

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

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Summary statement: Cold tolerance is linked to the ability to maintain ion balance in the cold. We demonstrate that chill tolerant *Drosophila* maintain ion balance through adaptive changes in epithelial K⁺ transport.

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 balancing reabsorption from the hindgut and passive leak arising from the gut lumen. Here, we used a scanning ion-selective electrode technique (SIET) system at benign (23°C) and low (6°C) temperature 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 increased 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 leakage 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.

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; Košťál et al., 2004; Košťál et al., 2006; MacMillan et al., 2015a; MacMillan and Sinclair, 2011a; MacMillan and Sinclair, 2011b; 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 the family Drosophilidae are also consistent with this: *D. melanogaster* acclimated to low temperature in the laboratory or *D. subobscura* acclimatized to cold field conditions have an improved capacity to maintain water and ion balance when chilled (MacMillan et al., 2015b; MacMillan et al., 2016). Similarly, cold adapted *Drosophila* species from temperate regions have higher capacity for ion balance regulation in the cold than sister species from tropical regions (MacMillan et al., 2015a).

The major organs that are 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; Phillips, 1970). At the Malpighian tubules, secretion of ions and water is driven by apical V-type H^+ ATPase activity, creating a favourable 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)). Combined, this active ion transport establishes an osmotic gradient for 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 at 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^+ , while Na^+ reabsorption is tied to apical Na^+/H^+ exchangers and basolateral Na^+/K^+ ATPases (Phillips, 1981; Phillips et al., 1987; Phillips et al., 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) 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 superior capacity to balance active transport with passive leak at low temperatures. Previous studies have investigated this paradigm by looking at the effect of cold in isolated organs: Maddrell (1964) found that cold lowered secretion of ions more than secretion of fluid in 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 in 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 latter study also found that cold-sensitive drosophilids secreted relatively more Na^+ at low temperature while 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 use 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 can measure the site-specific capacity for K^+ secretion/reabsorption in different organs of the same individual and thereby pinpoint if differences in the capacity to maintain low hemolymph $[K^+]$ is due to differences in ion transport of specific organs. We predicted that 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 at 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 leak. Thus, we assayed the passive movement of a paracellular marker from the gut to the hemolymph (indicative of leak) by feeding flies fluorescent FITC-dextran and measuring FITC-fluorescence in the hemolymph of control and cold-exposed flies. Here we hypothesized that the guts of chill susceptible species would leak gut contents into the hemolymph during cold exposure while chill-tolerant species would be able to prevent leak. Lastly, 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; Andersen et al., 2015b; Andersen et al., 2015c; MacMillan et al., 2015a; Olsson et al., 2016). Flies were maintained under “common garden” conditions at 21-22°C with a 12:12 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 one litre 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 approximately 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 done 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 of which temperature was lowered by $0.2^{\circ}\text{C min}^{-1}$. 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 adults were briefly submerged in 70 % ethanol for sedation, and females were quickly moved to a small glass dish containing *Drosophila* saline at 23°C (137 mM Na⁺, 15 mM K⁺, 158.5 mM Cl⁻, 8.5 mM Mg²⁺, 2 mM Ca²⁺, 10.2 mM HCO₃⁻, 4.3 mM H₂PO₄, 20 mM glucose, 10 mM glutamine, and 15 mM MOPS buffer, pH 7.0) with a layer of elastomer in the bottom (Sylgaard 184, Dow Corning Corp., 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: i) the secretory distal segment of the Malpighian tubules; ii) the reabsorptive proximal segment of the Malpighian tubules and iii) the reabsorptive rectal pads in the rectum. This was done 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 mM KCl backfill solution and K⁺ ionophore I cocktail B (Sigma Aldrich, St. Louis, MO, USA) and 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 mM KCl (osmolality adjusted with LiCl) and only microelectrodes with a slope between 50 and 62 mV were used (mean \pm standard deviation: 53.4 ± 2.1 mV, N = 40).

