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RESEARCH ARTICLE

Central nervous system shutdown underlies acute cold tolerance in tropical and temperate *Drosophila* species

Mads Kuhlmann Andersen[1,*], Nikolaj Johannes Skole Jensen[1], R. Meldrum Robertson[2] and Johannes Overgaard[1]

ABSTRACT

When cooled, insects first lose their ability to perform coordinated movements (CT_{min}) after which they enter chill coma (chill coma onset, CCO). Both these behaviours are popular measures of cold tolerance that correlate remarkably well with species distribution. To identify and understand the neuromuscular impairment that causes CT_{min} and CCO we used inter- and intraspecific model systems of *Drosophila* species that have varying cold tolerance as a consequence of adaptation or cold acclimation. Our results demonstrate that CT_{min} and CCO correlate strongly with a spreading depolarization (SD) within the central nervous system (CNS). We show that this SD is associated with a rapid increase in extracellular [K^+] within the CNS causing neuronal depolarization that silences the CNS. The CNS shutdown is likely to be caused by a mismatch between passive and active ion transport within the CNS and in a different set of experiments we examine inter- and intraspecific differences in sensitivity to SD events during anoxic exposure. These experiments show that cold adapted or acclimated flies are better able to maintain ionoregulatory balance when active transport is compromised within the CNS. Combined, we demonstrate that a key mechanism underlying chill coma entry of *Drosophila* is CNS shutdown, and the ability to prevent this CNS shutdown is therefore an important component of acute cold tolerance, thermal adaptation and cold acclimation in insects.

KEY WORDS: CNS, Spreading depression, Ion balance, Chill coma, Chill tolerance, CT_{min}

INTRODUCTION

Insect performance is heavily influenced by ambient temperature and when exposed to decreasing temperatures most insects will lose the ability to coordinate movement (CT_{min}) and ultimately enter a reversible state of complete neuromuscular paralysis (i.e. enter chill coma at the chill coma onset, CCO) (Mellanby, 1939; Hazell and Bale, 2011; Overgaard and MacMillan, 2017). Insects can recover from chill coma without injury if the coma is short and/or occurs at relatively high temperatures. Even so, chill coma represents an unresponsive behavioural stage where the animal is 'ecologically dead'. It is therefore not surprising that lower thermal limits are among the best predictors of insect distribution (Addo-Bediako et al., 2000; Sunday et al., 2011; Bale, 2002; Kimura, 2004). This also includes *Drosophila* where CT_{min} and CCO vary markedly between species and correlates well with the climatic and latitudinal variation among these species (Kellermann et al., 2012; Andersen et al., 2015c). These traits are also highly plastic such that cold acclimation lowers CT_{min} and CCO considerably (Mellanby, 1954; Lee et al., 1987; Kelty and Lee, 1999). Differences within and among *Drosophila* species are therefore clear signs of adaptation or acclimation to temperature variability over evolutionary and seasonal time scales. Considering the importance of cold tolerance trait and its close association with insect distribution, it is valuable to understand the physiological mechanisms setting lower thermal limits.

CT_{min} and CCO in insects are most likely to be caused by cold-induced impairment of neuromuscular function. However, there is still some controversy regarding the proximate cause, as some studies have highlighted muscle impairment over nervous impairment and vice versa. For example, Anderson and Mutchmor (1968) and Bradfisch et al. (1982) observed that nervous activity in insects could continue after chill coma onset, which indirectly indicates that cold coma is caused by reduced muscular function. Consistent with this idea, a number of studies have shown how cold-induced depolarization of the muscle membrane potential (V_m) impairs muscle function by interfering with action potential generation and contractility (Goller and Esch, 1990; Hosler et al., 2000; Findsen et al., 2014; MacMillan et al., 2014; Findsen et al., 2016; Overgaard and MacMillan, 2017). In contrast Andersen et al. (2015a) observed that muscle V_m was maintained at sub-critical temperatures in some cold-tolerant drosophilids even at temperatures below their CCO, and it was therefore suggested that impairment of nervous function could be the proximal cause of cold coma. This idea was not new, as other studies had already shown that the central nervous system (CNS) is silenced at critically low temperatures in insects, including *Drosophila* (Rodgers et al., 2010; Armstrong et al., 2012). Specifically, it has been shown that the CNS shuts down due to a spreading depolarization (SD) which is characterized by an abrupt surge in extracellular [K^+] within the CNS. The SD in cold-exposed insects is thought to develop when an initial cold-induced depolarization of nervous tissue causes an increase in neuronal activity. This increased activity results in excessive release of K^+ due to the repolarizing K^+ currents into the extracellular space. A rapid hyperkalemic (high $[K^+]_o$) situation therefore develops that further depolarizes the membrane, creating a positive feedback loop which eventually silences the CNS due to excessive depolarization (Armstrong et al., 2009; Rodgers et al., 2010). In other words, SD events are essentially caused by an imbalance between the accumulation of K^+ from cells relative to the active clearance back to the intracellular space. Similar SD events are also found in insects during anoxic exposure and during exposure to critically high temperatures and in all cases it is observed that the SD events lead to a comatose animal (Rodgers et al., 2007, 2010; Spong et al., 2016b).

[1]Zoophysiology, Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark. [2]Department of Biology, Queen's University, Kingston, ON, K7L 3N6, Canada.

