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RESEARCH ARTICLE

Maintenance of hindgut reabsorption during cold exposure is a key adaptation for *Drosophila* cold tolerance

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ABSTRACT

Maintaining extracellular osmotic and ionic homeostasis is crucial for organismal function. In insects, hemolymph volume and ion content is regulated by the secretory Malpighian tubules and reabsorptive hindgut. When exposed to stressful cold, homeostasis is gradually disrupted, characterized by a debilitating increase in extracellular K^+ concentration (hyperkalemia). Accordingly, studies have found a strong link between species-specific cold tolerance and the ability to maintain ion and water homeostasis at low temperature. This is also true for drosophilids where inter- and intra-specific differences in cold tolerance are linked to the secretory capacity of Malpighian tubules. There is, however, little information on the reabsorptive capacity of the hindgut in *Drosophila*. To address this, we developed a novel method that permits continuous measurements of hindgut ion and fluid reabsorption in *Drosophila*. We demonstrate that this assay is temporally stable (~2 h) and responsive to cAMP stimulation and pharmacological intervention in accordance with the current insect hindgut reabsorption model. We then investigated how cold acclimation or cold adaptation affected hindgut reabsorption at benign (24°C) and low temperature (3°C). Cold-tolerant *Drosophila* species and cold-acclimated *D. melanogaster* maintain superior fluid and Na^+ reabsorption at low temperature. Furthermore, cold adaptation and acclimation caused a relative reduction in K^+ reabsorption at low temperature. These characteristic responses of cold adaptation/acclimation will promote maintenance of ion and water homeostasis at low temperature. Our study of hindgut function therefore provides evidence that adaptations in the osmoregulatory capacity of insects are critical for their ability to tolerate cold.

KEY WORDS: Absorption, Acclimation, Adaptation, Assay, Ion transport, Rectum, Renal system, Temperature

INTRODUCTION

Maintenance of a relatively stable extracellular environment is essential for cell function and ultimately survival of most animals (Bernard, 1872). The concentrations of major ions and the volume of the extracellular fluid are maintained primarily through actions of the secretory Malpighian tubules and the absorptive hindgut in insects (Beyenbach and Piermarini, 2008; Edney, 1977; Phillips, 1970). These osmoregulatory organs act in synchrony to balance the passive movement of ions and water across gut epithelia and integument, whereby they help to secure ion and water homeostasis. Secretion and reabsorption of ions and water involve energy-demanding ion pumps that, accompanied by co-transporters and ion exchangers, regulate movement of ions and water (Beyenbach and Piermarini, 2011; O'Donnell et al., 2003; Phillips, 1981, 1970). Active transporters are temperature sensitive, and exposure to low temperature will therefore tend to reduce the capacity for secretion and reabsorption of ions (Gerber and Overgaard, 2018; MacMillan et al., 2015a; Yerushalmi et al., 2018). The loss of active transport can therefore ultimately lead to the characteristic, cold-induced loss of extracellular ion and water balance observed in chill-susceptible insects (Andersen et al., 2017b; Des Marteaux and Sinclair, 2016; Koštál et al., 2007, 2004, 2006; MacMillan et al., 2015a; MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017).

The loss of ion balance, particularly the increase in extracellular K^+, is linked to chill injury (Andersen et al., 2017a; Bayley et al., 2018; MacMillan et al., 2015c; Overgaard and MacMillan, 2017) and can be ascribed to two main processes: (1) K^+ leaks into the hemolymph via transcellular or paracellular pathways faster than it can be removed (by the Malpighian tubules), or (2) Na^+ and water leak into the gut faster than it can be reabsorbed resulting in a loss of hemolymph volume, which concentrates the K^+ remaining in the hemolymph (MacMillan et al., 2015b; MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). Consistent with this paradigm, there are now several studies that have shown how cold-adapted or cold-acclimated insects are characterized by superior osmoregulatory function at low temperature (Andersen et al., 2017c; Des Marteaux et al., 2017; Gerber and Overgaard, 2018; MacMillan et al., 2015a; Yerushalmi et al., 2018). Despite their improved tolerance, these chill-tolerant insects remain susceptible to cold and are merely categorized as being more tolerant to mild cold than their chill-sensitive counterparts.