For single-point measurements, the ion-selective microelectrode was placed immediately adjacent to the sampling sites (1 μ m from the organ). Here ("origin"), measurement of the K⁺ concentration gradient was performed by recording and averaging voltage for 1s after which the microelectrode was moved 100 μ m away perpendicular to the tissue ("away") and another voltage was recorded. A four second wait time was used after each movement of the electrode and for each single point measurement four repeats was 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 (see Donini and O'Donnell (2005) for details):

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

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

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

Where the flux of K^+ in $\text{pmol cm}^{-2} \text{s}^{-1}$ is dependent on D_{K^+} , the diffusion coefficient of K^+ ($1.92 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$), 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 the proximal segment, and lastly at one of the posterior rectal pads and measurement was started. Measurements were performed at 6-10 sites along each of the three areas with at least 32 μm intervals at the main segment of the Malpighian tubules and 16 μm at 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 with 16 μm intervals. After repeated measurements of all three sites, a reference measurement was performed at least 0.2 cm from all tissue to control for the effect/noise of moving the electrode. Measurements were performed on 6-10 flies per species per temperature. After measurement, gradients were converted into fluxes (as described above), 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.

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 (Sigma Aldrich, 3-8 kDa, #FD4-100MG), mixed with 50 μ L 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 x 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 fluorometrically 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 μ m intervals through the middle of the capillary tube. Image analysis was then performed in 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 done in all individual images per stack of images, and for stack (each hemolymph sample), the image with the maximal fluorescence was compared to 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 GmbH & Co. KG, 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 A/S, Birkerød, DK), 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 (R Core Team, 2016). The effects of temperature and species on K^+ flux and FITC-dextran leak were analysed using two-way ANOVA, with species and temperature as factors. Differences in organ sizes were analysed 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 means \pm 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 ($7.4 \pm 0.3^\circ\text{C}$ and $7.8 \pm 0.3^\circ\text{C}$, respectively). The most cold tolerant species were *D. persimilis* ($0.5 \pm 0.2^\circ\text{C}$) and *D. montana* ($-1.3 \pm 0.1^\circ\text{C}$), while the cosmopolitan *D. melanogaster* entered chill coma at intermediate temperatures ($3.1 \pm 0.1^\circ\text{C}$, Table 1).

K⁺ transport in the Malpighian tubules

Secretion and reabsorption of K^+ in 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^+ at 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 $\text{pmol cm}^{-2} \text{s}^{-1}$) than their chill-tolerant allospecifics (-200 to -120 $\text{pmol cm}^{-2} \text{s}^{-1}$) and with the cosmopolitan species *D. melanogaster* having intermediate secretion rate ($\sim -250 \text{ pmol cm}^{-2} \text{s}^{-1}$). 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 at the lower (reabsorbing) segment of the Malpighian tubules (Fig. 1B) (effect of species: $F_{4,61} = 2.5$, $P = 0.049$). However, the association to species cold tolerance was less clear for K^+ reabsorption of 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 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 amongst species after the 16 h feeding period ($64.3 \pm 35.7 \text{ ng } \mu\text{L}^{-1}$ for *D. montana* and $\sim 25 \text{ ng } \mu\text{L}^{-1}$ for the remaining four species) (Fig. 2). Hemolymph concentration of FITC-dextran increased significantly (effect of treatment: $F_{1,61} = 6.2$, $P = 0.015$) independent 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 due 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 LTE₅₀ (Fig. 2, insert). Here we found a significant correlation corroborating the tendency that chill tolerant species 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 find that the volume of the Malpighian tubules is larger in cold-adapted species (Fig. 3B; $F_{4,26} = 70.1$, $P < 0.001$), 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 (Fig. 3C; $F_{4,26} = 2.2$, $P = 0.092$, and insert). Similarly we observed no clear pattern in the size corrected cross-sectional area of the rectum despite statistical effect of species (Fig. 3E; $F_{4,24} = 12.8$, $P < 0.001$). For example we did not find that

