 0°C (a temperature that causes chill-coma, chilling injury and eventual death). Although there was a sharp (44%) drop in the rate of CO_2 emission at the CT_{min} and spiracular control was lost, there was a low, continuous rate of CO_2 release throughout chill-coma, indicating that the spiracles were open and gas exchange could occur through the tracheal system. Prolonged exposure to 0°C caused muscle ATP levels to increase marginally (rather than decrease as OCLT would predict), and there was no change in muscle lactate or alanine concentration. Thus, it appears that insects are not susceptible to OCLT at low temperatures but that the CT_{min} may instead be set by temperature effects on whole-animal ion homeostasis.

Key words: critical thermal minimum, thermal limit, oxygen- and capacity-limitation of thermal tolerance, chill-coma, chill susceptible, thermolimit respirometry.

INTRODUCTION

Critical thermal limits to activity and performance are used to study the adaptation of ectotherms to local climates, in part because such an approach allows for bottom-up predictions of climate-driven changes in animal distribution and abundance (Chown et al., 2010). These predictions depend on a clear understanding of the physiological underpinnings of critical thermal limits. In marine ectotherms, both the critical thermal minimum (CT_{min}) and the critical thermal maximum (CT_{max}) are set by an inability to supply sufficient oxygen to metabolically demanding tissues (oxygen- and capacity-limitation of thermal tolerance, OCLT) (reviewed by Pörtner, 2010). Beyond critical thermal limits, aerobic scope decreases, causing increased reliance on anaerobic metabolism to sustain ATP production, and an accumulation of harmful anaerobic byproducts (Zielinski and Pörtner, 1996). As a mechanism for setting critical thermal limits, OCLT is well supported in a wide variety of water-breathing animals, including marine invertebrates (Zielinski and Pörtner, 1996; Schröer et al., 2009; Frederich and Pörtner, 2000; Lannig et al., 2008) and vertebrates (Pörtner et al., 2004; Van Dijk et al., 1999). On a broader scale, OCLT predicts the effects of elevated temperatures on fish fitness and abundance (Pörtner and Knust, 2007; Farrell et al., 2008; Martins et al., 2011), thereby suggesting a mechanism for variation in thermal tolerance among wild populations (Eliason et al., 2011). When considered together (as an oxygen supply index), environmental oxygen supply and organismal oxygen demand can provide strong predictive power for aquatic ecological patterns in a changing climate (Verberk et al., 2011). Among air-breathing terrestrial insects, critical thermal limits have been similarly used in both theoretical and applied contexts to test hypotheses regarding variation in thermal tolerance (Klok and Chown, 2003; Terblanche et al., 2008; Nyamukondiwa and Terblanche, 2010). However, the current understanding of the physiological mechanisms underlying thermal thresholds in insects lags behind that of marine animals.

When cooled, insects cross their CT_{min}, defined as the temperature at which they lack the coordination to stand or cling to a surface, and are immobilized as they enter chill-coma (Hazell and Bale, 2011). Long-term exposure to temperatures below the CT_{min} results in the accumulation of injuries and eventual death (Koštál et al., 2006; Koštál and Tollarová-Borovanská, 2009; MacMillan and Sinclair, 2011a). Although the precise mechanisms underlying the insect CT_{min} are not known, onset of chill-coma is associated with a disruption of neuromuscular function. When cooled, muscle resting potentials of *Apis mellifera* (Hymenoptera: Apidae) and *Drosophila melanogaster* (Diptera: Drosophilidae) depolarize, and the muscles lose the ability to generate action potentials (Hosler et al., 2000). In the fall field cricket (*Gryllus pennsylvanicus* Burmeister, Orthoptera: Gryllidae), this loss of muscle membrane potential is driven by a failure of whole-animal osmoregulation as inorganic ions and water migrate from the haemolymph to the gut during chilling (MacMillan and Sinclair, 2011a). Membrane potentials are expected to decrease below a threshold where the rate of ion leak exceeds the active transport capacity of cells, and tissues and ions diffuse down their concentration gradients (MacMillan and Sinclair, 2011b). If the insect CT_{min} and/or chill-coma is caused by an inability to deliver oxygen to tissues (as predicted by OCLT), then reduced aerobic metabolism would decrease substrate (ATP) availability and, consequently, lower the rate of ATP-dependent ion transport.

