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RESEARCH ARTICLE

Cold tolerance of *Drosophila* species is tightly linked to the epithelial K^+ transport capacity of the Malpighian tubules and rectal pads

Mads Kuhlmann Andersen[1,‡], Heath A. MacMillan[2,*], Andrew Donini[2] and Johannes Overgaard[1]

ABSTRACT

Insect chill tolerance is strongly associated with the ability to maintain ion and water homeostasis during cold exposure. Maintenance of K^+ balance is particularly important due to its role in setting the cell membrane potential that is involved in many aspects of cellular function and viability. In most insects, K^+ balance is maintained through secretion at the Malpighian tubules, which balances reabsorption from the hindgut and passive leak arising from the gut lumen. Here, we used the scanning ion-selective electrode technique (SIET) at benign (23°C) and low (6°C) temperatures to examine K^+ flux across the Malpighian tubules and the rectal pads in the hindgut in five *Drosophila* species that differ in cold tolerance. We found that chill-tolerant species were better at maintaining K^+ secretion and suppressing reabsorption during cold exposure. In contrast, chill-susceptible species exhibited large reductions in secretion with no change, or a paradoxical increase, in K^+ reabsorption. Using an assay to measure paracellular leak, we found that chill-susceptible species experience a large increase in leak during cold exposure, which could explain the apparent increase in K^+ reabsorption found in these species. Our data therefore strongly support the hypothesis that cold-tolerant *Drosophila* species are better at maintaining K^+ homeostasis through an increased ability to maintain K^+ secretion rates and through reduced movement of K^+ towards the hemolymph. These adaptations are manifested both at the Malpighian tubule and at the rectal pads in the hindgut, and ensure that cold-tolerant species experience less perturbation of K^+ homeostasis during cold stress.

KEY WORDS: Chill tolerance, Ion regulation, Hindgut, SIET, Paracellular leak

INTRODUCTION

The physiological performance of insects is strongly influenced by temperature and it is therefore not surprising that insect cold tolerance, including that of drosophilids, is found to correlate strongly with geographical distribution (Addo-Bediako et al., 2000; Andersen et al., 2015c; Kellermann et al., 2012; Kimura, 2004; Sunday et al., 2011). Most insect species are chill susceptible, meaning they suffer from negative effects of low temperatures above the spontaneous freezing point of their body fluids (Bale, 2002; Overgaard and MacMillan, 2017; Sinclair et al., 2015). A major physiological challenge for these species is their capacity to maintain ion and water homeostasis during low-temperature exposure (Andersen et al., 2017a; Koštál et al., 2004, 2006; MacMillan et al., 2015a; MacMillan and Sinclair, 2011a,b; Overgaard and MacMillan, 2017; Zachariassen et al., 2004), and particularly loss of K^+ balance has been shown to cause membrane depolarization and cell death (Andersen et al., 2017b; MacMillan et al., 2015c; Overgaard and MacMillan, 2017). Observations from Drosophilidae are also consistent with this: *Drosophila melanogaster* that are acclimated to low temperature in the laboratory or *D. subobscura* that are acclimatized to cold-field conditions have an improved capacity to maintain water and ion balance when chilled (MacMillan et al., 2015b, 2016). Similarly, cold-adapted *Drosophila* species from temperate regions have higher capacity for ion balance regulation in the cold than do their sister species from tropical regions (MacMillan et al., 2015a).

The major organs responsible for the maintenance of extracellular ion and water balance in insects are the Malpighian tubules and hindgut. Through active transport mechanisms, these organs form a circuit of secretion (Malpighian tubules) and reabsorption (hindgut) of ions and water that constantly provide balance against the passive para- and/or transcellular ion movement (Beyenbach and Piermarini, 2011; Phillips, 1981, 1970). At the Malpighian tubules, secretion of ions and water is driven by apical V-type H^+ ATPase activity, creating a favorable gradient for K^+/H^+ and Na^+/H^+ exchange (Beyenbach et al., 2010). In *Drosophila*, this transport is also dependent on basolateral Na^+/K^+ ATPases for moving Na^+, K^+ and Cl^- into the cell (see Ianowski and O'Donnell, 2004, O'Donnell et al., 2003 and Ramsay, 1954). When combined, this active ion transport establishes an osmotic gradient that allows water to enter the tubule lumen from the hemolymph, aided by aquaporins expressed by principle and stellate cells (Kaufmann et al., 2005; Misyura et al., 2017; Spring et al., 2009; Tsujimoto et al., 2013). Some K^+ and water is reabsorbed in the lower segment of the Malpighian tubules, before passing into the hindgut through the ureter (O'Donnell and Maddrell, 1995; Rheault and O'Donnell, 2001). The rectal pads in the hindgut are the primary site for reabsorption of ions and water. Here, K^+ reabsorption is coupled to apical reabsorption of Cl^- and secretion of H^+, whereas Na^+ reabsorption is tied to apical Na^+/H^+ exchangers and basolateral Na^+/K^+ ATPases (Phillips, 1981; Phillips et al., 1987, 1996). Lowered temperature presumably challenges ion balance because it slows active ion transport. At a sufficiently low temperature, active transport can no longer compensate for the passive leak of ions and water (MacMillan et al., 2015b; MacMillan and Sinclair, 2011b; Overgaard and MacMillan, 2017; Zachariassen et al., 2004). In many insects, this is manifested in a net leak of Na^+ and water from the hemolymph compartment to the gut (and possibly other tissues),

[1]Zoophysiology, Department of Bioscience, Aarhus University, Aarhus 8000, Denmark. [2]Department of Biology, York University, Toronto M3J 1P3, Ontario, Canada.
*Present address: Department of Biology, Carleton University, Ottawa K1S 5B6, Ontario, Canada.

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

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

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List of symbols and abbreviations

C_B	background concentration
CCO	chill coma onset temperature
FITC	fluorescein isothiocyanate
LTe_{50}	temperature causing 50% mortality
S	electrode slope from a 10-fold difference in ion concentration
SIET	scanning ion-selective electrode technique
ΔC	concentration gradient
ΔV	voltage gradient
Δx	distance of electrode movement in the SIET system

causing an elevated concentration of K^+ in the reduced hemolymph volume (MacMillan et al., 2015b; MacMillan and Sinclair, 2011b). However, within *Drosophila*, MacMillan et al. (2015a) showed how hemolymph [K^+] in chill-susceptible species increased more than what would be expected from the decrease in hemolymph volume, clearly demonstrating the importance of passive leak of K^+ from other compartments into the hemolymph.