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

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 in 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; Košťál et al., 2004; Košťál et al., 2006; MacMillan et al., 2015a; MacMillan et al., 2015c). Because ion balance is related to the capacity of the osmoregulatory organs we hypothesised that cold tolerant *Drosophila* species would exhibit adaptive differences in K^+ transport during cold exposure that would favour maintenance of low hemolymph $[K^+]$. In contrast we hypothesised 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 this hypothesis 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, while 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 at 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 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 if 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, 2009; Edney, 1977; Phillips, 1981; Phillips et al., 1987) and here we show clear patterns in cold adaptation that links differences in rectal K^+ reabsorption to the species' cold tolerance. In accordance with our hypothesis, reabsorption of K^+ at the rectal pads was suppressed at low temperature in tolerant species while 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), and Phillips et al. (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 with low temperature have also been found for ion reabsorption in the locust hindgut (*Locusta migratoria*) (Houlihan and Sell (1984)) where low temperature greatly 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 Fig. 1C+E and Fig. 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 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) 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 of 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; MacMillan et al., 2015b; 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 does therefore not partition changes between species or temperature treatments in relation to alterations in passive or active transport. Clearly future studies should investigate this further, for example 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 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 analysing 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; Terhzaz et al., 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 especially Na^+ 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 ($5^{\circ}C$) greatly reduced urine production in *Locusta migratoria*. The remaining three studies we found in the literature are incomparable to 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)).

CONCLUSION

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 insect cold tolerance.

LIST OF SYMBOLS AND ABBREVIATIONS

[X]	Concentration of ion X
ΔC	Concentration gradient
ΔV	Voltage gradient
Δx	Distance of electrode movement in the SIET system
C_B	Background concentration
FITC	Fluorescein isothiocyanate
LT ₅₀	Temperature causing 50% mortality
S	Electrode slope from a 10-fold difference in ion concentration
SIET	Scanning ion-selective electrode technique

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COMPETING INTERESTS

None

AUTHOR CONTRIBUTIONS

The study was conceived and designed by all authors, MKA and HAM performed the experiments. MKA analysed the data and wrote first draft, and all authors edited and approved of the final version.

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Table 1

Origin and description of the five *Drosophila* populations used in the present study. All species were kept under “common garden” conditions of 21-22°C and a 12:12 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 centre, San Diego, USA (*D. equinoxialis* and *D. persimilis*); Prof. Volker Loeschcke, Aarhus University, Denmark (*D. melanogaster*) and Prof. Anneli Hoikkala, University of Jyväskylä, Finland (*D. montana*). The values measured for CCO in the present study were reminiscent of previously obtained values (Andersen et al., 2015c), and thus the LT_{e50} (the temperature causing 50 % mortality after a 1 h exposure) estimated by Andersen et al. (2015c) were assumed to be maintained as well (¹). LT_{e50} 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).

Species	Origin	Collection date	Distribution	Source	CCO (°C)	LT_{e50} (°C) ¹
<i>D. birchii</i>	Australia	2008	Tropical	Hoffmann, AUS	7.4 ± 0.3 ^a	-3.3 ± 0.2
<i>D. equinoxialis</i>	Honduras	< 1984	Tropical	DSSC, USA	7.8 ± 0.3 ^a	-4.6 ± 0.3
<i>D. melanogaster</i>	Denmark	2011	Cosmopolitan	Loeschcke, DNK	3.1 ± 0.1 ^b	-8.1 ± 0.2
<i>D. persimilis</i>	Canada	Unknown	Temperate	DSSC, USA	0.5 ± 0.2 ^c	-12.1 ± 0.5
<i>D. montana</i>	Finland	2008	Temperate	Hoikkala, FIN	-1.3 ± 0.1 ^d	-13.2 ± 0.3