There are consistent changes in insect ventilation patterns at thermal limits (Lighton and Turner, 2004), which allows one to test the effects of experimental manipulation of ambient oxygen levels during heating or cooling on critical thermal limits by flow-through respirometry and optical activity detection during a temperature ramp (Lighton and Turner, 2004; Stevens et al., 2010). Using such an approach, the CT_{max} of tenebrionid beetles (*Gonocephalum simplex*, Coleoptera: Tenebrionidae), does not decrease with oxygen concentration except under extreme hypoxia (2.5% O_2) (Klok et al., 2004). Similarly, oxygen concentration has no effect on CT_{min} of *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Stevens et al., 2010). This suggests that, unlike marine animals, upper and lower thermal limits of terrestrial insects are not tightly linked by a common mechanism of OCLT but are instead probably set by alternative mechanisms (Stevens et al., 2010), which is consistent with wide-scale observations of decoupling of upper and lower thermal limits in insects (Addo-Bediako et al., 2000). Such experimental manipulations provide strong evidence against oxygen limitation at low temperatures in insects in the short term. However, OCLT in marine invertebrates can occur in normoxia and over periods of days, and the relationship between oxygen availability and thermal tolerance has not been examined in insects under these conditions.

The insect tracheal system delivers oxygen directly to respiring cells *via* diffusion combined (when the insect is active) with convective ventilation. The tracheal system delivers oxygen efficiently even at ambient oxygen levels below 10% (Harrison et al., 2001), which means that oxygen supply to insect tissues is unlikely to be limited during low temperature exposure in normoxia. By contrast, marine invertebrates do not have direct delivery of oxygen, and seawater is relatively hypoxic compared with air. Among fish and marine invertebrates, OCLT appears to be linked to sensitivity of the circulatory system. In particular, temperature affects cardiac output and the capacity for oxygen-binding pigments to bind or release molecular oxygen (Lannig et al., 2004; Melzner et al., 2007). The limiting factors that may lead to OCLT – oxygen delivery by way of oxygen-binding pigments and a circulatory system – do not occur in insects, suggesting that oxygen delivery may not be crucial for aerobic metabolism in terrestrial insects in normoxia.

If the CT_{min} was set by an inability to effectively deliver oxygen to tissues (OCLT), we predicted that aerobic metabolism would fail at the CT_{min} and crickets would rely on increased anaerobic metabolism while in chill-coma. To test this, we used thermolimit respirometry on fall field crickets to characterize respiratory phenomena associated with crossing the CT_{min} and determine whether the spiracles remain open following chill-coma onset. We also quantified muscle concentrations of ATP and anaerobic end-products (lactate and alanine) during prolonged exposure to, and recovery from, a temperature below the CT_{min}.

MATERIALS AND METHODS

Fall field crickets (*G. pennsylvanicus*) were derived from a population collected on the University of Toronto at Mississauga campus (43.3°N, 79.4°W) in the summer of 2004 and were reared at a constant temperature (25°C), humidity (70% relative humidity, RH) and photoperiod (14 h:10 h L:D). Crickets were fed commercial rabbit feed (Little Friends Rabbit Food, Martin Mills Inc., Elmira, ON, Canada). Adult rearing and egg collection methods followed those previously described (Judge, 2010; MacMillan and Sinclair, 2011a). Eggs were stored at +4°C for 3 months between generations to accommodate an obligate diapause (Rakshpal, 1962). All experiments were completed on gravid adult females, approximately 3 weeks after the final moult.

Respirometry

CO_2 production and activity of individual crickets were measured using flow-through respirometry following previously described methods (Klok et al., 2004; Lighton and Turner, 2004; Williams et al., 2010). Crickets (N=8) were placed individually in a cylindrical glass respiration chamber (11 cm^3) inside a PTC-1 temperature-controlled cabinet [Sable Systems International (SSI), Las Vegas, NV, USA] where the temperature was held at 20°C for 30 min, then decreased at –0.25°C min^{-1} to 0°C, where it was held for 1 h. Dry, CO_2-free air was passed through the chamber at 50 ml min^{-1} and flow rate was controlled by mass flow valves (Sierra Instruments, Monterey, CA, USA) and an MFC-2 controller (SSI). The CO_2 and water vapour concentration of air exiting the animal chamber were determined with a Li7000 infrared gas analyser (LiCor, Lincoln, NE, USA). Throughout the period of data collection, activity was recorded using an AD-1 infrared activity detector (SSI). The temperature of the exterior of the animal chamber (the glass on which the insect was resting) was recorded using a type-T thermocouple and TC-2000 thermocouple meter (SSI). Carbon dioxide release and activity data were collected using a UI2 interface and Expedata software (SSI) and corrected by recording an empty chamber for 10 min at the beginning and end of each run and subtracting the baseline to correct for any instrument drift. Carbon dioxide data were corrected for the dilution of water vapour and converted into $\dot{V}_{CO_2}$ (ml min^{-1}) in Expedata software.