Given the effects of chilling described above, it may be expected that the ionoregulatory organs of chill-tolerant species have a superior capacity to balance passive leak with active transport at low temperatures. Previous studies have investigated this paradigm by looking at the effect of cold in isolated organs: Maddrell (1964) found that the cold lowered the secretion of ions more than the secretion of fluid at the Malpighian tubules of the blood-sucking *Rhodnius prolixus*; Houlihan and Sell (1984) showed that low temperature reduces reabsorption of Na^+ and K^+ in the locust (*Locusta migratoria*) rectum and that, in particular, Na^+ becomes energetically expensive to reabsorb; Yi and Lee (2005) found that winter acclimatization reduced fluid secretion rates at the Malpighian tubules of *Eurosta solidaginis*; and, more recently, MacMillan et al. (2015a) demonstrated how low temperature impairs ion (and water) secretion of entire Malpighian tubules in five species of *Drosophila* with widely different cold tolerances. Importantly, the last study also found that cold-sensitive drosophilids secreted relatively more Na^+ at low temperature, whereas cold-adapted species retained their preference for K^+ secretion, which would help mitigate cold-induced hyperkalemia. The putative importance of renal secretion in insect cold tolerance was also emphasized by the recent studies of Terhzaz et al. (2015, 2017), who demonstrated that diuretic neuropeptides (which act on the Malpighian tubules) had major impacts on the cold tolerance of *D. melanogaster*.

In the present study, we used an epithelial leak assay and a SIET system (scanning ion-selective electrode technique) to investigate the effects of temperature on epithelial K^+ transport at the Malpighian tubule and rectal pads in five *Drosophila* species that originate from different climatic regions. Using the SIET system, we measured the site-specific capacity for K^+ secretion/reabsorption in different organs of the same individual and thereby pinpoint whether differences in the capacity to maintain low hemolymph [K^+] are due to differences in ion transport of specific organs. We predicted that the cold would affect K^+ transport in such a manner that hemolymph [K^+] would increase in chill-susceptible species (as has been previously observed), while their chill-tolerant allospecifics would have superior capacity to maintain hemolymph K^+ homeostasis. Specifically, we predicted that K^+ secretion by Malpighian tubules of chill-susceptible species will be suppressed more than that of chill-tolerant species by low temperature, and K^+ reabsorption in lower segments of Malpighian tubules and rectal pads of chill-tolerant species will be suppressed more in chill-tolerant species. To test these hypotheses, we measured epithelial capacity for K^+ transport of the main and lower segments of the Malpighian tubules and of the rectal pads. These measurements were performed at benign and low temperature to assess initial differences between species, as well as their response to chilling. Paradoxically, these measurements revealed an increase in K^+ reabsorption at low temperature in the chill-sensitive species, which might suggest leakage. Thus, we assayed the passive movement of a paracellular marker from the gut to the hemolymph (which is indicative of leak) by feeding flies fluorescent FITC-dextran and measuring FITC fluorescence in the hemolymph of control and cold-exposed flies. Accordingly, we hypothesized that the guts of chill-susceptible species would leak gut contents into the hemolymph during cold exposure, whereas chill-tolerant species would be able to prevent leak. Last, we measured the size of the Malpighian tubules and hindgut to investigate whether any putative differences in ion transport capacity were associated with differences in organ size under the assumption that proportionally larger ionoregulatory organs provide higher capacity for transport.

MATERIALS AND METHODS

Model system and animal husbandry

The five drosophilid species used in the present study (*Drosophila montana*, *D. persimilis*, *D. melanogaster*, *D. equinoxialis* and *D. birchii*; see Table 1) are known to possess marked differences in cold tolerance (Andersen et al., 2015a,b,c; MacMillan et al., 2015a; Olsson et al., 2016). Flies were maintained under 'common garden'

Table 1. Origin and description of the five *Drosophila* populations used in the present study

Species	Origin	Collection date	Distribution	CCO (°C)	LTe_{50} (°C)*
D. birchii	Australia	2008	Tropical	7.4±0.3[a]	−3.3±0.2
D. equinoxialis	Honduras	<1984	Tropical	7.8±0.3[a]	−4.6±0.3
D. melanogaster	Denmark	2011	Cosmopolitan	3.1±0.1[b]	−8.1±0.2
D. persimilis	Canada	Unknown	Temperate	0.5±0.2[c]	−12.1±0.5
D. montana	Finland	2008	Temperate	−1.3±0.1[d]	−13.2±0.3

*The values measured for chill coma onset temperature (CCO) in the present study were reminiscent of previously obtained values (Andersen et al., 2015c) and, thus, the LTe_{50} values (the temperature causing 50% mortality after a 1 h exposure) estimated by Andersen et al. (2015c) were also assumed to be maintained. Species sharing superscript letters do not have statistical differences in CCO (Tukey's honest significant differences *post hoc* analysis).
All species were kept under 'common garden' conditions of 21–22°C and a 12 h:12 h diurnal cycle through all developmental stages. *Drosophila* stocks were provided from laboratory cultures by Prof. Ary Hoffmann (University of Melbourne, Australia; *D. birchii*); the *Drosophila* Species Stock Center (San Diego, CA, USA; *D. equinoxialis* and *D. persimilis*); Prof. Volker Loeschcke (Aarhus University, Denmark; *D. melanogaster*); and Prof. Anneli Hoikkala (University of Jyväskylä, Finland; *D. montana*).
LTe_{50} values were used for correlations as this measure is directly linked to the ability to maintain ion balance (specifically hemolymph [K^+]) during cold stress, whereas CCO is not (Overgaard and MacMillan, 2017).