*Author for correspondence (mads.andersen@bios.au.dk)

M.K.A., 0000-0002-6215-8941

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Robertson et al. (2017) recently demonstrated that the loss of coordination at CT_{min} was caused by SD events in *Locusta migratoria* and thus that CNS failure initiates the gradual loss of function when temperature is lowered. Although there is some evidence to suggest that the temperatures causing CT_{min} and SD are closely associated for locusts (Robertson et al., 2017), and that CT_{min} and CCO are separate thermal limits (Coleman et al., 2015), only one study has examined if variation in CT_{min} induced via rapid cold hardening was associated with improved homeostatic capacity in the CNS (Armstrong et al., 2012). However, to our knowledge, no study has investigated if adaptation elicits similar changes in the ionoregulatory capacity of the CNS, nor has developmental acclimation been examined. In this study we hypothesized that the entry into chill coma, initiated with a loss of coordination at CT_{min}, was caused by a SD event in the CNS. We investigated this hypothesis by measuring field potentials and extracellular K^+ concentrations in the CNS using direct current (DC) and K^+-sensitive microelectrodes during gradual cooling in five *Drosophila* species with varying cold tolerance. Using similar methods, we estimated CT_{min} in cold- and warm-acclimated *D. melanogaster* and investigated if cold acclimation changed the capacity of flies to maintain CNS ion homeostasis at low temperature. To gain further insight into possible mechanisms supporting difference in homeostatic capacity we also examined if differences in cold tolerance were associated with differences in homeostatic resilience during exposure to complete anoxia. With these experiments, we show that the onset of chill coma, an important phenotypic predictor of species distribution, is closely related to loss of CNS function. We also demonstrate that thermal adaptation and acclimation are associated with improved capacity to maintain homeostasis during anoxic exposure, which highlights the importance of regulated CNS function in both thermal adaptation and acclimation.

MATERIALS AND METHODS

Model organisms and animal husbandry

For experiments on interspecific differences, all five species of *Drosophila* (*Drosophila birchii* Dobzhansky & Mather 1961, *Drosophila equinoxialis* Dobzhansky 1946, *Drosophila melanogaster* Meigen 1830, *Drosophila persimilis* Dobzhansky and Epling 1944 and *Drosophila montana* Patterson 1943) were reared under 'common garden' conditions of 22–23°C and 12 h:12 h light: dark cycle. These five species were chosen as they are known to possess marked differences in cold tolerance and have been studied numerous times in recent literature (Andersen et al., 2015a,b,c; MacMillan et al., 2015; Olsson et al., 2016). Intraspecific differences were induced in *D. melanogaster* by rearing eggs of *D. melanogaster* to adults at either constant 15°C or constant 25°C with a 12 h:12 h light:dark cycle. All flies were fed on Leeds medium based on oatmeal (per litre of water: 60 g yeast, 40 g sucrose, 30 g oatmeal, 16 g agar, 12 g methyl paraben and 1.2 ml acetic acid), were kept in bottles with 40 ml media, and were allowed to oviposit for 2 h to 2 days depending on species, to control rearing densities of the next generation at around 100 individuals. Newly emerged flies were transferred to vials (7 ml media) and left to develop for 6–9 days, after which only females (considered non-virgin) were used for experiments.

All measurements regarding interspecific differences were conducted by Mads K. Andersen in the laboratory of R. M. Robertson (Queen's University, Canada) while all experiments regarding acclimation in *D. melanogaster* were performed by Nikolaj J. S. Jensen in the laboratory of J. Overgaard (Aarhus University, Denmark). Because minor differences in rearing conditions and experimental set-up could introduce 'noise', we discuss the effects of adaptation and acclimations separately and refrain from comparing *D. melanogaster* data from the interspecific experiments with those acclimated at 15 and 25°C, respectively.

Measurement of lower thermal limits

The temperatures of loss of coordination (CT_{min}) and complete cessation of movements (CCO) of all five species of *Drosophila* and acclimated *D. melanogaster* were estimated by placing individual flies in sealed 4 ml glass vials and submerging them in a bath containing a 1:1 (v/v) mixture of water and ethylene-glycol, which was then cooled by 1°C min^{-1}. During cooling, the glass vials were regularly tapped to provoke movement (to test the ability to move rather than the fly's inclination to move; Sinclair et al., 2015), and the temperatures for loss of the ability to right (proxy for loss of coordinated movements) and complete loss of muscular function (indicated by no observed movement) were noted as CT_{min} and CCO, respectively.

Preparation of electrodes and electrophysiological measurements

To estimate the failure temperature of the CNS we used pulled glass electrodes. Electrodes for the measurement of field potentials (DC electrodes) were made by pulling filamented borosilicate glass capillaries (1 mm diameter; 1B100F-4, World Precision Instruments, Sarasota, FL, USA) to a low tip resistance (5–7 MΩ) using a Flaming-Brown P87 micro-pipette puller (Sutter Instruments, Novato, CA, USA) and back-filled with 500 mmol l^{-1} KCl. The electrode was then placed in an electrode holder with a chlorinated silver wire, and connected to a DUO 773 two-channel intracellular/extracellular amplifier (World Precision Instruments). Raw voltages were digitized using a DigiData 1440A series interface (Axon Instruments, Union City, CA, USA) and recorded using AxoScope 9.0 (Axon Instruments). Electrophysiological measurements were conducted on two different, but qualitatively similar set-ups where only data acquisition differed; in the second set-up, raw voltages were digitized by a MP100A data acquisition system and recorded using Acqknowledge software (both from Biopack Systems, Goleta, CA, USA).