ATP, lactate and alanine quantification during cold exposure and recovery

The concentrations of ATP, lactate and alanine in cricket femur muscle were measured during prolonged chill-coma and chill-coma recovery as an index of energy status and reliance on anaerobic metabolism. A total of 112 crickets were placed individually into 14 ml plastic snap-top vials with lids loosely attached to allow for airflow. Crickets were exposed to a linear temperature decline from 20 to 0°C at a rate of –0.25°C min^{-1} and held at 0°C. Once 0°C was reached, eight crickets were removed from the cold and snap-frozen whole in liquid nitrogen; this was repeated every 6 h for 24 h, and every 24 h thereafter up to 120 h. In order to test for effects of cold exposure on metabolism during chill-coma recovery, a subset of crickets was exposed to the same temperature ramp followed by 24 h at 0°C before being removed to 20°C for 5 min, for 20 min, to the point of chill-coma recovery (when the animal could stand, ~70 min) or for 24 h, then snap-frozen in liquid nitrogen. Control crickets were snap-frozen directly from their rearing conditions. As a positive control for the effects of impaired aerobic respiration, six crickets were exposed to anoxia (N_2 gas) for 1 h at 20°C followed by snap-freezing without further exposure to air.

Frozen crickets were dissected on an aluminium plate cooled by liquid nitrogen. For each cricket, both hindfemurs were weighed and pooled to obtain a sample that was almost exclusively muscle tissue by mass. Tissue was immediately minced with scissors on ice in 1 ml of homogenization buffer (6% perchloric acid, 1 mmol l^{-1} EDTA). Minced samples were homogenized at 4°C with a bullet blender (Next Advance, Inc., Averill Park, NY, USA) using 1 mm glass beads and centrifuged at 20,000 ***g*** at 4°C for 20 min. The resulting supernatant was transferred to a new microcentrifuge tube and neutralized with 480 µl of a basic solution (2 mol l^{-1} KOH, 0.4 mol l^{-1} imidazole, 0.3 mol l^{-1} KCl). Samples were centrifuged again for 5 min to remove the resulting precipitate and the supernatant was stored at –80°C until use in biochemical assays.

ATP was quantified using an ATP-determination bioluminescence kit (Invitrogen Canada Inc., Burlington, ON,

Canada), which quantifies sample ATP concentration from the rate of ATP-dependent light production by firefly luciferase (EC number: 1.13.12.7, Invitrogen Canada Inc.). Light production was quantified using a Sirius single-tube luminometer (Berthold Systems Inc., Pforzheim, Germany), and sample ATP concentration determined by comparison to standards of known ATP concentration.

L-Lactate was quantified in 96-well assay plates using lactate dehydrogenase (EC number: 1.1.1.27, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) in an NADH-linked spectrophotometric assay (Noll, 1984), with sample concentrations determined from standard curves of known lactate concentrations. L-Alanine was quantified in the same manner, but using alanine transaminase (EC number: 2.6.1.2, Sigma-Aldrich Canada Ltd) in an alternative NADH-linked assay (Williamson, 1990).

