

Muscle membrane potential and insect chill coma

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Summary statement

Cold tolerant *Drosophila* species defend their muscle resting membrane potential at low temperatures and may enter chill coma as a result of different physiological mechanisms than less tolerant species.

Abstract

Chill susceptible insects enter a reversible paralytic state, termed chill coma, at mild low temperatures. Chill coma is caused by neuromuscular impairment, allegedly triggered by cold-induced depolarization of muscle resting membrane potential (V_m). We used five *Drosophila* species that vary in cold tolerance (chill coma temperature spanning approx. 11°C) and repeatedly measured muscle V_m during a downward temperature ramp (20 to -3°C). Cold tolerant species were able to defend their V_m down to lower temperatures. An ability not explained by species specific differences in initial V_m at 20°C, but by cold tolerant drosophilids defending V_m across a broad range of temperatures. We found support for a previously suggested “critical threshold” of V_m related to chill coma, in three of the five species, interestingly, the cold tolerant *Drosophila* species may enter coma due to processes unrelated to muscle depolarization as their V_m was not significantly depolarized at their chill coma temperatures.

Keywords

chill tolerance, critical thermal minimum, cold exposure, comparative, inter-species

Abbreviations

CCRT	Chill coma recovery time
CNS	Central nervous system
CT _{min}	Critical thermal minimum
V_m	Resting membrane potential
$[K^+]_{ext}$	Extracellular K^+ concentration

Introduction

Many insects succumb to the effects of cold at temperatures considerably higher than those causing them to freeze (Hosler et al., 2000; Košťál et al., 2006; MacMillan and Sinclair, 2011a; Andersen et al., 2013; Andersen et al., 2015). Many insects enter a reversible paralytic state at low temperature, termed chill coma, which is caused by neuromuscular impairment. Insects in chill coma may further develop chill injury and ultimately die if the cold exposure is severe (Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair, 2011a). The onset temperature of chill coma (the critical thermal minimum, CT_{min}) and chill coma recovery time upon rewarming (CCRT) are both metrics that correlate closely to interspecific variance in insect distribution, and are widely used to assess insect cold tolerance (Hori and Kimura, 1998; MacMillan and Sinclair, 2011b; Andersen et al., 2015).

The physiological mechanisms of chill coma remain largely unresolved. However, cold-induced neuromuscular impairment has been associated with chill coma and linked to a decrease of excitability due to a depolarization of either the central nervous system (CNS) and/or the muscle tissue (Wareham et al., 1974; Hosler et al., 2000; Rodgers et al., 2010; MacMillan et al., 2014). Low temperatures induce muscle depolarization in several chill sensitive insects, including the hawk moth (Wareham et al., 1975), American cockroach (Wareham et al., 1974), vinegar fly, honey bee (Hosler et al., 2000) and migratory locust (MacMillan et al., 2014). *In vitro*, such depolarization can directly decrease muscle force production, even at high temperatures (Findsen et al., 2014), possibly due to a decrease in voltage sensitive Ca^{2+} -channel currents (Salkoff and Wyman, 1983). Accordingly, it has been suggested that cold-induced depolarization directly causes chill coma (Hosler et al., 2000; Findsen et al., 2014; MacMillan et al., 2014). Chill coma onset has been repeatedly found to coincide with a “critical threshold” muscle V_m , between -35 to -45 mV (Wareham et al., 1974; Hosler et al., 2000; MacMillan et al., 2014). Similarly, there seems to be a tight association between the recovery from chill coma and the recovery of membrane potential when the insect is returned to normal temperatures after cold exposure. Prolonged cold exposure causes increased $[K^+]_{ext}$, which depolarizes muscle cells, and insects that lose K^+ balance during cold exposure only recover from chill coma when K^+ balance is restored (MacMillan et al., 2012; Andersen et al., 2013; Findsen et al., 2013).