For experiments, flies were gently immobilized in a bed of wax on a glass coverslide or directly on top of a controllable thermoelectrically cooled stage. After immobilization, a small hole was made in the abdomen between the second and third-to-last segment with a micro pen, and a small incision was made in the head cuticle along the midline using a pair of capillary scissors to access the brain. The coverslide was then secured on the temperature-controlled stage, the glass electrode inserted into the brain using micromanipulators and a tungsten (or Ag/AgCl) electrode was inserted into the abdomen for grounding, after which the voltage was zeroed. Temperature of the plate was then lowered from room temperature by 1°C min^{-1} until a drop in DC field potential (indicative of a spreading depolarization having occurred) was recorded (see representative traces in Fig. 1). The temperature of CNS failure (i.e. when the spreading depolarization occurred) was estimated as the temperature of half-amplitude of the drop in DC potential.

To measure the extracellular K^+ concentration ($[K^+]_o$) in the fly CNS, K^+-sensitive electrodes were constructed from unfilamented borosilicate glass capillaries (1 mm diameter; 1B100-4, World Precision Instruments). Glass capillaries were pulled to form a tip resistance of 5–7 MΩ, and were subsequently heated to 100°C and silanized for 1 h in an atmosphere of dichlorodimethylsilane or *N*,*N*-dimethyltrimethylsilylamine (Sigma-Aldrich, St Louis, MO, USA) under a glass Petri dish. After salinization, electrodes were first

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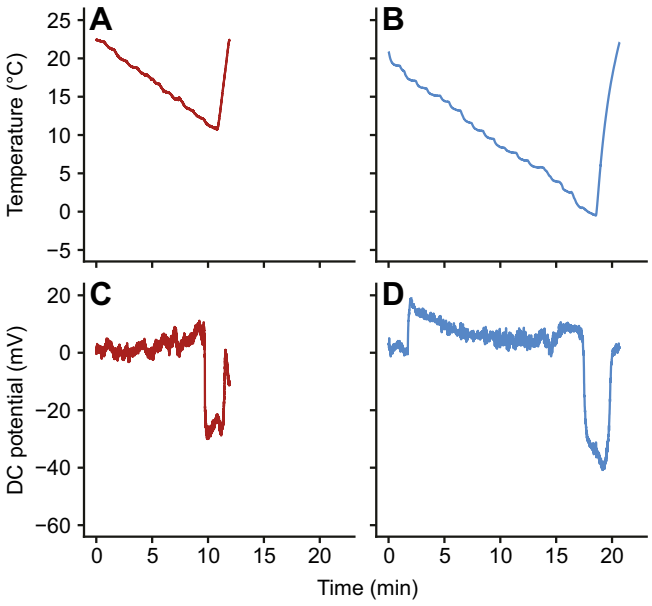

Fig. 1. Representative traces of temperature and DC field potential for cold-sensitive *Drosophila birchii* and cold-tolerant *D. montana*. (A,B) Temperature; (C,D) DC field potential; *D. birchii* traces are in red (A,C) and *D. montana* traces are in blue (B,D). Temperature was gradually reduced by 1°C min^{-1} while a DC microelectrode was used to measure the field potential in the CNS. Note the different temperature for SD occurrence (abrupt drop in DC potential at ∼12 and ∼1°C, respectively).

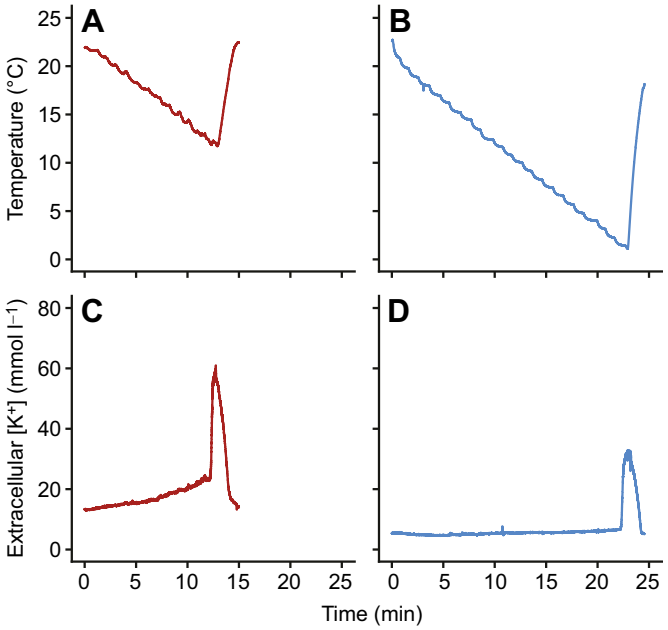

Fig. 2. Representative temperature traces and measurement of extracellular K^+ concentration in the CNS of cold-sensitive *D. birchii* and cold-tolerant *D. montana*. (A,B) Temperature; (C,D) K^+ concentration; *D. birchii* traces are in red (A,C) and *D. montana* traces are in blue (B,D). Temperature was lowered by 1°C min^{-1} while a K^+-sensitive microelectrode measured $[K^+]_o$ in the CNS. Note the different peaks in K^+ concentrations during the rapid surge (indicative of the SD occurring); ∼60 mmol l^{-1} for *D. birchii* and ∼35 mmol l^{-1} for *D. montana*. In addition, the small gradual increase in extracellular $[K^+]$ for *D. birchii* during cooling (before the rapid surge) was not observed in all *D. birchii* tested.