Data analysis

Respirometry data were managed in Expedata and statistical analyses were conducted with R version 2.13 (R Development Core Team, 2011). Thermolimit respirometry estimates of the CT_{min}, identified as a cessation of activity or spiracular control, were determined from the respirometry data as has previously been described (Lighton and Turner, 2004; Stevens et al., 2010). Briefly, absolute difference sums (ADS; a cumulative sum of absolute differences between consecutive data points) of cricket activity (a unit-less measure) and $\dot{V}_{CO_2}$ were calculated using the ADS function in Expedata. Five-minute periods surrounding the cessation of spiracular control ($\dot{V}_{CO_2}$) and activity were selected manually and the ADS regressed against time. The highest five consecutive residuals of the regression were selected and the mean temperature at the inflection point of activity (aADS) or $\dot{V}_{CO_2}$ (vADS), which were considered as two separate estimates of CT_{min}, were recorded. Stevens et al. also used the final burst of CO_2 prior to the cessation of activity as a third method of estimating the CT_{min} (Stevens et al., 2010). However, in *G. pennsylvanicus* the final burst of CO_2 was indistinguishable from the bursts that preceded it (Fig. 1), so this method was not used. aADS and vADS CT_{min} values for each cricket were compared using a paired *t*-test. The estimates of CT_{min} derived from aADS and vADS did not significantly differ (see Results); thus, aADS-derived CT_{min} was used in subsequent analyses.

The slope of the aADS provides an index of activity level (Lighton and Turner, 2004), so aADS slopes and $\dot{V}_{CO_2}$ from the duration of the temperature ramp (excluding the holds at 20 and 0°C) were binned into 1°C increments to estimate the thermal dependence of CO_2 release and activity (Fig. 2A). Because each cricket had a different CT_{min}, binning of $\dot{V}_{CO_2}$ data directly by temperature did not allow for estimation of the magnitude effect of crossing the CT_{min} on the proportional rate of CO_2 release. To make such an estimate, $\dot{V}_{CO_2}$ data were shifted such that temperatures were expressed as a positive or negative value relative to the CT_{min} of the individual cricket (such that 0 represents the CT_{min}). We then corrected for the resulting shift in the rate–temperature curve by expressing mass-specific rates of CO_2 release proportional to the mean rate of each individual at 20°C (Fig. 2B).

We then examined the thermal sensitivity of metabolic rate before and after the CT_{min}. Rates of CO_2 release from each individual cricket were split into three stages of cooling, and temperature coefficient (Q_{10}) values over those temperature ranges determined as $10^{(10\times slope)}$ of a line of best fit of $\log_{10}(\dot{V}_{CO_2})$ regressed against temperature. The first segment, from 15 to 0.5°C above the CT_{min} of each individual (determined from activity ADS data), was used to examine the effect of temperature on $\dot{V}_{CO_2}$ above the CT_{min}. The second segment, from 0.5°C below the CT_{min} of each individual to the previously reported chill-coma temperature of 2.3°C determined from visual observation (MacMillan and Sinclair, 2011a), represented the effect of temperature on the $\dot{V}_{CO_2}$ between the CT_{min} and chill-coma onset. The last period, from 2.3°C to the end of the temperature ramp (0°C), represents the effect of further temperature decline on $\dot{V}_{CO_2}$ release while in chill-coma. Q_{10} values from different periods of the temperature ramp were compared using a repeated measures ANOVA using the lme() function of the nlme package in R.

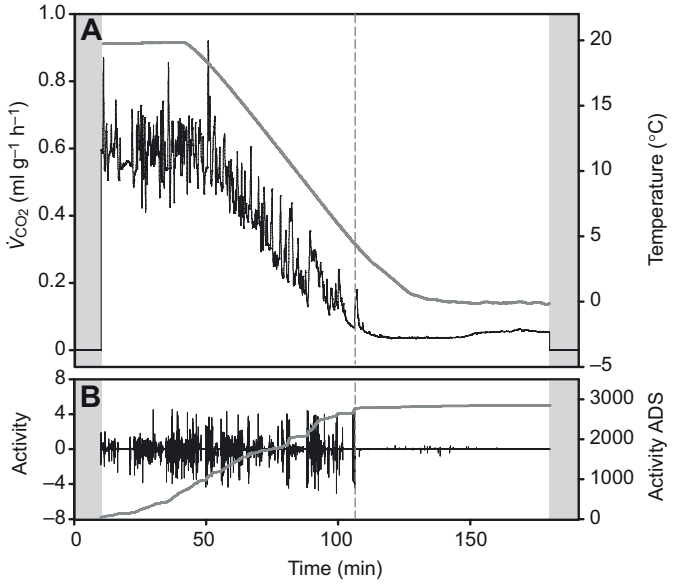

Fig. 1. An example of respirometry and activity data from one adult female *Gryllus pennsylvanicus* during a temperature ramp. (A) CO_2 emission (black line) and temperature (grey line). (B) Activity (black line) and activity absolute difference sum (ADS; grey line) while maintained at 20°C for 1 h, and during cooling from 20 to 0°C at 0.25°C min^{-1}. Vertical grey bands at each end of the trace indicate the baseline recording from an empty chamber. The dashed vertical line indicates the CT_{min} temperature determined as the point of highest residuals of a regression of activity ADS over time (see Materials and methods for details).