Figures

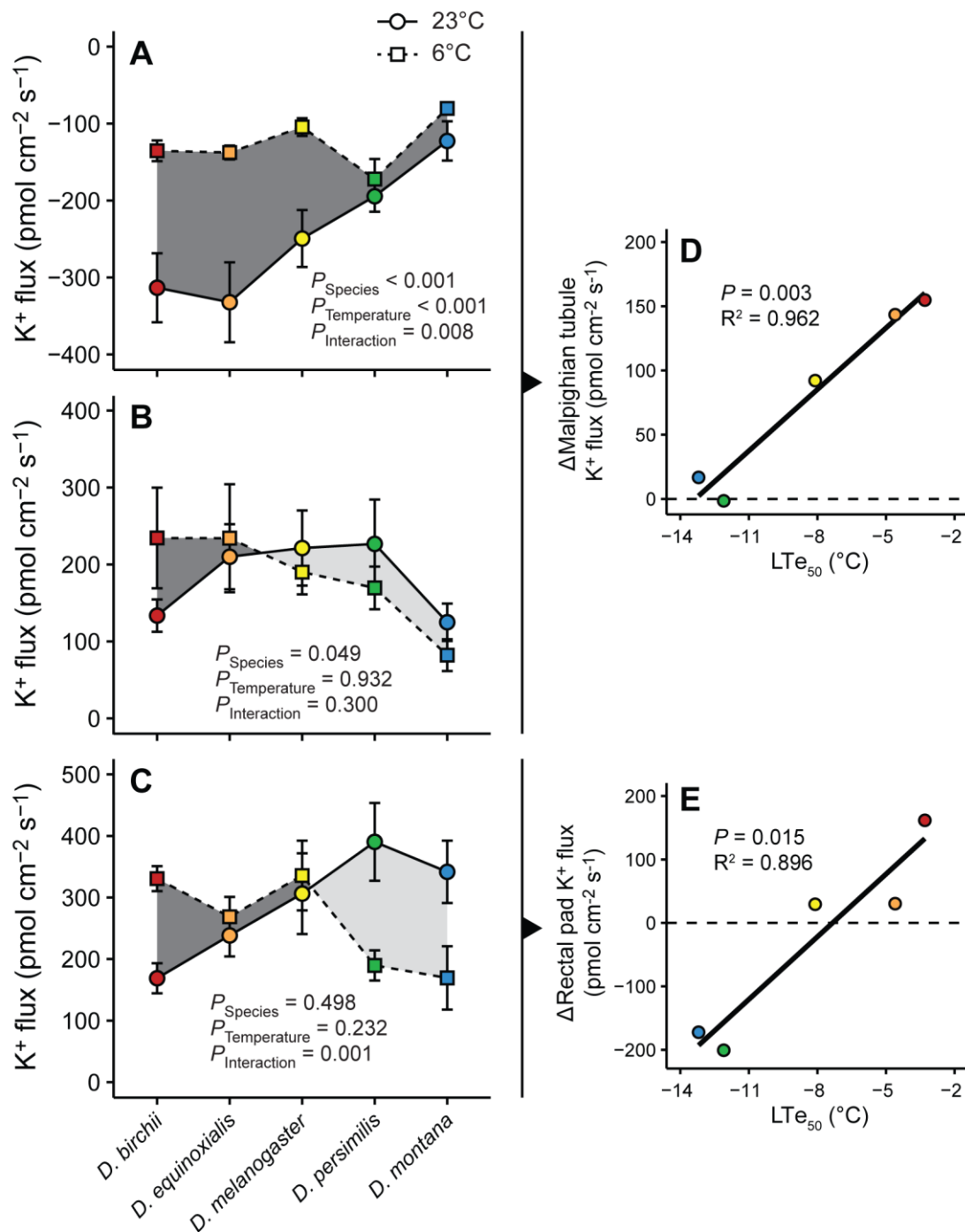


Figure 1 – The capacity for epithelial K⁺ flux measured at the main ionoregulatory organs in *Drosophila*. Fluxes of K⁺ were measured at the (A) distal and (B) proximal segments of the Malpighian tubules, and (C) at the rectal pads in five species of *Drosophila* at benign (23°C, circles) and low (6°C, squares) temperatures. Negative values indicate secretion of K⁺ while positive values indicate reabsorption of K⁺ into the hemolymph. Areas