back-filled with K^+ ionophore (Potassium Ionophore I, cocktail B, Sigma-Aldrich) and subsequently backfilled with 500 mmol l^{-1} KCl to form a K^+-sensitive membrane. A DC electrode was used for reference and fashioned as described in the previous paragraph. K^+-sensitive electrodes were calibrated in standards of 15 and 150 mmol l^{-1} KCl (difference made up of LiCl) and the voltage was zeroed in the lowest standard. Only electrodes with a slope between 50 and 62 were used (mean±s.e.m.: 54.3±0.5 mV, N=37). Subsequently, both electrodes were inserted into the CNS along with a grounding electrode in the abdomen. Voltages measured by the K^+-sensitive electrode were then converted into K^+ concentration as follows:

$$\text{Extracellular } [K^+] = c \cdot 10^{\left(\frac{V}{S}\right)}, \quad (1)$$

where c is the concentration of K^+ in the low standard buffer (15 mmol l^{-1} K^+), V is the voltage measured from the K^+-sensitive electrode in the fly brain, and S is the slope of the electrode, which was corrected for changes in temperature using the Nernst equation. In these experiments we made sure to continue the cooling until a peak in extracellular $[K^+]$ had been observed (typically 1–2°C below the onset of the K^+ increase). Previous studies of SD events in *Drosophila* have shown that the initial K^+ surge is the largest, and that continued cooling leads to oscillating $[K^+]$ in the extracellular space rather than further increases in $[K^+]$ (Armstrong et al., 2012). Accordingly, the flies were reheated to room temperature shortly after the peak was detected. Representative traces of extracellular $[K^+]$ are shown in Fig. 2.

In a separate set of experiments, all five species and warm- and cold-acclimated *D. melanogaster* were tested for the ability to resist anoxic depolarization: instead of being mounted on a glass coverslide, flies were immobilized in the middle of a custom-made funnel connected to a nitrogen tank with a regulator. After insertion of a single DC electrode in the head and grounding in the abdomen, flies were exposed to pure N_2 from below. The time to anoxic depolarization was estimated as the time to half-amplitude of the drop in DC potential.

Statistics

All statistical analyses were performed in R 3.4.2 software (R Core Team, 2017). Differences between lower thermal limits in the five species and between acclimation groups was analysed using a linear mixed effects model using the lme() function with species or acclimation temperature and the thermal limit (CT_{min} or CCO) as fixed factors, and the individual fly as a random factor. Interspecific differences in CNS shutdown temperature were analysed using a one-way ANOVA, while differences between warm- and cold-acclimated flies were analysed using Student's t-tests. Extracellular $[K^+]$ levels (control and at peak $[K^+]_o$ during the SD events) were analysed between species and between acclimation temperatures using linear mixed effects models with species or acclimation temperature and K^+ level (control or peak) as fixed factors and the fly as a random factor. The effect of species and acclimation on the time to anoxic depolarization was tested using a one-way ANOVA and a Student's t-test, respectively. Correlations were tested using linear regression. All values are presented as means±s.e.m. unless otherwise stated, and the critical level for significance was 0.05 in all analyses.

RESULTS

Temperature causing loss of CNS function

The temperature of CNS shutdown was estimated as the temperature of SD occurrence (Fig. 3). Large differences in CNS shutdown

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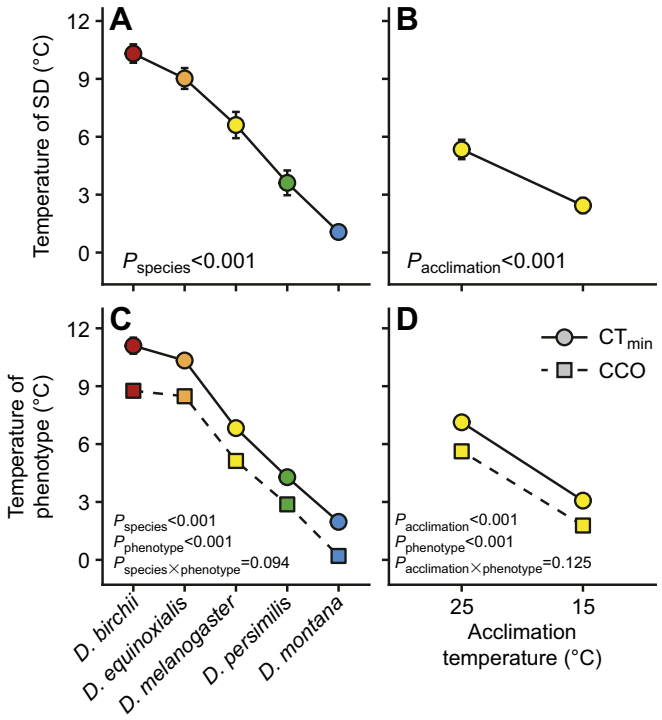

Fig. 3. Lower thermal limits of CNS function and estimates of lower thermal limits for five species of *Drosophila* and cold- and warm-acclimated *D. melanogaster*, respectively. (A,B) Thermal limits of CNS function; (C,D) estimates of thermal limits. Loss of CNS function was noted as the temperature of a SD occurrence, while the lower thermal limits were estimated as the temperature for loss of coordinated movements (CT$_{min}$) and as the temperature of complete neuromuscular paralysis (chill coma onset, CCO), respectively. Error bars not visible are obscured by the symbols. Sample sizes are as follows: (A) *N*=8–10; (B) *N*=10; (C) *N*=6–9; (D) *N*=16–20.

temperature were found between species ($F_{4,42}$=50.4, P<0.001; Fig. 3A) and ranged from 10.3±0.5°C in *D. birchii* to 1.1±0.3°C in *D. montana*. Acclimating *D. melanogaster* to constant 15 or 25°C also elicited marked differences; cold acclimation lowered the temperature of SD occurrence by 2.9°C from 5.3±0.5°C in 25°C-acclimated flies to 2.4±0.4°C in 15°C-acclimated flies (t_{18}=−4.6, P<0.001; Fig. 3B).