Here, we test three hypotheses regarding chill coma onset and muscle membrane potential: 1) More cold tolerant species defend V_m during cold exposure, either by having a more polarized baseline (initial) V_m or by an improved ability to maintain V_m when exposed to low temperatures. 2) Chill coma onset occurs at a critical depolarization of muscle V_m in insects. 3) Cold sensitive species lose K^+ balance during a cold exposure, which leaves the muscle membranes in a depolarized state after rewarming such that chill coma recovery is slowed. To test these hypotheses, we used five chill sensitive *Drosophila* species with markedly different cold tolerance; *D. birchii* (Dobzhansky & Mather, 1961), *D. equinoxialis* (Dobzhansky, 1946), *D. melanogaster* (Meigen, 1830), *D. persimilis* (Dobzhansky and Epling, 1944) and *D. montana*

(Patterson and Wheeler, 1942). For all species, we repeatedly measured muscle V_m while exposing them to a temperature ramp from the rearing temperature (20°C) to -3°C and following return to 20°C.

Results & Discussion

The five species of *Drosophila* examined varied widely in cold tolerance (Andersen et al. 2015), and their chill coma onset temperatures (CT_{min} s) were significantly different ($H = 93.38$; $P < 0.001$, post-hoc tests; $P < 0.001$, in all cases) and ranged almost 11°C between the least and most cold tolerant (from 8.8 ± 0.2 to -2.0 ± 0.1 °C; Fig. 1 and Table S1). As hypothesized, we found that the more cold tolerant species were better able to defend their V_m at lower temperatures. The rank-order of significant depolarization was similar to the rank-order of cold sensitivity (Fig. 1); a significant depolarization occurred at 10°C for *D. birchii*, followed by *D. equinoxialis* (5°C), *D. melanogaster* (0°C), *D. persimilis* (-3°C) and *D. montana* (which did not significantly depolarize, even at -3°C). The variation in CT_{min} among species was not explained by a higher polarization of muscle V_m at 20°C. If chill coma occurs when the membrane potential passes a certain critical threshold, then possessing a more polarized “baseline” V_m would increase the change in V_m necessary to pass the threshold. Although the baseline V_m (at 20°C) was most polarized for the most cold tolerant species (-67.4 ± 2.9 mV, *D. montana*), the second most cold tolerant species had the least polarized V_m of all the species at 20°C (-55.5 ± 2.5 mV, *D. persimilis*). Accordingly, there was no significant tendency for the baseline V_m to decrease with decreasing CT_{min} among species ($r = 0.593$, $P = 0.292$, Fig. 2A). Though, there seems to be a tendency when ignoring *D. persimilis*, a more polarized baseline V_m is clearly not a general strategy among all cold tolerant drosophilids and an alternative strategy may therefore be to defend V_m across a broad range of temperatures, as *D. persimilis* does. These two strategies are not mutually exclusive, and may contribute differently across the genus where cold tolerance has evolved several times (Kellermann et al., 2012; MacMillan et al., 2015).

Earlier observations have indicated a “critical threshold” V_m of -35 to -45 mV associated with onset of chill coma in insects (Wareham et al., 1974; Hosler et al., 2000; MacMillan et al., 2014). In the present study we found support for such a critical threshold in three of the five species; *D. birchii*, *D. equinoxialis* and *D. melanogaster* did depolarize to around -40 to -50 mV at their CT_{min} . These species may therefore experience a decrease or absence of action potentials as a result of the depolarization (Wareham et al., 1974; Salkoff and Wyman, 1983; Hosler et al., 2000; Findsen et al., 2014). By contrast, *D. persimilis* and *D. montana* suffered no significant depolarization at their CT_{min} . Thus, cold adapted *Drosophila* species may enter coma as a result of processes unrelated to depolarization of muscle V_m . For these species, coma could result from a direct effect of temperature (not related to depolarization) on voltage sensitive Ca^{2+} -channels, hindering propagation of action potentials (Frolov and Singh, 2013) or to a failure of CNS conduction (Rodgers et al., 2010).