The majority of studies investigating osmoregulatory organs in insects have used model species such as *Drosophila*, *Locusta* and *Rhodnius*, and collectively, these studies have been biased towards epithelial transport in Malpighian tubules. This 'bias' is likely influenced by the availability of the 'Ramsay assay', which is an easy and effective method to study epithelial transport and regulation in insects (see Ramsay, 1954, but also Dow et al., 1994; Rheault and O'Donnell, 2004; Davies et al., 2019, for more recent applications). However, Malpighian tubules only represent the 'secretory half' of the osmoregulatory organs and historically, there has been a shortage of studies investigating hindgut reabsorption in small model insects such as *Drosophila* (Black et al., 1987; Hanrahan et al., 1984; Phillips et al., 1987, 1996). Recent studies of osmoregulatory function of *Drosophila* have become possible with the use of scanning ion-selective electrode technique (SIET) which estimates the net ion flux, but SIET cannot quantify bulk ion or fluid transport in these small species (Andersen et al., 2017c; D'Silva et al., 2017; Donini and O'Donnell, 2005; Yerushalmi et al., 2018). Studies using SIET have already indicated

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the influence of cold acclimation and adaptation on ion and water reabsorption capacity in *Drosophila* (Andersen et al., 2017c; Yerushalmi et al., 2018), but considering the important role of the insect hindgut, it would be valuable to develop a system that can directly measure the combined actions of active and passive ion and water movements under *in vitro* conditions that mimic *in vivo* ion and temperature conditions.

The transport mechanisms currently known to occur in the hindgut (i.e. the rectal pads) of herbivorous insects include basolateral Na^+/K^+ ATPases, which maintain a favorable gradient for Na^+ reabsorption across the apical membrane aided by H^+/Na^+, amino acid/Na^+ and NH_4^+/Na^+ exchangers. Reabsorption of K^+ is intimately linked to apical Cl^- transport, and although the exact mechanism is still debated, two main non-mutually exclusive hypotheses suggest that Cl^- is reabsorbed via: (1) H^+ recycling through H^+/Cl^- symporters and apical V-type H^+ ATPase activity and/or (2) direct Cl^- reabsorption via an electrogenic Cl^- pump (see Gerencser and Zhang, 2003; Hanrahan and Phillips, 1983; Phillips, 1981; Phillips et al., 1987; Phillips et al., 1996). Fluid reabsorption is thought to occur through the scalariform complex in the hindgut (Phillips, 1981; Wall and Oschman, 1975, 1970). Here, the combination of a highly convoluted intercellular space and a high density of energy-demanding Na^+/K^+ ATPases in the intercellular membrane creates an osmotic gradient through the paracellular space of the rectal pads, drawing water through to the basolateral side of the epithelium (Gupta et al., 1980; Phillips et al., 1987). Despite this knowledge of transport mechanisms, few studies have actually measured the transport of fluid and ions across the hindgut of small insects, and no study has so far been able to measure the net movement of ions and water across the *Drosophila* hindgut. It has therefore also been difficult to study the thermal dependency of net transport and evaluate putative role of hindgut capacity in relation to inter- and intra-specific differences in cold tolerance of this genus.

In the present study, we introduce a novel assay capable of measuring *in vitro* ion and water reabsorption across the *Drosophila* hindgut. In our initial experiments, we demonstrate the temporal stability of this assay and investigate how pharmacological blockade of central ion transporters and channels inhibit reabsorption. After these descriptive experiments, we use the assay to compare hindgut reabsorption at benign (24°C) and low temperature (3°C) in three species – *Drosophila montana*, *Drosophila melanogaster* and *Drosophila birchii* – that are characterized by large differences in cold tolerance. Similarly, we use this assay to evaluate the effects of cold acclimation by investigating hindgut transport in cold- and warm-acclimated *D. melanogaster*. With these experiments, we test the hypothesis that cold-tolerant species and cold-acclimated *D. melanogaster* exhibit adaptive changes in reabsorption capacity that will help them to prevent hemolymph hyperkalemia during cold exposure. Specifically, we hypothesize that cold acclimation and cold adaptation are associated with the ability to suppress K^+ reabsorption, and to maintain Na^+ and water reabsorption during exposure to low temperature.