Lower thermal limits

When estimating the lower thermal tolerance of the five *Drosophila* species we distinguished between the loss of coordinated movement (CT$_{min}$) and the temperature for complete neuromuscular paralysis (chill coma onset, CCO) (Fig. 3C,D). We found that these two measures differed by approximately 1.8°C ($F_{1,34}$=291.4, P<0.001). The temperature of both these measures also differed considerably between species ($F_{4,34}$=374.0, P<0.001) ranging from 11.1±0.4 and 8.8±0.1°C (CT$_{min}$ and CCO, respectively) in the tropical *D. birchii* down to 2.0±0.1°C (CT$_{min}$) and 0.2±0.1°C (CCO) in the temperate *D. montana* (Fig. 3C).

Cold acclimating *D. melanogaster* lowered both CT$_{min}$ and CCO by ∼4°C compared with the warm-acclimated conspecifics ($F_{1,34}$=2583.0, P<0.001, see Fig. 3D). Thus, warm-acclimated *D. melanogaster* had a CT$_{min}$ of 7.1±0.0°C and a CCO of 5.6±0.1°C while cold-acclimated flies had CT$_{min}$ and CCO temperatures of 3.1±0.1 and 1.8±0.1°C, respectively, with a significant difference of approximately 1.4°C between the two measures ($F_{1,34}$=438.3, P<0.001), which remained the same between acclimation regimes (i.e. no interaction; $F_{1,34}$=2.5, P=0.125).

Extracellular K$^+$ in the drosophilid brain

The cold-induced spreading depolarization observed was always associated with a rapid surge in extracellular [K$^+$] in the CNS. Resting extracellular [K$^+$] in the CNS was around 10 mmol l^{-1} K$^+$ in all five *Drosophila* species and in both cold- and warm-acclimated *D. melanogaster*, and extracellular [K$^+$] increased significantly during the cold-induced SD in both model systems [effect of treatment (resting [K$^+$] vs peak [K$^+$] during the SD); interspecific experiments: $F_{1,22}$=315.1, P<0.001; intraspecific experiments: $F_{1,8}$=134.7, P<0.001]. In the interspecific comparison we found statistical differences between species ($F_{4,22}$=9.6, P<0.001) and a significant interaction with treatment (resting [K$^+$] vs peak SD [K$^+$]; $F_{4,22}$=6.8, P=0.001), illustrating that species differences were associated with the [K$^+$] surge and not differences in resting values (Fig. 4A). Extracellular [K$^+$] in the CNS increased approximately threefold (to 29.5±2.7 mmol l^{-1} K$^+$ and 36.0±3.6 mmol l^{-1} K$^+$) in chill-tolerant species (*D. montana* and *D. persimilis*) but almost fivefold (to 57.9±3.3 mmol l^{-1} K$^+$) in the least tolerant species (*D. birchii*).

Similar patterns were observed with acclimation (Fig. 4B) where the extracellular [K$^+$] tended to differ between acclimation treatments, but this was not significant ($F_{1,8}$=4.5, P=0.097). As with the interspecific model system, this trend was attributable to a tendency for a dampened surge in extracellular [K$^+$] in cold-acclimated flies (44.4±5.7 mmol l^{-1} K$^+$) relative to their warm-acclimated conspecifics (58.3±3.7 mmol l^{-1} K$^+$) (interaction: $F_{1,8}$=4.7, P=0.063).

Resistance to anoxic depolarization

Exposure to complete anoxia was used to estimate the flies' ability to maintain CNS ion balance in the absence of aerobic ATP production. Immersing flies in 100% N_2 gas caused a rapid anoxic depolarization in the CNS of all animals tested (Fig. 5A). The time until anoxia caused SD differed between species ($F_{4,24}$=12.0,

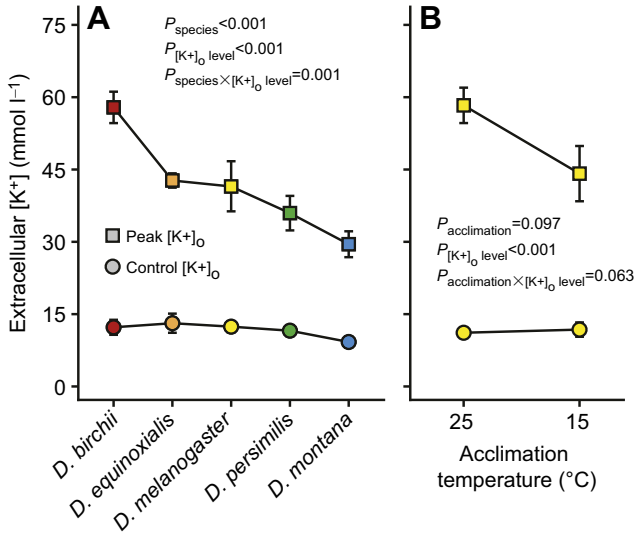

Fig. 4. Extracellular K$^+$ concentration measured in the CNS of five *Drosophila* species with varying cold tolerance and *D. melanogaster* acclimated to high or low temperature. (A) Five *Drosophila* species with varying cold tolerance; (B) acclimated *D. melanogaster*. Both control (circles, measured at room temperature of ∼23°C) and peak SD extracellular [K$^+$] (squares, at the respective SD temperatures) are shown. Error bars not visible are obscured by the symbols. *N*=5–6 per species and acclimation temperature.