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conditions at 21-22°C with a 12 h:12 h light:dark cycle and, under these conditions, all five lab populations reproduced continuously. Parental flies were kept in bottles and fed on oat-based Leeds medium (for 1 l of water: 60 g yeast, 40 g sucrose, 30 g oat meal, 16 g agar, 12 g methyl paraben and 1.2 ml acetic acid) and were allowed to oviposit for between 2 h and 2 days (depending on species) before being moved to separate bottles. This allowed us to maintain rearing densities at ∼100 individuals per bottle. Emerging flies were transferred to 50 ml vials containing 10–15 ml of the same medium, and were left to mature for 6–10 days before being used in experiments (only females were used, and were considered non-virgin).

The relative cold tolerance of these five species has been measured multiple times, but was reconfirmed by measuring the temperature of chill coma onset (i.e. complete cessation of movement). This was carried out by placing individual flies in sealed 4 ml glass vials that were submerged in a 1:1 (v/v) mixture of water and ethylene glycol the temperature of which was lowered by 0.2°C min^{-1}. The glass vials were gently tapped at regular intervals to test for the ability to move (Sinclair et al., 2015) and when no movement was observed this was noted as the chill coma onset temperature (CCO).

Fly preparation

On the day of the experiment, mature females were briefly submerged in 70% ethanol for sedation and were quickly moved to a small glass dish containing *Drosophila* saline at 23°C [137 mmol l^{-1} Na^+, 15 mmol l^{-1} K^+, 158.5 mmol l^{-1} Cl^-, 8.5 mmol l^{-1} Mg^{2+}, 2 mmol l^{-1} Ca^{2+}, 10.2 mmol l^{-1} HCO_3^-, 4.3 mmol l^{-1} H_2PO_4, 20 mmol l^{-1} glucose, 10 mmol l^{-1} glutamine and 15 mmol l^{-1} MOPS buffer (pH 7.0)] with a layer of elastomer in the bottom (Sylgaard 184, Dow Corning, Midland, MI, USA). The head was removed and the whole gut with the Malpighian tubules attached was gently extracted. The isolated gut was then transferred to a plastic petri dish and placed such that the main and lower segments of the Malpighian tubules and rectum were accessible to the ion-selective electrode used in the SIET system. The preparation was then placed either directly under the microscope (for measurements at room temperature, 23°C) or in a water-cooled stage where temperature was maintained by circulating a 1:1 (v:v) mixture of water and ethylene-glycol (for measurements at 6°C). Cooling and stabilizing at 6°C usually took 10–15 min and temperature was monitored regularly using a type K thermocouple connected to a computer with a TC-08 interface (Pico Technologies, St Neots, UK).

Measurement of epithelial K^+ transport

We used the scanning ion-selective electrode technique (SIET) to measure the flux of K^+ at three ion-transporting epithelia: (1) the secretory distal segment of the Malpighian tubules; (2) the reabsorptive proximal segment of the Malpighian tubules; and (3) the reabsorptive rectal pads in the rectum. This was carried out for all five species of *Drosophila* at both experimental temperatures (23°C and 6°C). Briefly, the SIET measures the concentration gradient of ions in the unstirred boundary layer adjacent to an ion-transporting epithelia, from which an estimate of ion flux can be calculated using Fick's law of diffusion. Our K^+ selective microelectrodes were constructed using 100 mmol l^{-1} KCl backfill solution and K^+ ionophore I cocktail B (Sigma Aldrich, St Louis, MO, USA). Details of the SIET system have been described previously (Donini and O'Donnell, 2005; Nguyen and Donini, 2010). Microelectrodes were calibrated in standards of 5 and 50 mmol l^{-1} KCl (osmolality adjusted with LiCl) and only microelectrodes with a slope between 50 and 62 mV were used (mean±s.d.: 53.4±2.1 mV, N=40).

For single-point measurements, the ion-selective microelectrode was placed immediately adjacent to the sampling sites (1 µm from the organ). Here ('origin'), measurement of the K^+ concentration gradient was performed by recording and averaging voltage for 1 s after which the microelectrode was moved 100 µm away perpendicular to the tissue ('away') and another voltage was recorded. A 4 s wait time was used after each movement of the electrode and for each single-point measurement, four repeats were averaged into a single measurement using the ASET software (Automated Scanning Electrode Technique version 2.0, Science Wares, East Falmouth, MA, USA).

Calculation of ion fluxes

Concentration gradients were calculated from the voltage gradients obtained from the SIET system using the following formula (for details, see Donini and O'Donnell, 2005):

$$\Delta C = C_B \cdot 10^{\frac{\Delta V}{S}} - C_B, \quad (1)$$

where ΔC is the concentration gradient in µmol cm^{-3}, C_B is the background ion concentration (the average of the concentration at the two points of measurement, 'origin' and 'away', in µmol cm^{-3}; derived from the equations of Smith et al., 1999), ΔV is the calculated voltage gradient (µV) and S is the slope of the electrode. Concentration gradients were then converted into ion fluxes using Fick's law of diffusion:

$$K^+ \text{ flux} = \frac{(D_{K^+} \cdot \Delta C)}{\Delta x}, \quad (2)$$

where the flux of K^+ in pmol cm^{-2} s^{-1} is dependent on D_{K^+} [the diffusion coefficient of K^+ (1.92×10^{-5} cm^2 s^{-1})], ΔC (the concentration gradient calculated above) and Δx [the distance between the measurement points 'origin' and 'away' in cm (here 0.01 cm)].

Experimental protocol

On the day of the experiment, the SIET system was prepared, after which individual flies were dissected and placed under the microscope in the SIET system. After stabilization of temperature, the ion-selective microelectrode was placed adjacent to the distal segment of a single tubule of the anterior pair of Malpighian tubules, then adjacent to the proximal segment and, finally, at one of the posterior rectal pads, and measurement was started. Measurements were performed at six to 10 sites along each of the three areas, with at least 32 µm intervals in the main segment of the Malpighian tubules and 16 µm in the lower segment of the Malpighian tubules. At the rectum, a hotspot for reabsorption of K^+ was found along the midline of the individual rectal pads, and measurements were performed in and around this area at 16 µm intervals. After repeated measurements of all three sites, a reference measurement was taken at least 0.2 cm from all tissue to control for the effect/noise of moving the electrode. Measurements were performed on six to 10 flies per species per temperature. After measurement, gradients were converted into fluxes (as described above) and corrected for the effect of electrode movement, and flux was then calculated as the mean of the three highest representative values. Values for flux were discarded if these were within a cut-off limit of less than five times the effect of electrode movement.