The cold-induced depolarization of muscle tissue in insects can be caused by a number of factors, including reduced activity of electrogenic pumps, direct temperature effects on membrane permeability and conductance as well as increases in $[K^+]_{\text{ext}}$ following cold exposure (Košťál et al., 2004; MacMillan and Sinclair, 2011a; Andersen et al., 2013; Finsen et al., 2013; MacMillan et al., 2014). The prevailing data suggest that this occurs during a two-step process where a direct temperature related depolarization is followed by a further gradual depolarization caused by perturbation of ion and water balance (MacMillan et al., 2014). In the present study we examined the contribution of ionic perturbation to V_m by measuring membrane potential after the temperature ramp, when the flies were returned to 20°C. Reverting the temperature dependent depolarization meant that any remaining difference (ΔV_m) between the baseline V_m at 20°C and the measurement after cold exposure (also 20°C) is likely caused by an increased $[K^+]_{\text{ext}}$. We related ΔV_m to the CCRT, since this is considered to be a good measure of an animal's ability to recover from increased $[K^+]_{\text{ext}}$ caused by cold exposure (MacMillan et al., 2012; Andersen et al., 2015). Although not significant, we noted that *D. persimilis* and *D. montana* were very similar or slightly hyperpolarized when returned to 20°C while the remaining (less cold tolerant) species tended to be slightly depolarized (Fig. 2B). This depolarization was only significant in *D. birchii* ($t = -3.74$, $df_{19.52}$, $P = 0.001$), and there was no overall relationship between CCRT and ΔV_m among species ($r=0.207$, $P=0.679$, Fig. 2C). It is possible that the association between ΔV_m and CCRT is obscured by the experimental design, where V_m during recovery was measured 13 minutes after the return to 20°C. All species had recovered from chill coma at this time (Fig. 2C), and any K^+ dependent depolarization (which we infer from ΔV_m) may have been partially recovered.

In conclusion, more cold tolerant *Drosophila* species are able to maintain muscle V_m at lower temperatures than their cold sensitive congeners. Also, cold tolerant species may have evolved the ability to circumvent the “critical threshold” of muscle depolarization related to chill coma in the chill sensitive *Drosophila* (and other insect species). Some chill sensitive species experience an increased $[K^+]_{\text{ext}}$ during cold exposure that augments the cold-induced depolarization, whereas the cold tolerant flies avoid this.

Materials and methods

Experimental animals

Five *Drosophila* species were provided from laboratory cultures by: Professor Anneli Hoikkala, University of Jyväskylä, Finland (*D. montana*); Professor Volker Loeschcke, Aarhus University, Denmark (*D. melanogaster*); the *Drosophila* Species Stock Center, San Diego, USA (*D. equinoxialis* and *D. persimilis*) and Professor Ary Hoffmann, University of Melbourne, Australia (*D. birchii*) (Table 1). Experimental flies were raised as described in Andersen et al. (in press). All experimental flies were 6-9 day old non-virgin female flies, raised under low density conditions at $20 \pm 1^\circ\text{C}$.

Measurements of chill coma onset and recovery

To associate the species' physiological differences to their cold tolerance phenotype we used two measures associated with chill coma, as both CCRT and CT_{min} strongly correlate to lethal temperature in these species (Andersen et al. 2015). In a parallel study, conducted on the same populations, we measured the critical thermal minimum (CT_{min}) (Andersen et al., in press). Briefly, CT_{min} was scored by submerging individual flies in 5 ml vials (N=20 per species) in an ethylene-glycol and water solution (1:2) and progressively cooling at a rate of $0.2^{\circ}\text{C min}^{-1}$ from 20°C . Once spontaneous movement ceased, flies were motivated to move by tapping the vial, and the CT_{min} was recorded when all capacity for movement stopped. Another measure of cold tolerance, chill coma recovery time (CCRT), was assessed from the time it took the flies recover from chill coma and regain standing position following a similar temperature ramp. Flies (N=10 per species) were placed individually in 5 ml sealed containers and submerged in an cooling bath for the duration of the temperature ramp (20 to -3.5°C at $0.2^{\circ}\text{C min}^{-1}$), after which they were quickly returned to room temperature to allow for visual recording of CCRT, while tapping the vials every 30 s to motivate standing as fast as physiologically possible.