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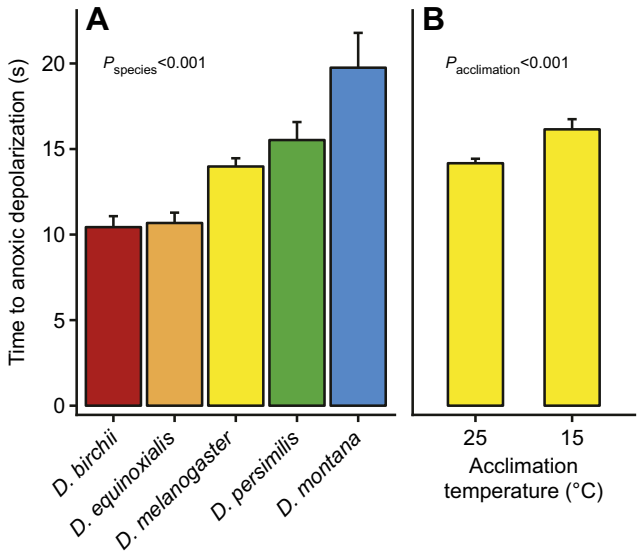

Fig. 5. The time to anoxic depolarization in five species of *Drosophila* and acclimated *D. melanogaster*. (A) Five *Drosophila* species; (B) acclimated *D. melanogaster*. Complete anoxia was induced using N_2 exposure while field potentials were measured. Cold-tolerant flies generally tolerated anoxia for longer durations irrespective of whether differences in cold tolerance were due to (A) adaptation or (B) acclimation. Sample sizes are: (A) 5–6; (B) 13–14.

P<0.001), ranging from approximately 10.5 s in the cold-sensitive *D. birchii* and *D. equinoxialis* to almost twice that (19.8±2.0 s) in the most cold-tolerant species, *D. montana*. A similar pattern was again observed for cold- and warm-acclimated *D. melanogaster* where the CNS of cold-acclimated flies resisted complete anoxia ∼2 s longer (16.2±0.6 s relative to 14.2±0.3 s; t_{25}=3.1, P=0.004; Fig. 5B).

DISCUSSION

Evolutionary differences in lower thermal limits have been demonstrated numerous times for drosophilids (Hoffmann et al., 2002; Kimura, 2004; Overgaard et al., 2011; Kellermann et al., 2012; Andersen et al., 2015c). Such differences can be estimated in several ways including lethal limits or chill coma recovery time [see reviews by Terblanche et al. (2011) and Sinclair et al. (2015)]; however, measures of acute cold tolerance (CT_{min} and CCO) are particularly good predictors for species distribution in drosophilids (Kellermann et al., 2012; Andersen et al., 2015c). In accordance with previous studies we also found considerable variation between species with CT_{min} ranging from 11.1±0.4°C in the tropical *D. birchii* to 2.0±0.1°C in the temperate *D. montana* (with similar variation in CCO). CT_{min} and CCO represent physiological states where the organisms are 'ecologically dead' and this may be the foundation for the strong correlation with distribution limits. Along the same lines, there have been many studies that demonstrate how CT_{min} and CCO vary with acclimation (Lee et al., 1987; Kelty and Lee, 1999; Schou et al., 2017). In *Drosophila* it is typical that a change in acclimation temperature of 1°C elicits an approximate 0.4°C change in CT_{min} and CCO (Overgaard et al., 2011; Sørensen et al., 2016), which is exactly what we observe here for warm- and cold-acclimated *D. melanogaster* (CT_{min} changed from 7.1±0.0 to 3.1±0.1°C and CCO from 5.6±0.1 to 1.8±0.1°C when flies were acclimated to 25 and 15°C, respectively). Again, this strong acclimation response suggests that maintaining behaviour and neuromuscular performance represents an important capacity when flies acclimate to a colder environment. Considering the popularity and utility of these acute cold tolerance traits, it is interesting to note that relatively little is known regarding the physiological mechanisms causing these characteristic phenotypes [but see MacMillan and Sinclair (2011), Andersen et al. (2015a), Overgaard and MacMillan (2017) and Robertson et al. (2017) for discussions].

Central nervous system shutdown is caused by a spreading depolarization

The loss of organismal function during entry into chill coma (initiated by a loss of coordinated movements at CT_{min} resulting in complete neuromuscular paralysis at CCO) must be caused by some malfunction of the neuromuscular system. This could be failure in the CNS, failure of peripheral nerves and synapses, or failure of the excitation–contraction coupling at the muscles. Previous insect studies have focused on the muscular side of this physiological pathway and have found inconclusive evidence. For example, it has been shown that the muscle membrane depolarizes and loses excitability at temperatures close to CT_{min} (Hosler et al., 2000) and that the degree of cold-induced depolarization correlates well with CCO in some species (see Fig. 3 in Andersen et al., 2017a). However, some drosophilids are able to maintain muscle membrane polarization at temperatures below CCO (Andersen et al., 2015a). More recently, using the locust as a model organism, it was proposed that nervous failure caused the loss of coordinated movements at CT_{min} (see Robertson et al., 2017) such that the loss of 'ecological function' was due to CNS shutdown.