MATERIALS AND METHODS

Animal husbandry

The present study investigated three species of *Drosophila* (*Drosophila montana* Stone, Griffen and Patterson 1941, *Drosophila melanogaster* Meigen 1830 and *Drosophila birchii* Dobzhansky and Mather, 1961; see Table S1 for their origins) that are characterized by considerable differences in cold tolerance. Thus, *D. montana* is a cold-tolerant species [critical thermal minimum (CT_{min})=−0.3±0.1°C, temperature causing 50% cold mortality in the population following a 2 h exposure (LT_{50})=−13.2±0.3°C], *D. melanogaster* has intermediate tolerance (CT_{min}=3.0±0.2°C, LT_{50}=−8.1±0.2°C) and *D. birchii* is a cold-sensitive species (CT_{min}=7.7±0.2°C, LT_{50}=−3.3±0.2°C) (Andersen et al., 2015; Andersen and Overgaard, 2019; MacMillan et al., 2015a). All species were reared under common-garden conditions at 19°C where they were fed on oatmeal-based Leeds medium (for one liter of water: 60 g yeast, 40 g sucrose, 30 g oatmeal, 16 g agar, 12 g methyl paraben and 1.2 ml acetic acid). Fly populations were kept in 200 ml bottles containing 40 ml of medium. To produce experimental flies, adults were allowed to oviposit for 2 h to 2 days (depending on the species) in bottles with medium to ensure a rearing density of 100–200 individuals per bottle. Newly emerged flies were transferred to vials (7 ml medium) and left to mature for 6–9 days before experiments; all experiments were conducted on adult females.

For *D. melanogaster*, we performed additional experiments on flies developmentally acclimated to 15, 19 or 23°C. These flies were produced by having adults oviposit in bottles for 2 h at 19°C and then transferring the egg-containing bottle to thermal cabinets that were maintained at 15, 19 or 23°C. Newly emerged flies were collected in vials and stored at their developmental temperature before being used for experiments at the age of 6–9 days. Again, only adult females were used for experiments.

Quantitative measurement of ion and water reabsorption in *Drosophila* hindgut

Individual flies were briefly submerged in 70% ethanol for sedation and females were then transferred to a glass Petri dish containing standard *Drosophila* saline with a layer of silicone elastomer at the bottom (Sylgaard 184, Dow Corning Corp., Midland, MI, USA) (saline: 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). The head, legs and wings were quickly removed, and the intact gut (crop, foregut, midgut, Malpighian tubules and hindgut) was carefully dissected out, while ensuring that a small piece of cuticle remained around the anus (a representative drawing and a photo of the assay is shown in Fig. 1). Here, it is important to note that the Malpighian tubules and the entire gut (fore-, mid- and hindgut) must be intact so that they display their usual peristaltic contractions. The isolated and still moving gut was then gently transferred to a 50 µl droplet of *Drosophila* saline kept under paraffin oil. Using fine forceps (Dumont #5, 0.05×0.02 mm tips, Fine Science Tools Inc., Foster City, CA, USA) attached to a micromanipulator, the hindgut was carefully drawn horizontally out of the droplet by gripping the small piece of cuticle still attached to the anus. This procedure isolates the hindgut from the remaining gut and Malpighian tubules (which remains immersed in the 50 µl saline droplet). Any saline that still clings to the hindgut after this procedure is removed using a pulled glass capillary. Next, the isolated hindgut is superfused with a smaller droplet of saline (0.2 µl for *D. birchii* and *D. melanogaster* and 0.3 µl for *D. montana*). This droplet contains 100 µmol l^{-1} of the food dye amaranth (Sigma-Aldrich, St Louis, MO, USA), so it is easy to see under the microscope, and the amaranth also makes it easy to see if the small droplet (0.2–0.3 µl) fuses with the larger droplet (which did not contain any amaranth dye). The small droplet clings to the hindgut because of surface tension, and will not envelop the anus because of the hydrophobic nature of the cuticle that still remains (Fig. 1). This preparation therefore has the added benefit that any discharge from the gut will be delivered outside the saline droplet as fecal droplets, which sink to the bottom of the Petri dish. Over time, the isolated hindgut will reabsorb ions and water