Resting membrane potential

Single flies were placed at 0°C for 25 seconds, to induce a brief paralysis allowing us to place the flies directly on a custom-built glass plate connected to a programmable cooling bath preset at 20°C . For each experimental round, 16 flies were mounted and immobilized on the glass plate using a thin layer of sports resin. To obtain muscle resting membrane potential (V_m) a reference electrode ($\text{Ø} = 0.05 \text{ mm}$, 99.99% hard platinum, Pg Metal Shop, London, United Kingdom) was placed in the hemolymph by puncturing the carapace directly. Immediately before measuring, a small window was cut in the dorsolateral anterior half of the thorax with the tip of a $0.45 \times 12 \text{ mm}$ syringe needle, and a glass micro electrode (Clark borosilicate glass microelectrodes, GC100TF; Warner Instruments, Hamden, CT, USA) was then inserted into the flight muscle (Fig. S1), both electrodes were mounted on micro manipulators (World Precision Instruments Inc., Berlin, Germany). The glass electrode was pulled to a tip resistance of 5-10 $\text{M}\Omega$ using a Flaming-Brown P-97 electrode puller (Sutter Instruments Co., Novato, CA, USA) and both the reference and glass electrode were connected to an Electro 705 differential electrometer (World Precision Instruments Inc., Sarasota, FL, USA) such that data could be obtained through a 1401 Micro3 data acquisition system connected to a PC running Spike2 (v8, Cambridge Electronic Design, Cambridge, UK). The glass microelectrode was gently moved inward at a $25\text{-}40^{\circ}$ angle aiming at the dorsolateral flight muscles. A flight muscle fiber was assumed to be penetrated once an instant drop in the measured potential was registered on the screen. Each fly was used to assess V_m at one temperature and for each fly a minimum of 3 "repeatable" (all within a 10mV span) membrane potentials were collected in succession (assumed to be from the same muscle fiber). After a minimum of 3 repeatable measurements the electrode was moved deeper into the flight muscle and the

practice was repeated on a different muscle fiber if possible (*i.e.* we obtained V_m from one or two muscle fibers per animal). In cases where we succeeded in measuring two fibers we used the most negative V_m from the animal. In cases where we were unable to obtain repeatable measurements, the fly was rejected from the dataset. After measuring V_m at 20°C (baseline) the temperature ramp was initiated ($-0.2^\circ\text{C min}^{-1}$), and V_m was measured in two new flies for every $5\pm 0.5^\circ\text{C}$ down to 0°C, and at $-3\pm 0.5^\circ\text{C}$. After the measurement at -3°C the temperature bath was quickly reset to 20°C and 15 min later (after 13 minutes at 20°C) two more flies were measured (20°C return). We were unable to decrease temperatures further to -3.5°C , since this exposed the experimental setup to the risk of ice formation. The ΔV_m was calculated for each species as the difference between the mean of the baseline V_m (at 20°C) minus the mean of the return V_m (also at 20°C). We collected V_m from between 8 and 14 animals per temperature per species (5 species x 7 temperatures, resulting in 35 species-temperature combinations).

Statistics and Analyses

Data was deemed normally distributed by investigating the boxplots with superimposed data points for the different experimental groups. This was generally confirmed as Shapiro-Wilks tests only rejected normality in 4 of the 35 groups. Differences in V_m were investigated using a generalized linear model, examining the effect of temperature on V_m for each species individually. To test for differences in critical thermal minimum (CT_{\min}) among species (data previously published in Andersen et. al. (in press)) we used a non-parametric Kruskal-Wallis test (H test) and a Dunn's multiple post hoc comparison test. Initial (baseline) and return to 20°C V_m values were analyzed by Welch two-sample unpaired t-tests. The regression between the baseline V_m and CT_{\min} as well as the relationship between CCRT and ΔV_m were conducted using a linear regression. All statistics were done in R 3.1.2 (R Core Team, 2014) and values reported as means \pm s.e.m. unless otherwise stated.