The effects of 1 h anoxia, cold exposure duration and recovery time following 24 h at 0°C on muscle metabolite quantity were independently analysed using the lm() function (Pinheiro et al., 2011), with tissue mass treated as a covariate in all cases. As tissue mass was always a significant covariate ($P<0.05$), only parameters from exposure or recovery time are reported. All values reported are means ± 1 s.e.m.

RESULTS

A representative respirometry trace is presented in (Fig. 1). All crickets displayed continuous respiratory patterns without discontinuous or cyclic gas exchange. Rates of CO_2 release were stable at 20°C (before the temperature ramp began) and decreased with the decreasing temperature. Activity was often detected in concert with spikes in CO_2 release, indicating that the activity detector was probably recording abdominal contractions (active ventilation). Activity was detected throughout the temperature ramp until it ceased completely at an individual-specific threshold temperature (dashed vertical line in Fig. 1).

Thermolimit respirometry estimates of the CT_{min} determined by the aADS method (6.5±0.5°C) and vADS method (6.2±0.4°C) did not differ significantly (t_7=1.48, P=0.183). CT_{min} estimates obtained

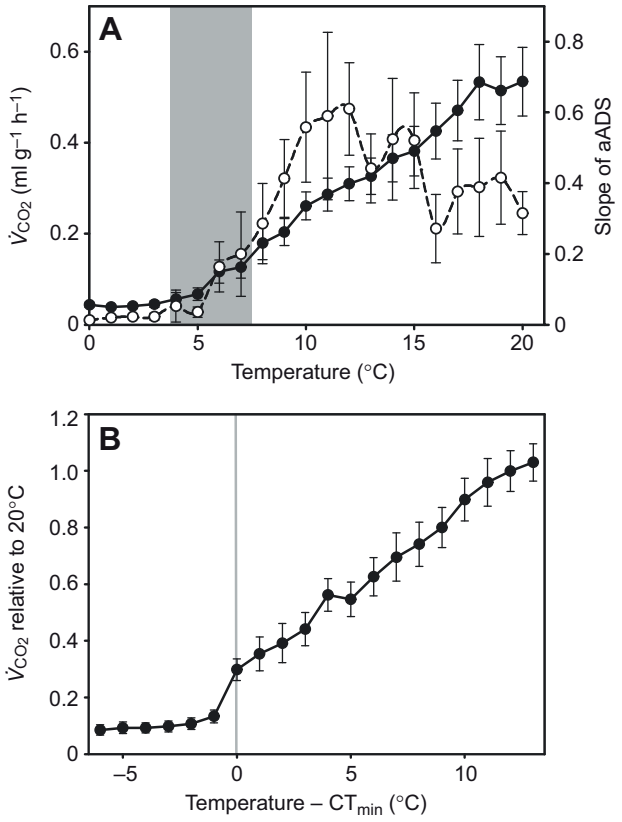

Fig. 2. CO_2 release and slope of activity absolute difference sum (aADS) of female *G. pennsylvanicus* during a linear temperature ramp from 20 to 0°C at 0.25°C min^{-1}. Data for each cricket were averaged at 1°C increments. (A) Data expressed as mass-specific rate of CO_2 release (filled circles) and slope of aADS (open circles). The shaded region covers the range of temperatures over which individual crickets crossed their aADS CT_{min} (see Results for details). (B) The same $\dot{V}_{CO_2}$ data as in A, standardized to individual CT_{min} (such that 0 represents the CT_{min}, vertical grey line) and individual metabolic rate at 20°C (see Materials and methods for details). Values are means (±s.e.m.), N=8 crickets.

by aADS were broadly distributed between 4.3 and 8.6°C (Fig. 2A). When expressed as a proportional rate of CO_2 release at temperatures relative to the aADS CT_{min} (see Materials and methods for details), the rate of CO_2 release dropped by approximately 44% between the CT_{min} and 1°C below the CT_{min} (Fig. 2B).