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The five species studied do not have the same hemolymph ion composition under benign conditions and hemolymph ion composition is also differently affected by cold exposure in these five species (MacMillan et al., 2015a). Nevertheless, all experiments were conducted in the same standard saline such that any differences in flux among species or temperature treatments are directly comparable.

Leak assay

To assess the amount of paracellular leak, we used the method recently described by MacMillan et al. (2017). Briefly, flies were fed for 16 h on a diet containing FITC-dextran [1.25 mg of FITC-dextran (3–8 kDa, FD4-100MG Sigma-Aldrich, St Louis, MO, USA), mixed with 50 µl milliQ water and 10 mg of active yeast]. FITC-dextran is not metabolized when flies are feeding on this diet and it can only cross epithelial barriers via the paracellular pathway. After the feeding period, female flies of each species were randomly exposed to either a cold stress (4 h at 0°C) or sampled directly from control conditions (21-22°C). After treatment, hemolymph was collected in rectangular microcapillary tubes (0.2×0.02 mm; VitroTubes, VitroCom, Mountain Lakes, NJ, USA) from individual flies (see details in MacMillan and Hughson, 2014). The ends of the capillary tubes were quickly dipped in hydrated paraffin oil to prevent evaporation. FITC-dextran concentrations in the hemolymph were measured flourometrically using the methods of Leader and O'Donnell (2005). Briefly, fluorescence intensity was imaged on a Fluoview 300 confocal microscope (Olympus America, Center Valley, CA, USA; excitation 488 nm, emission 535–565 nm) along the *z*-axis at 1 µm intervals through the middle of the capillary tube. Image analysis was then performed using ImageJ (Schindelin et al., 2015) by selecting three regions from the inside of the capillary tube and one region outside (for background fluorescence). Fluorescence intensity in the three regions inside the tube was measured and averaged, after which background fluorescence was subtracted. This was carried out in all individual images per stack of images, and for each stack (each hemolymph sample), the image with the maximal fluorescence was compared with a set of standards of known concentration (measured using the same procedure).

Morphometrics of the ionoregulatory organs

To estimate the size of the Malpighian tubules and the rectum, flies were carefully dissected as described above, except flies were weighed on a Sartorius micro balance (MSE6.6S000DM; Sartorius Lab Instruments, Goettingen, Germany) prior to the dissection. The excised guts were transferred to plastic petri dishes, placed under a stereomicroscope (Carl Zeiss Stemi 2000-CS, Carl Zeiss, Birkerød, Denmark), and photographed using a Sony α NEX 7 digital camera. Images were then imported into ImageJ (Schindelin et al., 2015), where the length (from the end of the tubule to the ureter junction) and average width (mean of five evenly spaced measurements along the length of the tubule) of the anterior Malpighian tubules were measured and used to calculate surface area and volume. To estimate the size of the rectum, it was placed flat in the dish and the cross-sectional area was measured in the frontal plane (the rectum was outlined and the area measured), along with the maximum width and length.

Data analysis

All statistical analyses were performed using R version 3.3.1 software (http://r-project.org). The effects of temperature and species on K^+ flux and FITC-dextran leak were analyzed using two-way ANOVA, with species and temperature as factors. Differences in organ sizes were analyzed using one-way ANOVA, and all correlations with cold tolerance were made using linear regressions. The level of statistical significance was 0.05 in all analyses and all values presented are mean±s.e.m.

RESULTS

Cold-tolerance measurements

The temperature for onset of chill coma differed among species with *D. birchii* and *D. equinoxialis* going into coma at the highest temperatures (7.4±0.3°C and 7.8±0.3°C, respectively). The most cold-tolerant species were *D. persimilis* (0.5±0.2°C) and *D. montana* (−1.3±0.1°C), whereas the cosmopolitan *D. melanogaster* entered chill coma at intermediate temperatures (3.1±0.1°C; Table 1).

K^+ transport at the Malpighian tubules

Secretion and reabsorption of K^+ at the Malpighian tubules was approximated in all five species by measuring the maximal site-specific K^+ flux at the main and lower segments at both benign (23°C) and low (6°C) temperature (Fig. 1). Secretion of K^+ in the main segment of the Malpighian tubules (Fig. 1A) varied among species (effect of species: $F_{4,70}$=7.1, P<0.001) with chill-sensitive species having considerably higher rates of K^+ secretion (−350 to −300 pmol cm^{-2} s^{-1}) than their chill-tolerant allospecifics (−200 to −120 pmol cm^{-2} s^{-1}), and with the cosmopolitan species *D. melanogaster* having an intermediate secretion rate (~−250 pmol cm^{-2} s^{-1}). As expected, exposure to low temperature tended to suppress K^+ secretion rate (effect of temperature: $F_{1,70}$=42.2, P<0.001). However, this reduction differed among species (interaction: $F_{4,70}$=3.8, P=0.008), such that low temperature suppressed secretion by more than 50% in *D. birchii*, *D. equinoxialis* and *D. melanogaster*, whereas K^+ secretion rates were suppressed by only 12% and 35% in the chill-tolerant species *D. persimilis* and *D. montana*, respectively.

We also found species-specific differences in K^+ reabsorption in the lower (reabsorbing) segment of the Malpighian tubules (effect of species: $F_{4,61}$=2.5, P=0.049; Fig. 1B). However, the association with species cold tolerance was less clear for K^+ reabsorption in this segment. For example there was no significant effect of lowering temperature (effect of temperature: $F_{1,61}$<0.1, P=0.932). Although we observed a trend for increases in reabsorption associated with low temperature in chill-sensitive species and the opposite trend for chill-tolerant species, this was not manifested in a significant interaction (interaction: $F_{4,61}$=1.3, P=0.300).