tinted in dark grey in panels A-C indicate changes that favor a (detrimental) increase in hemolymph $[K^+]$ and light grey 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 LT_{e50} -values (D). This calculation assumes a 70/30 percent 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 panels D and E indicate no effect of temperature in K^+ flux. Error bars not visible are occluded by the symbols. $N = 6-10$ per species per temperature in A-C, and values in D and E are based on means.

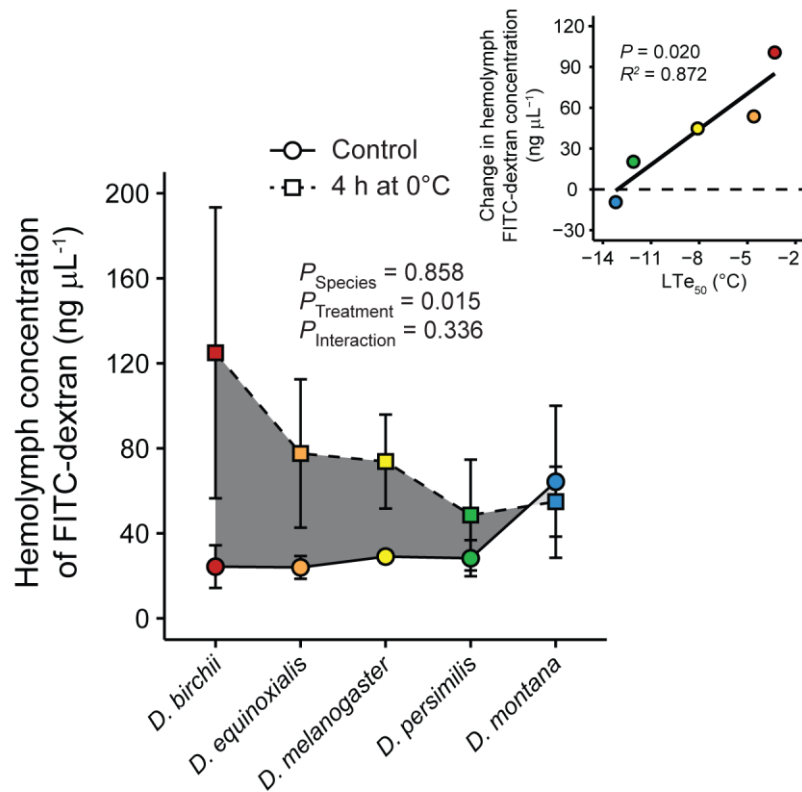


Figure 2 – The effect of cold exposure on paracellular leak from gut to hemolymph in five species of *Drosophila*. Leak from the gut into the hemolymph was estimated by feeding flies fluorescent FITC-dextran and measuring the concentration in the hemolymph in controls maintained at 23°C for 4 h (circles)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