Q_{10} values above the CT_{min} (3.3±0.3) did not significantly differ from those between the CT_{min} and chill-coma onset (3.4±0.6, t_{10}=0.23, P=0.822). However, the Q_{10} of $\dot{V}_{CO_2}$ below the chill-coma onset temperature was 1.5±0.2, significantly lower than the Q_{10} above 2.3°C (t_{10}=2.87, P=0.016).

Following prolonged (120 h) exposure to 0°C, muscle ATP concentration increased slightly but significantly from 8.4±0.6 to 10.5±0.5 µmol g^{-1} ($F_{2,55}$=19.02, P<0.001; Fig. 3A). Low temperature exposure had no significant effect on muscle lactate ($F_{2,52}$=1.32, P=0.256; Fig. 3A) or alanine ($F_{2,52}$=0.22, P=0.641; Fig. 3A) concentration. No significant changes in muscle ATP ($F_{4,21}$=0.49, P=0.488) or lactate ($F_{4,21}$=0.13, P=0.717) concentration occurred during recovery from 24 h at 0°C (Fig. 3B). Following 24 h of recovery, muscle alanine concentration was significantly lower than when measured at the point at which crickets could stand ($F_{4,25}$=5.45, P=0.003; Fig. 3B, 'Flip'). No other significant differences were found in muscle alanine concentration during recovery.

During 1 h of hypoxia at 20°C, muscle ATP concentration decreased significantly from 8.4±0.60 to 1.8±0.8 µmol g^{-1} ($F_{2,9}$=21.35, P=0.002), while muscle lactate concentration significantly increased from 0.23±0.04 to 0.31±0.03 µmol g^{-1} ($F_{2,9}$=9.54, P=0.013) (Fig. 4). While alanine was detectable in muscles of control and cold-exposed crickets, muscle alanine was below detectable levels in five of six anoxia-exposed crickets, and low in the sixth (0.89 µmol g^{-1}), compared with the control.

DISCUSSION

During a linear temperature decline, crickets ceased activity at individual-specific temperatures in a manner consistent with the CT_{min} (Hazell and Bale, 2011). Below this threshold, crickets did not actively ventilate, but released CO_2 continuously, typical of insects exposed to temperatures beyond critical thermal limits or to anoxia (Sinclair et al., 2004; Lighton and Schilman, 2007; Lighton and Turner, 2004; Kovac et al., 2007; Stevens et al., 2010). These thermolimit respirometry estimates of CT_{min} are lower and more variable than those determined as the complete absence of movement in response to a stimulus (2.3±0.1°C) by crickets from the same population (MacMillan and Sinclair, 2011a). Estimates of CT_{min} often differ by several degrees among methods (Terblanche et al., 2007), and thermolimit respirometry also yielded significantly higher CT_{min} estimates than visual methods in tenebrionid beetles (*Tenebrio molitor*, Coleoptera: Tenebrionidae) and a terrestrial isopod (*Porcellio scaber*, Porcellionidae; Isopoda) under similar conditions (Stevens et al., 2010). In *Periplaneta americana* (Blattaria: Blattidae), movement of the coxae can continue several degrees below the arrest of many other regions (Staszak and Mutchmor, 1973), possibly because thermal sensitivity of the nerves and muscles servicing the abdomen and limbs differ, and this could explain why crickets could continue to move their limbs to a lower temperature. However, estimates of CT_{min} determined by activity (aADS) did not differ significantly from those determined from ventilation (vADS), meaning all movement ceased when the crickets ceased control of ventilation in the respirometry chamber. This suggests that if the crickets remain untouched (as in the respirometry chamber), rather than being pestered by a scientist with a stick, all voluntary movement ceases with the cessation of ventilation, although the ability to move the limbs in response to stimulation is not yet lost. As such, thermolimit respirometry may better estimate limits to mobility in the wild than CT_{min} determination methods that require an operator to prod the insect under study. These results highlight the importance of establishing consistent terminology regarding the sequence of events leading to chill-coma, discussed extensively elsewhere (Hazell and Bale, 2011).