Reabsorption of K^+ at the rectal pads

The rectal pads of the insect hindgut are the major sites of active ion and water reabsorption. There were no consistent significant differences in K^+ fluxes (reabsorption) at the rectal pads related to species or temperature (Fig. 1C) (effect of species: $F_{4,65}$=0.9, P=0.498; effect of temperature: $F_{1,65}$=1.5, P=0.232). However, we found a highly significant interaction (interaction: $F_{4,65}$=5.2, P=0.001) demonstrating a species-specific response to temperature. Specifically, we found that K^+ reabsorption (into the hemolymph) decreased with low temperature exposure in chill-tolerant species (*D. montana* and *D. persimilis*) but increased during low temperature exposure in the most-sensitive species, *D. birchii*.

Leak from the gut to the hemolymph

To obtain a qualitative estimate of paracellular leak from the gut to the hemolymph compartment, we fed flies with fluorescent FITC-dextran and measured the concentration in the hemolymph

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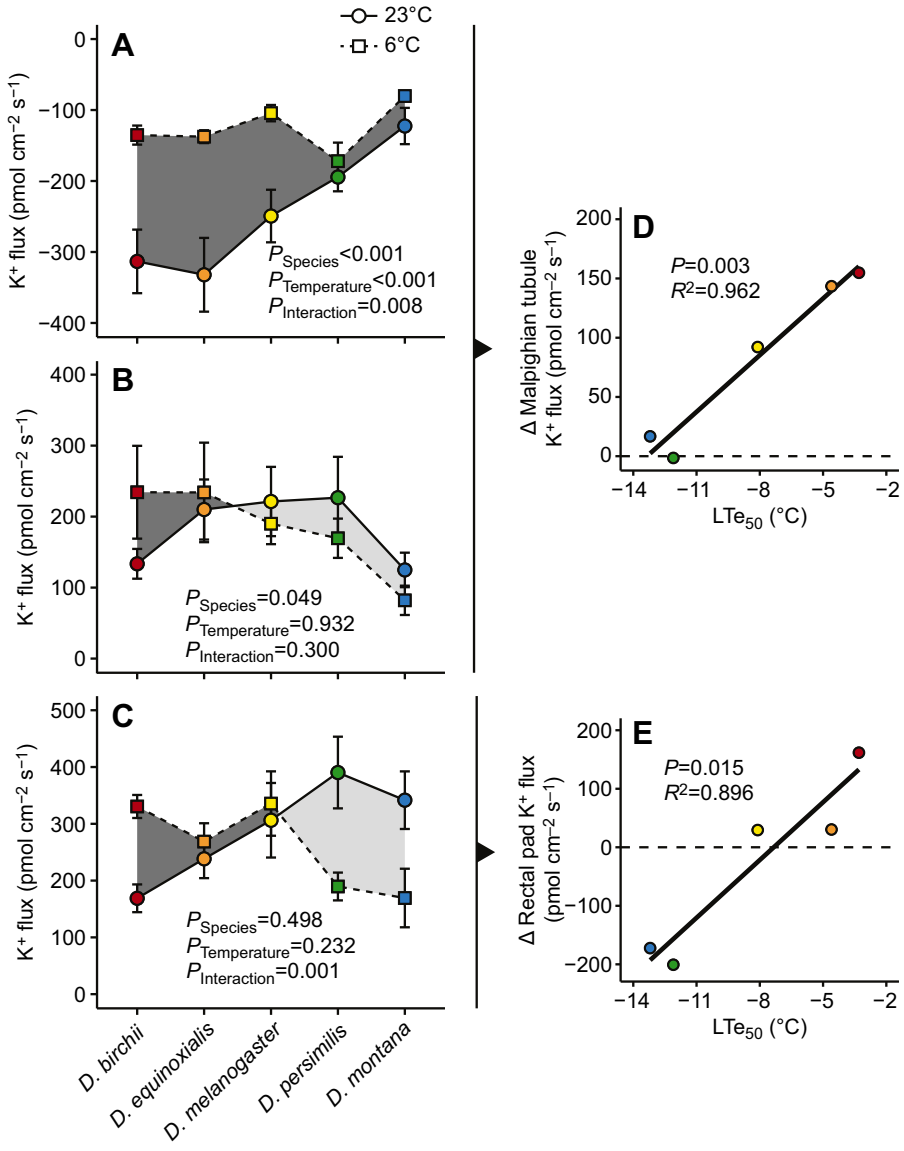

Fig. 1. The capacity for epithelial K^+ flux measured at the main ionoregulatory organs in *Drosophila*. Fluxes of K^+ were measured in the (A) distal and (B) proximal segments of the Malpighian tubules, and (C) at the rectal pads in five species of *Drosophila* at benign (23°C, circles) and low (6°C, squares) temperatures. Negative values indicate secretion of K^+, and positive values indicate reabsorption of K^+ into the hemolymph. Areas tinted in dark gray in A–C indicate changes that favor a (detrimental) increase in hemolymph [K^+] and light gray areas represent changes that will act to reduce hemolymph [K^+]. The effect of low temperature on K^+ flux of the entire Malpighian tubule was calculated and correlated with species-specific LTe_{50} (temperature causing 50% mortality) values (D). This calculation assumes a 70/30% distribution of distal and proximal tubule in all five species (see Maddrell, 1978). A similar graphic shows the change in flux with lowering of temperature at the rectal pads (E). The dashed lines in D and E indicate no effect of temperature in K^+ flux. Error bars that are not visible are occluded by the symbols. N=6–10 per species per temperature in A–C; values in D and E are based on means.

immediately after the feeding period (at 21–22°C) or after flies had been exposed to 4 h at 0°C after the feeding period. With the exception of *D. montana*, hemolymph concentrations of FITC-dextran were similar among species after the 16 h feeding period (64.3±35.7 ng μl^{-1} for *D. montana* and ~25 ng μl^{-1} for the remaining four species; Fig. 2). Hemolymph concentration of FITC-dextran increased significantly (effect of treatment: $F_{1,61}$=6.2, P=0.015) independently of species (effect of species: $F_{4,61}$=0.3, P=0.859) when flies were exposed to 4 h at 0°C. There was no significant interaction between species and temperature (interaction: $F_{4,61}$=1.2, P=0.336), possibly owing to the large variance in estimates from cold-exposed flies. However, we observed a tendency for chill-susceptible species to leak more than their cold-adapted allospecifics during cold exposure. To investigate this pattern further, we correlated the difference in leak between cold and benign temperature against the species LTe_{50} (temperature causing 50% mortality) (Fig. 2, inset). Here, we found a significant correlation corroborating the tendency of chill-tolerant species to change little in leak rate during cold exposure, while the gut becomes considerably leakier in warm-adapted species.