Here, we investigated the role of this CNS shutdown in setting the lower thermal limits in *Drosophila* using inter- and intraspecific model systems by measuring extracellular field potentials and extracellular [K^+] in the CNS. We found that CNS shutdown was associated with a spreading depolarization event. Cold-induced shutdown of the CNS has been demonstrated previously in *D. melanogaster* and locusts (Rodgers et al., 2010; Armstrong et al., 2012), and here we present evidence of it occurring in four additional species where CNS shutdown was always caused by an SD event. The SD event occurred over a wide range of temperatures in the five *Drosophila* species and at different temperatures in cold- and warm-acclimated *D. melanogaster*, reflecting their relative cold tolerances. Importantly, the temperature at which we observed SD events matches the temperatures for both CT_{min} and CCO (see Figs 3 and 6). These findings clearly demonstrate that CNS failure is a key mechanism setting the acute lower thermal limit in *Drosophila*. The relationship between onset of chill coma and CNS failure is correlative in nature and does not necessarily imply a causal relationship, as other physiological processes could also cause coma. Nevertheless, we note that this correlation is rather strong and is observed both in relation to cold acclimation and adaptation (see Fig. 6). Furthermore, SD events and coma also coincide if flies are exposed to anoxia, extreme heat or artificial application of ouabain in the CNS (Rodgers et al., 2007). In all cases, this leads to an SD event in the brain and the animal entering a comatose state. Although these observations indicate a central role for the CNS in the onset of cold coma, they do not exclude the possibility that other physiological processes are also failing at similar low temperatures.

The SD event is thought to occur due to an imbalance between active transport and passive movement of ions (particularly K^+) in the CNS. At benign temperatures, there is a continued flux of K^+ from the intracellular compartment to the small extracellular volume in the CNS. This flux is caused by repolarizing currents associated with neural activity, as well as passive leak. Low temperature

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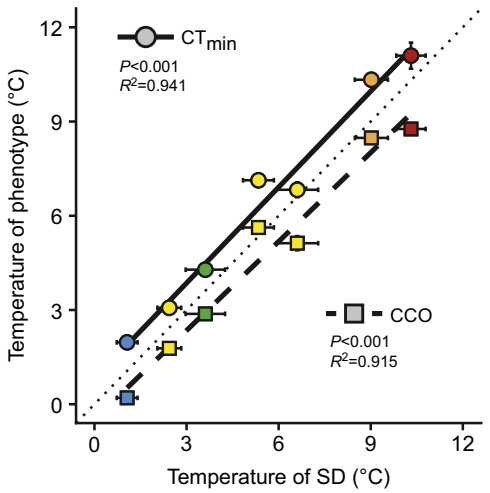

Fig. 6. The relationship between the temperature of loss of CNS function and the two parameters of lower thermal limits. CT_{min}, circles and continuous line; CCO, squares and dashed line; the dotted line represents the line of unity. Colour codes are the same as for Figs 3–5; the two leftmost yellow points represent cold- and warm-acclimated *D. melanogaster*, respectively. Error bars not visible are obscured by the symbols.

reduces the active transport responsible for maintaining K^+ balance, and at a certain point K^+ will start accumulating when the passive leak surpasses active clearance (Rodgers et al., 2010). This rise in extracellular [K^+] depolarizes cell membranes, which initially causes neurons to fire and creates a positive feedback loop where extracellular [K^+] increases rapidly and causes a dramatic depolarization of the nervous cells. This depolarization spreads across the CNS and eventually causes the neurons to silence via slow inactivation of voltage-gated channels, thereby silencing the CNS [see Rodgers et al. (2010) and Armstrong et al. (2009)]. An alternative hypothesis for the rapid K^+ accumulation was recently proposed in which the rapid rise of [K^+] is caused by the transformation of Na^+/K^+-ATPase into a large ion conduction channel at the temperature of SD occurrence (see Brisson et al., 2014) as it was observed that neurons depolarized and stopped firing before the K^+ surge (Robertson et al., 2017). Nevertheless, both hypotheses agree that the proximal cause of SD events is linked to a mismatch between active and passive transport within the CNS and both hypotheses agree that the surge in extracellular [K^+] is the cause of the CNS failure that leads to a loss of organismal function.

Mechanisms of variation in CNS shutdown

As mentioned above, we always observed a rapid increase in extracellular [K^+] when SD events were observed. Interestingly, the peak [K^+] reflected the adaptive and plastic differences in cold tolerance such that more cold-tolerant animals exhibited a lower peak than their cold-sensitive conspecifics or allospecifics (see Figs 2 and 4). This difference could relate to variation in a number of physiological parameters including: (1) increased extracellular volume (i.e. reduces the change in concentration elicited by a given K^+ leakage; see Spong et al., 2015), (2) increased resistance of glial cell membranes (i.e. reduce the leakage of K^+ from the intracellular space), (3) reduced neural activity (i.e. reduced accumulation of K^+ caused by neurons firing; see Spong et al., 2016a), and (4) increased Na^+/K^+-ATPase activity (i.e. increasing the rate of K^+ clearance; see Armstrong et al., 2011). All these processes might therefore also be responsible for the differences observed in SD temperature.