Upon crossing the thermolimit respirometry CT_{min}, there was an abrupt decline in the rate of CO_2 release by *G. pennsylvanicus*. A similar breakpoint occurs at the CT_{min} of the sub-Antarctic caterpillar *Pringleophaga marioni* (Lepidoptera: Tineidae) during cooling and has been suggested to reflect kinetic failure of ATP-dependent enzymes maintaining ionic homeostasis (Sinclair et al., 2004). While we cannot rule out the possibility that this reduction in metabolic rate is related to changes in the metabolic supply of ATP, its magnitude is such that it may also be explained by cessation of the metabolic demands of neuromuscular processes. For example, when an insect crosses its CT_{min} and enters chill-coma, it no longer maintains posture, ventilation or gut motility. It is also difficult to determine whether thermolimit respirometry at low temperatures identifies physiological failure of the ventilatory system or a behaviour (the complete cessation of movement) in response to declining temperatures, which may have higher inter-individual

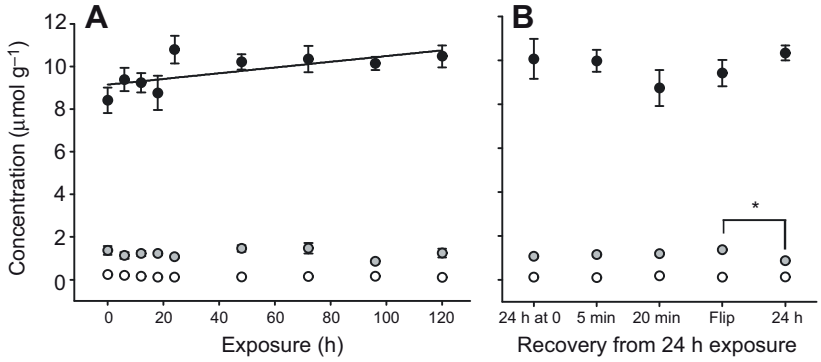

Fig. 3. Concentration of ATP (black), lactate (white) and alanine (grey) in the hindfemur of female adult *G. pennsylvanicus* (A) during exposure to 0°C and (B) during recovery at 20°C from 24 h in chill-coma at 0°C. Crickets recovering from 24 h at 0°C were removed to 20°C for 5 min, for 20 min, to the point of chill-coma recovery (when the cricket flipped onto its feet; Flip) or for 24 h (with access to food and water *ad libitum*) prior to sampling. Values are means (±s.e.m.) for N=8 crickets per sampling period. Error bars that are not visible are obscured by the symbols. ATP concentration significantly increased during cold exposure (linear regression; P<0.001). Asterisk denotes a significant difference in alanine concentration between the point crickets could stand and 24 h following removal from 0°C (Tukey HSD; P=0.003). No other significant changes in ATP, lactate or alanine concentration were observed during cold exposure or recovery.

variation than physiological failure of the neuromuscular system (Chown et al., 2009; Stevens et al., 2010). Indeed, coma in response to environmental stress could be adaptive, as it may act to protect against cellular damage (Rodgers et al., 2010). Temperature sensors have been confirmed in the third antennal segment and brain of *Drosophila* (Hamada et al., 2008). Future studies could assess whether the CT_{min} represents a direct consequence of temperature or a response to it, by testing the effects of excision of thermosensors on thermolimit respirometry estimates of the CT_{min}.

Although the rate of CO_2 release by *G. pennsylvanicus* was low in chill-coma, it was clearly above baseline levels, suggesting that the spiracles were open, and that CO_2 and O_2 were being exchanged with the environment. If the crickets in the current study had been oxygen limited while in chill-coma, we predicted that ATP availability would decline at the onset of chill-coma and remain low, accompanied by the accumulation of anaerobic end-products. From other studies on terrestrial insects, we predicted lactate and alanine to be the primary end-products of anaerobic metabolism in crickets (Hoback et al., 2000; Hoback and Stanley, 2001; Feala et al., 2007). During prolonged chill-coma, however, muscle ATP concentrations significantly increased, and no increase in lactate or alanine concentration was observed over the same period. Even when maintained at 0°C for 5 days, which results in 100% mortality in this species (MacMillan and Sinclair, 2011a), no increase in muscle lactate or alanine was observed. This is in stark contrast to the effects of anoxia, which decreased muscle ATP levels by 79%, increased muscle lactate by 35% and reduced muscle alanine concentration to undetectable levels within 1 h at 20°C. These results suggest that cold exposure and anoxia differ in their effects on aerobic and anaerobic metabolism.