Morphometrics of the ionoregulatory organs

Differences in the ability to regulate ion balance in the cold could potentially relate to the size of the ionoregulatory organs. To investigate this, we assayed aspects of size in both Malpighian tubule and hindgut of all five species (Fig. 3). We found that the volume of the Malpighian tubules is larger in cold-adapted species ($F_{4,26}$=70.1, P<0.001; Fig. 3B), a difference generally driven by chill-tolerant species having wider tubules, and, in the case of *D. montana*, significantly longer Malpighian tubules (see Fig. S1). Cross-sectional area of the rectum followed similar trends (Fig. 3D), with *D. birchii* and *D. equinoxialis* having the smallest rectums, *D. melanogaster* and *D. persimilis* with intermediate sized rectums, and *D. montana* with the largest rectums ($F_{4,24}$=11.9, P<0.001). These differences were driven primarily by differences in rectum length (Fig. S1). Chill-tolerant flies were also considerably larger (Fig. 3A) and when corrected for body mass we observed no differences or trends in the sizes of Malpighian tubule volume ($F_{4,26}$=2.2, P=0.092; Fig. 3C and inset). Similarly, we observed no clear pattern in the size-corrected cross-sectional area of the rectum, despite the statistical effect of species ($F_{4,24}$=12.8, P<0.001; Fig. 3E). For example, we did not find that differences in the

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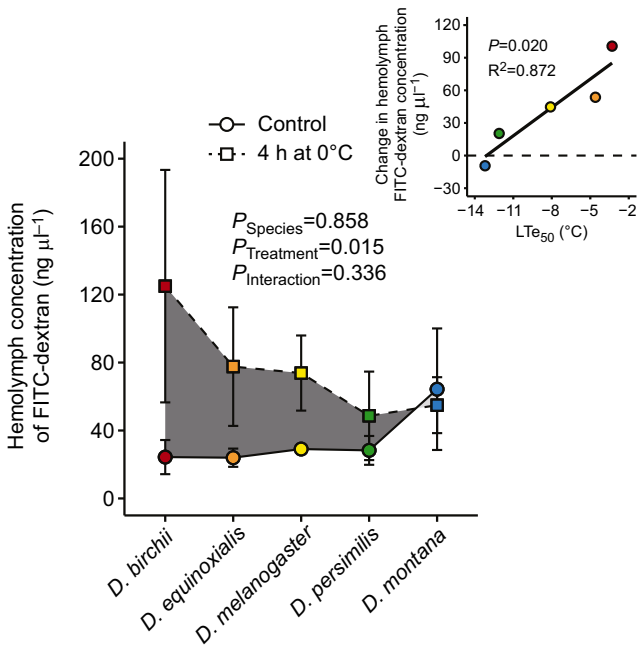

Fig. 2. The effect of cold exposure on paracellular leak from gut to hemolymph in five species of *Drosophila*. Leak from the gut into the hemolymph was estimated by feeding flies fluorescent FITC-dextran and measuring the concentration in the hemolymph in controls maintained at 23°C for 4 h (circles) and in flies exposed to 0°C for 4 h (squares). *N*=6–9 per species per treatment and error bars that are not visible are occluded by the symbols. Inset: the mean difference in leak between control and cold-exposed flies is plotted against their respective lower lethal temperature limits (LTe_{50}).

mass-corrected size of osmoregulatory organs were correlated to species differences in cold tolerance (Fig. 3C,E, inset).

DISCUSSION

Studies across the insect phylogeny and studies concerning insects with different acclimation treatments continue to find a very tight physiological link between cold tolerance and the ability of the insects to maintain ion and water balance (reviewed by Overgaard and MacMillan, 2017). This is also true for drosophilids, including the species system used here (MacMillan et al., 2015a; Olsson et al., 2016). Specifically, there is strong evidence indicating that the capacity to maintain low extracellular K^+ is important for low temperature survival in insects (Andersen et al., 2017b; Coello Alvarado et al., 2015; Koštál et al., 2004, 2006; MacMillan et al., 2015a,c). Because ion balance is related to the capacity of the osmoregulatory organs, we hypothesized that cold-tolerant *Drosophila* species would exhibit adaptive differences in K^+ transport during cold exposure that would favor maintenance of low hemolymph [K^+]. In contrast, we hypothesized that K^+ transport in intolerant species would change in a manner that would explain their tendency for hemolymph hyperkalemia in the cold.

Using five species of *Drosophila* with markedly different cold tolerance, we confirmed these hypotheses by measuring K^+ fluxes at sites along the Malpighian tubules and the rectal pads, showing that chill-tolerant species were able to maintain K^+ secretion in response to low temperature, whereas the cold treatment severely suppressed secretion of K^+ in chill-susceptible species (distal segment of the Malpighian tubule, Fig. 1A). Seemingly adaptive differences were also found in the proximal segment of the Malpighian tubule, where we found a tendency for suppressed reabsorption in cold-tolerant species and increased reabsorption in their susceptible allospecifics (Fig. 1B). The combined effect of cold on K^+ flux at the Malpighian tubules therefore shows a strong correlation between species-specific cold tolerance and their ability to maintain K^+ secretion at the Malpighian tubules at low temperature (Fig. 1D). These findings are qualitatively consistent with findings of MacMillan et al. (2015a), who used Ramsay assays to measure rates of primary urine production and ion secretion of whole Malpighian tubules in the same five *Drosophila* species used here. MacMillan et al. (2015a) found that the rate of primary urine production was markedly reduced in all five species when they were exposed to a temperature of 3°C but also revealed that K^+ secretion from chill-susceptible species was impaired much more by low temperature exposure than in the tolerant species. However, the Ramsay assay represents an open-ended system and MacMillan et al. (2015a) were unable to (1) distinguish between effects of cold on the distal and proximal segments (where we show clear differences in response), and (2) observe whether changes in Malpighian tubule transport could be countered by other reabsorptive parts of the ionoregulatory circuit (i.e. the hindgut, which we investigated here).