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Competing interests

None of the authors of this manuscript have any competing interests to declare.

Author contributions

All authors designed the experiments, J.L.A. performed the experiments and analyzed the data, and all authors wrote the manuscript.

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Figures

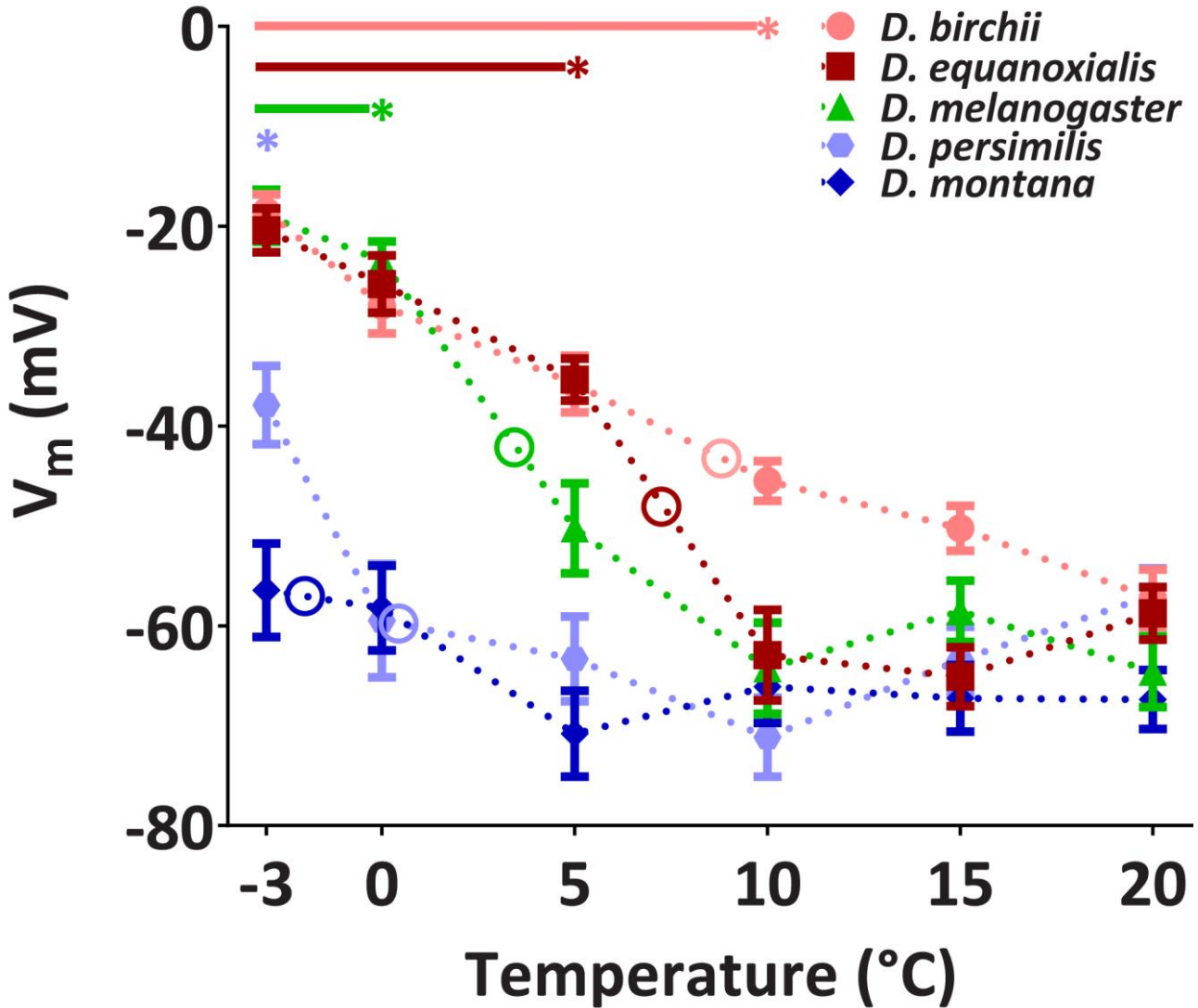


Figure 1. The resting membrane potential (V_m) of five species of *Drosophila* collected during cold-exposure. Flight muscle resting membrane potential collected for every 5°C from 20 to 0°C and at -3°C during a 0.2°C min⁻¹ downwards temperature ramp. Open circles indicate the species' mean critical thermal minimum (CT_{min}) collected during a similar temperature ramp using different animals. The asterisk and solid lines aligned with the legend cover the temperatures where the V_m is statistically different from the baseline values (20°C) for each species. Data is presented as means \pm s.e.m.