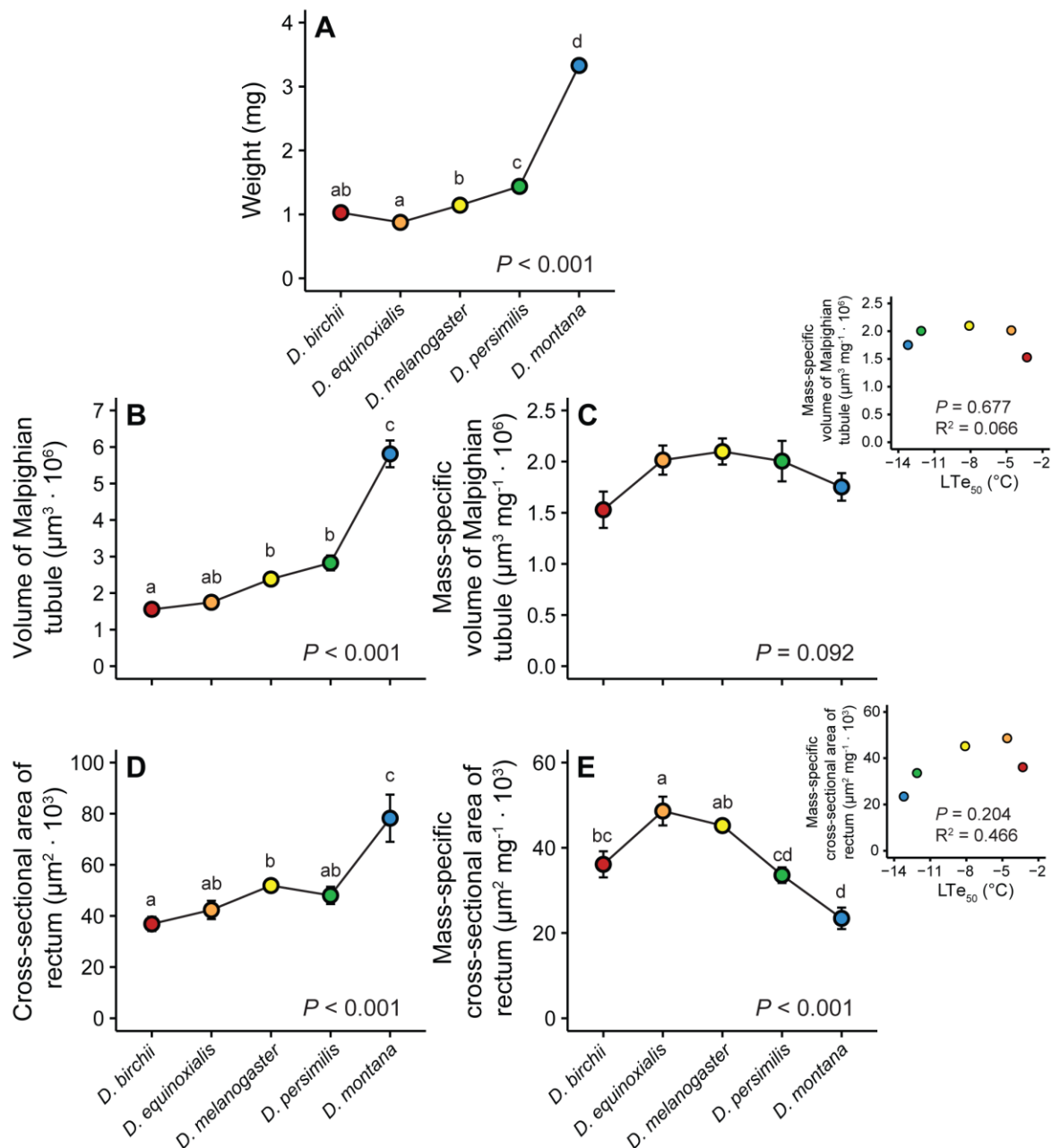


Figure 3 – Size of the ionoregulatory organs in five species of *Drosophila*. Mass of flies (A), was determined before the guts were dissected out in order to calculate the volume of the Malpighian tubules (B) and area of the rectum (D) (see fig. S1 for estimates of length and width). Malpighian tubule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

SUPPLEMENTARY MATERIAL

The supplementary material contains morphological measurements of the ionoregulatory organs, namely the Malpighian tubules and the rectum.