Ion regulation in insects is also highly dependent on the hindgut (Beyenbach and Piermarini, 2008; Edney, 1977; Phillips, 1981; Phillips et al., 1987) and here we show clear patterns in cold adaptation that link differences in rectal K^+ reabsorption to the cold tolerance of a species. In accordance with our hypothesis, reabsorption of K^+ at the rectal pads was suppressed at low temperature in tolerant species, whereas reabsorptive K^+ flux tended to increase for susceptible species (Fig. 1C,E). To our knowledge, the present study is the first to use an interspecific model system to investigate the effect of temperature on ion reabsorption and barrier function in the insect hindgut/rectum. Reabsorption of K^+ at the rectal pads is driven by active transport (see Phillips, 1981; Phillips et al., 1987, 1996), and the reduction in active K^+ reabsorption observed for the more chill-tolerant drosophilids is likely to be caused by reduced active transport. Similar reductions in response to low temperature have also been found for ion reabsorption in the locust (*Locusta migratoria*) hindgut (Houlihan and Sell, 1984), where low temperature reduced the amounts of Na^+, K^+ and water reabsorbed. In contrast to these findings, we observed an apparent increase in K^+ reabsorption in the least cold tolerant of the five species, *D. birchii*. Although it is possible that this represents a counterintuitive increase in active ion reabsorption, we propose that it is instead a product of increased ion leak. Ion fluxes measured by SIET are the cumulative result of passive and active ion transport, and the increased reabsorption of K^+ at the hindgut could therefore represent increased passive leak (see Figs 1C,E and 2). In the present study, we assayed the paracellular permeability of the entire digestive system and found that all species have similar levels of leakiness at benign temperatures. However, when exposed to chronic cold, chill-sensitive species had higher rates of paracellular leak, whereas little change was found in the chill-tolerant species (Fig. 2). These findings suggest that cold causes the gut to lose barrier function, leading to large uncharged molecules and ions moving down their electrochemical and osmotic gradients. Thus, the apparent increase in rectal pad K^+ reabsorption could at least in part be attributed to a cold-induced leak.

MacMillan et al. (2017) recently investigated gut leakiness in cold- and warm-acclimated *D. melanogaster* using a similar FITC protocol. They found that cold acclimation greatly reduced leakiness in cold-acclimated *D. melanogaster*, which they ascribed to differences in septate junction morphology and a marked increase in the length of cell-cell contact regions. Des Marteaux et al. (2017)

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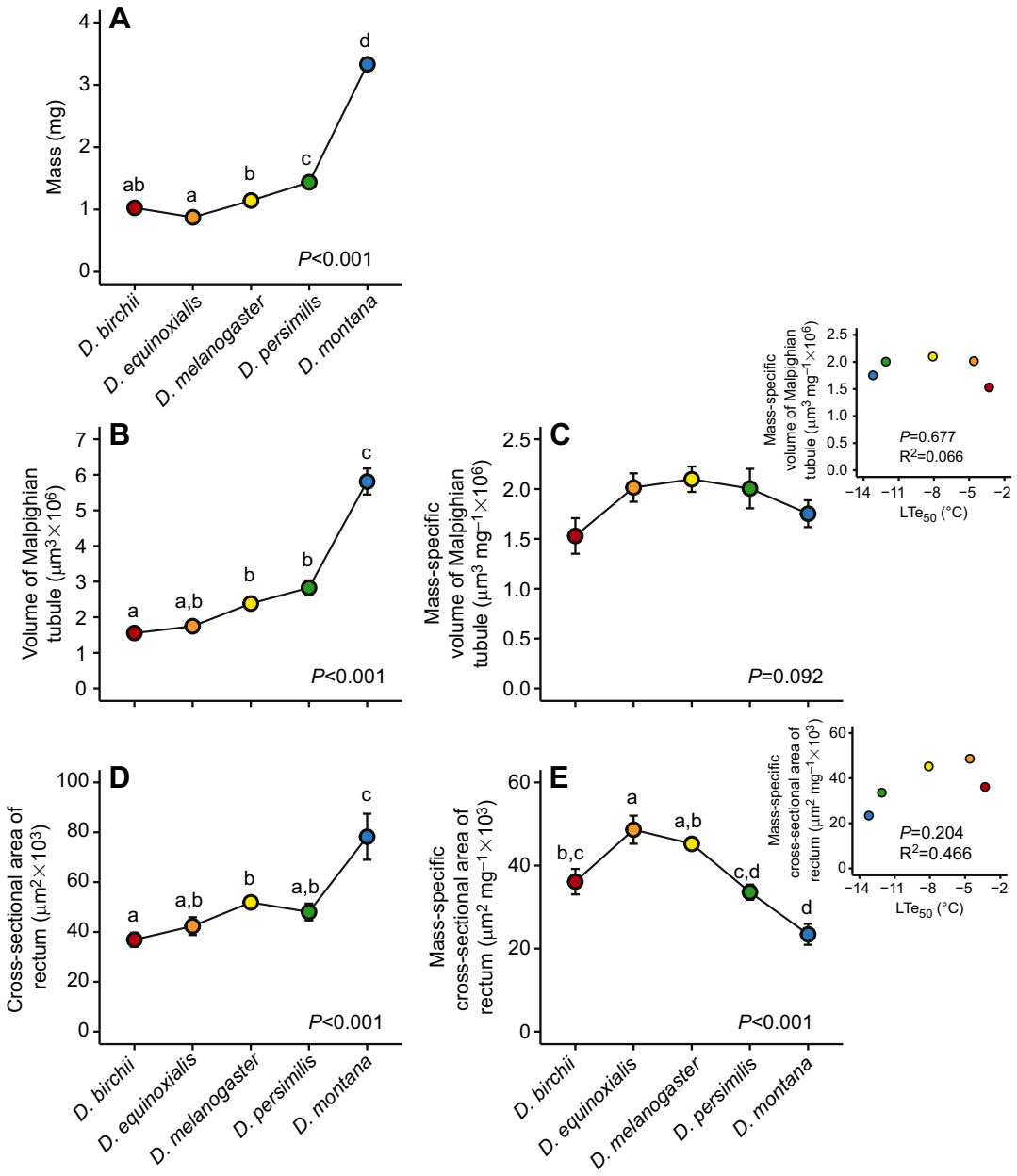