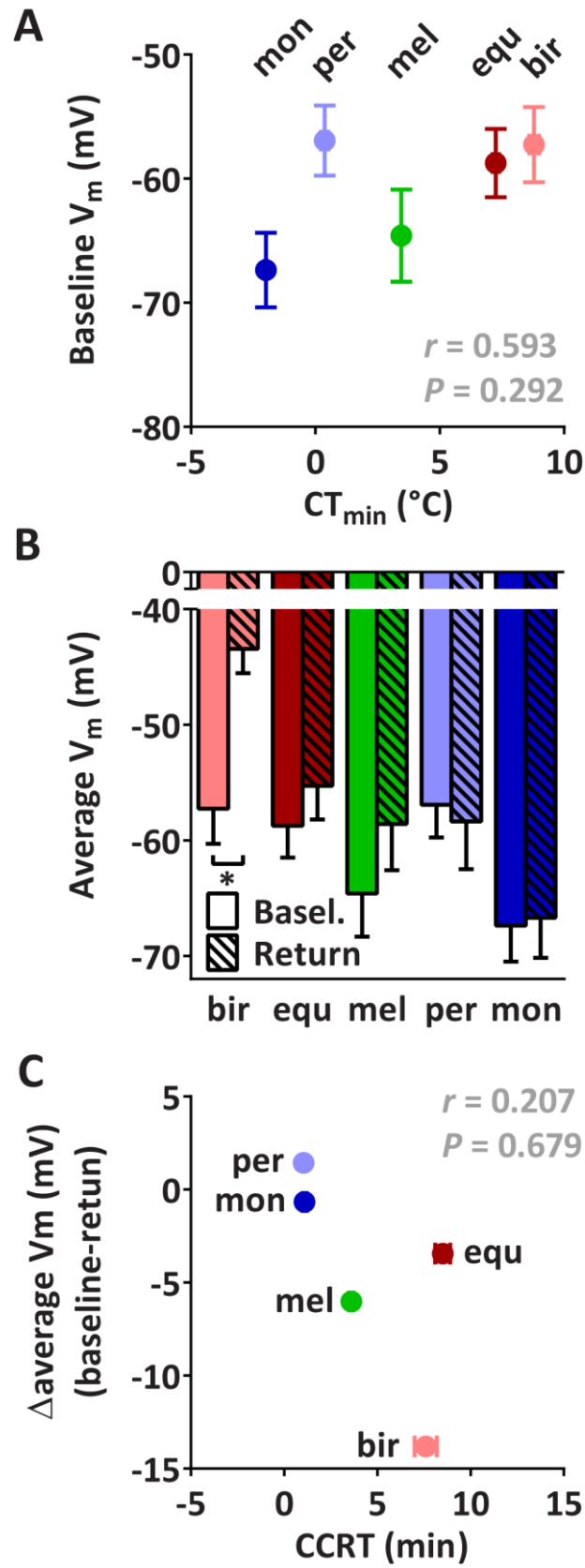


Figure 2. Initial muscle membrane potential (V_m) at 20°C and after returning to 20°C following a cold exposure in five species of *Drosophila*. **A)** Average baseline resting membrane potential (V_m) plotted against CT_{min} of the five species of *Drosophila*. **B)** Baseline (Basel.) and return 20°C resting membrane potentials (V_m), asterisks denote statistical differences within species, **C)** The relationship between Δ average V_m and chill coma recovery time (CCRT), ΔV_m is calculated as baseline mean V_m minus return mean V_m . The chill coma recovery time was collected after a 0.2°C min⁻¹ ramp from 20 to -3.5°C (N=10 for all species). Data is represented as means \pm s.e.m. (if possible) and species name abbreviations is shown in association with the data points/columns.

Tables

Table 1. Details of the five *Drosophila* species used for experiments. All species were kept in a $20\pm 1^\circ\text{C}$ room for at least a year before experiments. Table contains; Species name (abbreviation), country of origin, year of collection, species distribution profile, critical thermal minimum (CT_{min} , means \pm s.e.m.), the chill coma recovery time (CCRT, means \pm s.e.m.) along with the source where the flies have been kept since collection, before being brought to our lab (see methods).