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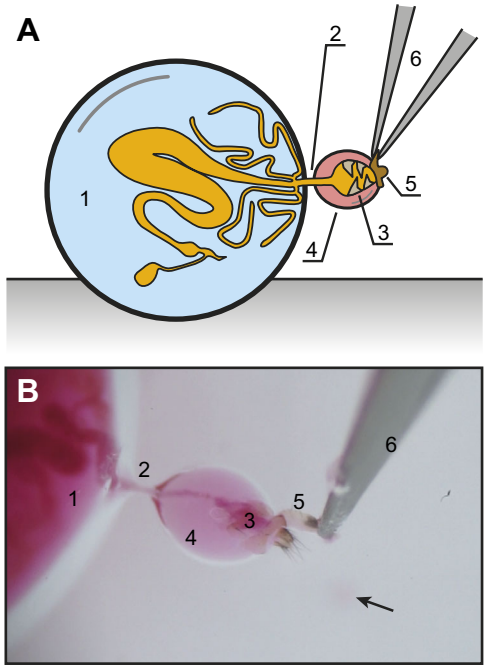

Fig. 1. Hindgut assay setup for *Drosophila melanogaster*. (A) Schematic drawing of the hindgut assay and (B) a picture of the assay applied to a specimen taken through a microscope. 1, A 50 µl droplet of standard *Drosophila* saline containing the fore- and midgut as well as the Malpighian tubules and most of the ileum; 2, ileum; 3, rectum with rectal pads; 4, small droplet of standard *Drosophila* saline dyed with amaranth; 5, anus with cuticle still attached; 6, fine forceps. In B, where parts of the midgut and the Malpighian tubules are also visible, amaranth dye was also added to the large droplet to visualize the Malpighian tubules, midgut and hindgut, and to demonstrate that the primary urine and gut content moves through the ileum and into the hindgut to supply fluid and ions for reabsorption (amaranth dye was not added to the large droplet in any experiments). A small pink droplet of fecal matter can be seen in the background out of focus (arrow).

and the small droplet surrounding the hindgut will gradually change its volume and ion concentrations as the initial saline is altered by the reabsorbate. After a given time period (typically 1 h at 24°C), we extracted the sample from the hindgut using glass capillaries pulled to a fine tip over a flame. This was done by moving the fine tip of the pulled glass capillary immediately next to the hindgut such that it would take up the fluid using capillary pressure. To ensure that all fluid was extracted, the tip of the pulled glass electrode was then gently moved along and across the surface of the hindgut to take up any residual fluid (the small cavity created between the remaining cuticle and the hindgut tends to 'hide' residual fluid). The extracted sample can then be transferred to a dish with hydrated paraffin oil by gently applying pressure to the other end of the capillary tube and can be stored under the hydrated paraffin oil for later estimations of volume and ion concentration. Here, it should be noted that volume estimation should be done before the sample forms a contact surface with the bottom of the dish as this will make the sample non-spherical.

Calculations of fluid and ion reabsorption

Estimates of fluid- and ion-reabsorption rates were derived from the measurements of changes in ion concentrations and volume of the droplet surrounding the hindgut. Volume of the mixture was estimated by imaging under a stereomicroscope (Carl Zeiss Stemi 2000-CS, Carl Zeiss A/S, Birkerød, Denmark) using a Sony α NEX 7 digital camera. After import into ImageJ (Schindelin et al., 2015), the diameter of the sample (d) was measured, and volume of the saline-reabsorbate mixture was calculated using the formula for the volume (v) of a sphere:

$$v = \frac{\pi \times d^3}{6}.$$

Water reabsorption rate was then calculated from the change in volume relative to the initial volume (0.2 or 0.3 µl) as follows:

$$J_{\text{fluid}} = \frac{\Delta v}{\Delta t},$$

where J_{fluid} is the fluid reabsorption rate (nl min^{-1}), Δv is the change in volume (in nl), and Δt is the duration of the experiment (in minutes).