Fig. 3. Size of the ionoregulatory organs in five species of *Drosophila*. (A,B,D) Mass of flies (A) was determined before the guts were dissected out in order to calculate the volume of the Malpighian tubules (B) and area of the rectum (D) (see Fig. S1 for estimates of length and width). (C,E) Malpighian tubule volume (C) and rectum cross-sectional area (E) were corrected for body mass. Points that share letters are not statistically different (Tukey's honest significant differences *post hoc* test). Error bars that are not visible are occluded by the symbol; *N*=5–7 per species. Insets in C and E represent correlations with the species-specific lower lethal thermal limit (LTe_{50}).

found alterations in gene expression levels after cold acclimation in *Gryllus pennsylvanicus* that suggested similar changes. Our findings in the present study suggest that increased paracellular resistance could also play a role in reducing leak in cold-resistant species but further studies are needed to verify this possibility. Differences in leak rates could also contribute indirectly to hyperkalemia, as it has been shown that cold is associated with leak of Na^+ (and osmotically accompanying water) towards the gut in many chill-sensitive insects (Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; MacMillan et al., 2015a,b; MacMillan and Sinclair, 2011b; Olsson et al., 2016). This movement of water causes a reduction in hemolymph volume that contributes markedly to the characteristic rise in hemolymph [K^+] during cold exposure. The SIET system used in the present study measures net ion transport and therefore does not partition changes between species or temperature treatments in relation to alterations in passive or active transport. Clearly future studies should investigate this further, e.g. by examining how pharmacological stimulation or blockade impacts ion transport.

In the present study, we also investigated the hypothesis that species with superior ionoregulatory capacity at low temperatures were endowed with proportionally larger ionoregulatory organs. However, there was no indication that this was the case, as size of both the Malpighian tubule and hindgut was found to be unrelated to species cold tolerance (Fig. 3 and Fig. S1). It must therefore be differences in epithelial properties that are driving the variation in

homeostatic capacity. In our analysis, we have focused on how species differ with respect to the change in epithelial K^+ flux with low temperature exposure rather than analyzing the data in relation to absolute values of flux. The *ex vivo* SIET measurements used in the present study do not necessarily represent the precise ion flux found in the living animal. *In vivo* fluxes would be affected by neuroendocrine regulation (Terhzaz et al., 2017, 2015) as well as the dynamic changes in hemolymph and in the lumen of the gut and Malpighian tubule (Maddrell, 1969; Ramsay, 1955). Nevertheless, we use the simple assumption that K^+ fluxes measured at benign temperature (when all species are able to maintain ion and water balance) represent a capacity needed to maintain homeostasis for that particular species. Accordingly, any change in K^+ flux will act to improve or reduce the homeostatic capacity.

Despite the compelling evidence to support an important role of iono- and osmoregulation for insect cold tolerance, few previous studies have investigated how cold affects the ion transport capacity of the iono- and osmoregulatory organs in insects, and even fewer have done so in the context of cold adaptation or cold acclimation in insects: In a study on the hindgut of the Pyrenean grasshopper (*Oedipoda germanica pyrenaica*), Houlihan and Sell (1984) found that lowering temperature generally suppressed reabsorption of Na^+, K^+ and Cl^-, and that the uptake of Na^+ in particular became energetically inefficient at the lowest experimental temperatures, which may indicate the onset of transepithelial leak. Four additional studies have investigated secretion of primary urine; Anstee et al. (1979) found that lowering temperature to stressful levels (5°C) greatly reduced urine production in *Locusta migratoria*. The remaining three studies we found in the literature are incomparable with our study due to differences in cold-tolerance strategy and entry into dormancy (*Eurosta solidaginis*; Yi and Lee, 2005) or because they describe non-stressful temperatures in a blood-feeding insect (*Rhodnius prolixus*; Maddrell, 1964) and a butterfly (*Pieris brassicae*; Nicolson, 1976).

Conclusions

In the present study, we show that cold-adapted species are able to maintain K^+ balance during cold exposure due to an innate ability to maintain secretion rates of K^+ at the Malpighian tubules and through suppressed K^+ reabsorption at the rectal pads at low temperature. Warm-adapted species, on the other hand, succumb to detrimental hyperkalemia as K^+ secretion is greatly reduced and through a paradoxical increase in K^+ reabsorption. We further demonstrate how these differences are, in part, related to the ability of cold-adapted species to avoid passive paracellular leak and not to differences in organ size. The underlying mechanisms behind these physiological differences in epithelial flux still remain unknown and could relate to the thermal sensitivity of active or passive ion transporters/exchangers involved in iono- and osmoregulation. Given the importance of Na^+ balance in setting cold tolerance, it would also be important to investigate how Na^+ transport is affected by low temperature. Future studies should therefore aim to investigate how epithelial transport mechanisms are maintained at low temperature, by measuring the activity of active transporters at low temperature, by directly measuring leak of Na^+ and K^+ and/or epithelial resistances, and by examining the roles of neuronal and humoral regulation, as the neuropeptide *capa* has been found to impact cold tolerance (Terhzaz et al., 2015). Investigating these mechanisms would provide valuable insight into basic mechanisms of cold tolerance in insects.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.K.A., H.A.M., A.D., J.O.; Methodology: M.K.A., H.A.M., A.D., J.O.; Formal analysis: M.K.A.; Investigation: M.K.A., H.A.M.; Resources: H.A.M., A.D., J.O.; Writing - original draft: M.K.A.; Writing - review & editing: M.K.A., H.A.M., A.D., J.O.; Visualization: M.K.A.; Supervision: H.A.M., A.D., J.O.; Project administration: M.K.A., H.A.M., A.D., J.O.; Funding acquisition: M.K.A., H.A.M., A.D., J.O.

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Supplementary information

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