Interestingly, the SD temperature in itself has been shown to alter the peak [K^+] during the SD event such that events occurring at a lower temperature had lower peak [K^+] (Rodríguez and Robertson, 2012). Our findings are, however, in contrast to the findings of Armstrong et al. (2012), who found that cold tolerance acquired through rapid cold hardening tended to increase the peak K^+ concentration whilst simultaneously lowering the temperature threshold for SD initiation. This difference between responses might relate to rapid trafficking of Na^+/K^+-ATPases to the membrane, which has previously been shown in relation to heat hardening (see Hou et al., 2014). A higher abundance of Na^+/K^+-ATPases could lead to the maintenance of CNS ion balance to lower temperature, but a larger perturbation of K^+ balance should they fail. Investigating these putative mechanisms in each species is beyond the scope of this study, but our experiment on resistance to anoxic depolarization allows for some insight into why some species succumb while others are able to maintain function at low temperature. When we exposed flies to anoxia at room temperature and recorded the time to SD, we found that cold-tolerant flies exhibited an increased resistance to anoxic depolarization (see Fig. 5). This indicates either increased buffer capacity (e.g. larger extracellular volume), decreased leakage of K^+ from the intracellular compartments, higher capacity for ATP production in a hypoenergetic state, reduced perturbation of Na^+/K^+-ATPase activity, or more likely a combination of these. Reduced membrane conductance (channel arrest) or nervous activity (spike arrest) are classical adaptations to hypoxia and anoxia in many ectotherms (Hochachka, 1986; Nilsson, 2001; Jackson, 2002) and similar mechanisms are likely to also be adaptive for species exposed to hypothermia (Hochachka, 1986; Boutilier, 2001).

CT_{min} and chill coma relate to distinct physiological events

The terms CT_{min} and CCO have long been used interchangeably in the literature under the common term CT_{min} (see reviews by Hazell and Bale, 2011; MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). However, it was recently proposed that these two measures arise from different physiological mechanisms such that the loss of coordinated movements at CT_{min} was caused by a shutdown of central nervous integration due to an SD event, while CCO was caused by muscular dysfunction such that sporadic, uncoordinated movements are possible below CT_{min} but cease completely at CCO (see Robertson et al., 2017). Despite the strong correlation between the temperature for SD and both acute measures of cold tolerance in our data, it is difficult to discern if the SD relates to the loss of coordination at CT_{min} or complete paralysis at CCO (Fig. 6). This is primarily due to the small temperature difference in our data (~1.5°C difference between CT_{min} and CCO; Fig. 6). However, in *L. migratoria* where the difference is larger (~4°C), the SD temperature closely matched the temperature of CT_{min} (Robertson et al., 2017). It is, of course, also possible that several neuromuscular systems fail at similar temperatures. For example, it was shown that V_m (which is important for muscle excitability) is lost in three of the five species used in this study (see Andersen et al., 2015a) such that *D. persimilis* and *D. montana* were able to maintain polarization. In addition, the ability to maintain V_m correlates strongly with acute cold tolerance across several insect species (see Andersen et al., 2017a), indicating that the muscles of cold-tolerant species have adapted to maintain function during cold exposure. In other words, it is possible that some (cold-tolerant) species succumb to CNS failure, while other (cold-sensitive) species succumb to a simultaneous shutdown of several physiological systems.

Conclusion

We demonstrate a central role of CNS function in relation to acute cold coma in *Drosophila*. We show this using both inter- and intraspecific model systems and demonstrate how CNS function was lost due to an SD event in the brain of all flies tested. The SD event occurred at temperatures reflecting their adaptive or plastic differences in acute cold tolerances and was always associated with a rapid, accelerating surge in extracellular [K^+] in the CNS. Lastly, experiments using anoxic exposure revealed that cold sensitivity of *Drosophila* is associated with an innate ability to resist ionic imbalance in the CNS whilst in a hypo-energetic state. Given that *Drosophila* represents important model organisms in the realms of genetics, physiology and ecology, these findings are of major importance in order to gain an intricate understanding of the physiological mechanisms setting species' thermal niches. Here we demonstrate the primacy of neural events in shaping lower thermal limits, and our findings highlight the importance of organ-specific approaches. Thus, we argue that future studies on variation in acute cold tolerance of *Drosophila* should consider more carefully how the general patterns (transcriptomics, metabolomics, etc.) may relate specifically to organ-specific changes. Such considerations are likely to reveal in more detail how adaptation and phenotypic plasticity allow insects to acquire thermal tolerance and maintain function, while others succumb.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.K.A., R.M.R., J.O.; Methodology: M.K.A., R.M.R., J.O.; Validation: M.K.A., N.J.S.J.; Formal analysis: M.K.A., N.J.S.J.; Investigation: M.K.A., N.J.S.J.; Resources: R.M.R., J.O.; Data curation: M.K.A., N.J.S.J.; Writing - original draft: M.K.A.; Writing - review & editing: M.K.A., N.J.S.J., R.M.R., J.O.; Visualization: M.K.A.; Supervision: M.K.A., R.M.R., J.O.; Project administration: M.K.A., N.J.S.J., R.M.R., J.O.; Funding acquisition: M.K.A., R.M.R., J.O.

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