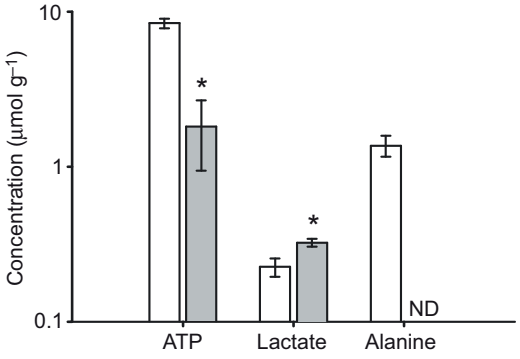

Fig. 4. Concentrations of ATP, lactate and alanine in the hindfemur of female adult *G. pennsylvanicus* under normoxic conditions (white bars) and following 1 h of anoxia at 20°C (grey bars). Asterisks indicate that ATP concentration significantly decreased (P=0.002) and lactate concentration significantly increased (P=0.013); alanine concentration fell below detectable levels (ND) in 5 of 6 individuals during exposure to anoxia.

Although the increase in ATP during cold exposure was statistically significant, it was small (25% over 5 days) in magnitude, and may not represent a biologically significant change. An accumulation of ATP following cold exposure has been noted in the chill-susceptible beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) (Colinet, 2011). Accumulation of ATP suggests that processes utilizing ATP (such as ion-motive enzymes) are slowed to a greater extent than oxidative phosphorylation. In contrast, total adenylate energy charge in the fat body cells of *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae) was unchanged during long-term cold exposure (Kostal et al., 2004). Disruption of metabolic processes is a common consequence of cold exposure, and ATP-producing pathways are targets for differential regulation in response to thermal challenges (Overgaard et al., 2007). Following removal from the cold and recovery from chill-coma, no change in ATP or lactate concentrations was noted. Thus, crickets probably do not rely on anaerobic metabolism to provide energy during long-term cold exposure that is known to cause injury, or during rewarming and chill-coma recovery.

Organisms faced with environmental stress often demonstrate a reduced thermal sensitivity of metabolism (Makarieva et al., 2006). To test for differences in thermal sensitivity of metabolic rate surrounding the thermolimit respirometry CT_{min} and the complete inability to move, we calculated temperature coefficients (Q_{10}) in three separate regions of the temperature ramp for each individual. The mean Q_{10} of cricket CO_2 emission prior to crossing the thermolimit respirometry CT_{min} was approximately 3, and did not differ significantly from Q_{10} values from below this breakpoint down to the chill-coma temperature of 2.3°C. Thus, metabolic rate remains temperature sensitive beyond the cessation of ventilation and voluntary movement. Interestingly, between 2.3°C (the point

at which crickets cease responding to physical stimulation) (MacMillan and Sinclair, 2011a) and 0°C, the mean Q_{10} significantly decreased to 1.5, indicating that rates of CO_2 emission are less temperature sensitive below the temperature at which no movement can be elicited from a cricket. Similarly, honeybees demonstrate low rates of CO_2 emission that are largely temperature independent while in chill-coma (Lighton and Lovegrove, 1990), and animals that exhibit minimum life-supporting metabolic rates during times of stress have a mean Q_{10} of metabolic rate of 1.1 (relatively close to the Q_{10} of 1.5 we observed during chill-coma) (Makarieva et al., 2006). However, why metabolic rate would be less dependent on temperature when insects are in chill-coma is not immediately clear. Unlike those under normoxic conditions, animals in anoxia on average maintain a relatively normal Q_{10} of 2.75 (Makarieva et al., 2006), which further supports the suggestion that crickets in chill-coma were not oxygen limited.

OCLT is widely supported in marine ectotherms and has provided a useful conceptual framework for investigating the effects of environmental temperature on species abundance (Pörtner, 2010). We, however, found no evidence that crickets lack adequate oxygen when in chill-coma up to the point of death. We therefore posit that the insect CT_{min} is more likely to be set by the direct effects of temperature on reaction rates of ionoregulatory enzymes such as Na^+,K^+-ATPase.

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