Species (abbreviation)	Origin	Collection year	Distribution profile	CT_{min} ($^\circ\text{C}$)	CRRT (min)	Source laboratory
<i>D. birchii</i> (bir)	Australia	2008	Tropical	8.79 ± 0.19	62.5 ± 4.6	Hoffmann, AU
<i>D. equinoxialis</i> (equ)	Honduras	<1984	Tropical	7.25 ± 0.13	42.3 ± 2.0	DSSC, US
<i>D. melanogaster</i> (mel)	Denmark	2011	Widespread	3.46 ± 0.07	15.7 ± 0.9	Loeschcke, DK
<i>D. persimilis</i> (per)	Canada	Unknown	Temperate	0.38 ± 0.14	1.1 ± 0.2	DSSC, US
<i>D. montana</i> (mon)	Finland	2008	Temperate	-1.99 ± 0.05	1.8 ± 0.4	Hoikkala, FI

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<i>D. melanogaster</i> (mel)	Denmark	2011	Widespread	3.46 ± 0.07	15.7 ± 0.9	Loeschcke, DK
<i>D. persimilis</i> (per)	Canada	Unknown	Temperate	0.38 ± 0.14	1.1 ± 0.2	DSSC, US
<i>D. montana</i> (mon)	Finland	2008	Temperate	-1.99 ± 0.05	1.8 ± 0.4	Hoikkala, FI

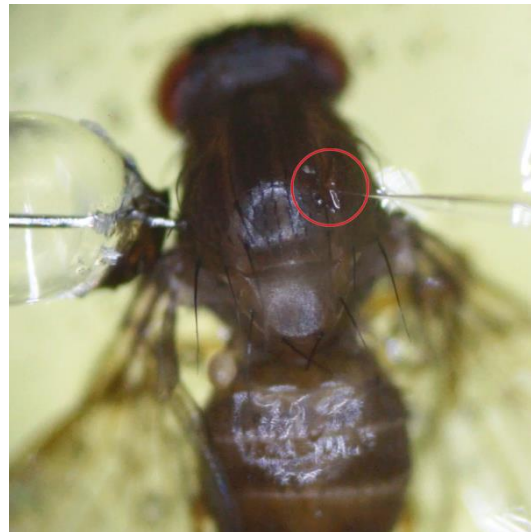


Fig. S1. Photo showing the placement of the electrodes. The reference electrode were inserted directly through the carapace (left) and glass electrode inserted into a flight muscle through a small window in the carapace cut with the tip of a syringe-needle (circled).