Figure S1

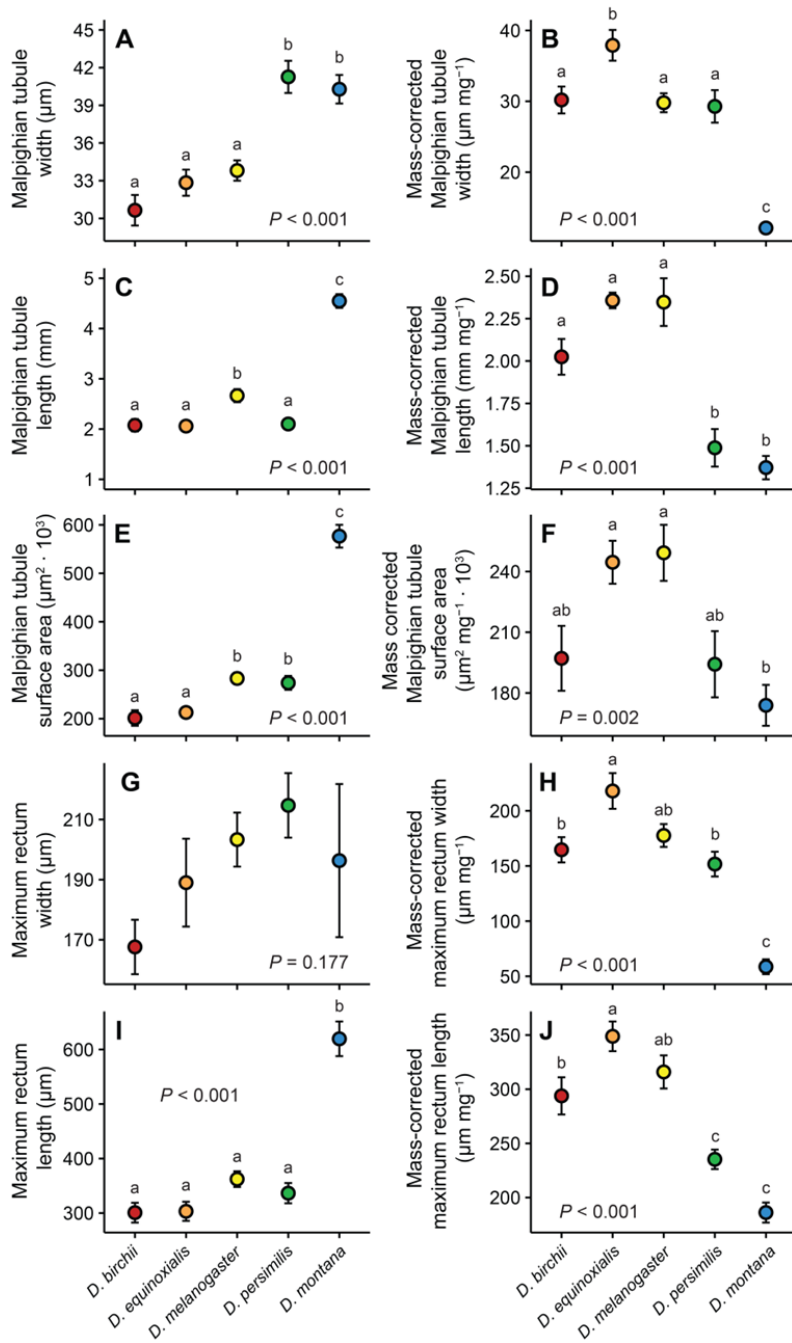


Figure S1 – Morphological measurements of the ionoregulatory organs in five species of *Drosophila*. To investigate if differences in ionoregulatory capacity related to differences in organ size, we measured (A) width and (C) length of the Malpighian tubules to calculate (E) surface area and (Fig. 3B) volume, along with the (G) width and (I) length of the rectum. Additionally, these measurements were corrected for body mass (right column; values for mass found in Fig. 3A). Data points sharing a letter are not statistically different (Tukey's Honest Significant Differences *post hoc* test) and error bars not visible are occluded by the symbols. N = 5-7 per species.