Ion concentrations (Na^+ and K^+) in matching control saline and the saline-reabsorbate mixture droplets were measured using ion-sensitive microelectrodes that were constructed as described in MacMillan et al. (2015a). Before measurements, the electrodes were calibrated in buffers with an order of magnitude difference in concentration (10 and 100 mmol l^{-1} for K^+, and 30 and 300 mmol l^{-1} for Na^+; osmolality was maintained by adding LiCl to the lower concentration standards). The recorded voltage change in these electrodes follows a Nernstian relationship with concentration (58.2 mV per 10-fold change in concentration). Only electrodes where a 10-fold change in concentration elicited a voltage change between 52 and 62 mV (means±s.d.; K^+: 56.3±1.9 mV, Na^+: 55.7±1.7 mV) were deemed useful and outliers were discarded. Raw voltages were digitized using a MP100A data acquisition system and recorded using AcqKnowledge software (Biopac Systems, Goleta, CA, USA). Using these electrodes ion concentrations were calculated as follows:

$$[\text{ion}] = [c] \cdot 10^{\Delta V/S},$$

where [ion] is the concentration of either Na^+ or K^+ in the sample reabsorbate in mmol l^{-1}, [c] is the concentration in the calibration buffer with the lowest calibration (30 or 10 mmol l^{-1}, for Na^+ and K^+, respectively), S is the voltage change observed with a 10-fold change in concentration, and ΔV is the difference between the voltage measured in the lower calibration fluid and the saline-reabsorbate mixture.

Measurement of ion reabsorption rate was calculated based on differences in ion concentrations and volume of the initial and final droplet surrounding the hindgut. To do this, the following formula was used:

$$J_{\text{ion}} = \frac{[\text{ion}]_{\text{end}} \cdot v_{\text{end}} - [\text{ion}]_{\text{start}} \cdot v_{\text{start}}}{\Delta t},$$

where J_{ion} is the rate of ion reabsorption (in pmol min^{-1}), $[\text{ion}]_{\text{end}}$ and $[\text{ion}]_{\text{start}}$ are the concentrations of either Na^+ or K^+ in the saline-reabsorbate mixture at the experiment end and start, respectively (in mmol l^{-1}). v_{start} is the volume of saline supplied to the hindgut in the beginning of the experiment (0.2 or 0.3 µl) and v_{end} is the volume of the saline-reabsorbate mixture at the end of the experiment. Δt is the duration of the experiment (min).

Experimental protocol and validation of assay

The primary scientific aim of the present study was to investigate if/how thermal adaptation and acclimation affects the hindgut capacity to maintain ion balance in *Drosophila* exposed to low temperature. To address this question, a novel method was developed to assess quantitatively the movements of fluid and

ions over the hindgut epithelia of *Drosophila*. To validate this new methodology, a series of experiments was run to investigate the temporal stability of the assay and to briefly examine how active transport could be manipulated both at the hindgut and upstream at the level of the Malpighian tubule. These experiments were all performed in *D. melanogaster* at room temperature (22–23°C).

Initially, the temporal stability of the assay was tested by measuring ion and fluid reabsorption rates every hour for 4 h (N=4). After ensuring the temporal stability of the assay, the effect of NaCN inhibition was investigated to examine if ion and water flux was related to active transport. These experiments were performed by initially measuring flux under control conditions over the first hour and subsequent measure flux following addition of 1 mmol l^{-1} NaCN (N=5). NaCN was added to both the large and small droplet to block all aerobically driven active transport in the Malpighian tubules, foregut and midgut (large droplet), as well as in the hindgut (small droplet). In two additional sets of experiments, the effects of partial blockade of active transport was tested by adding NaCN (1 mmol l^{-1}) to either the small droplet (to inhibit only the hindgut) or the large droplet (to inhibit only the upstream secretion) (N=3 for each experiment).

To examine the role of major transporters involved in hindgut reabsorption, the hindgut of *D. melanogaster* was subjected to three pharmacological agents that inhibit transporters and channels involved in ion and fluid reabsorption: (1) Na^+/K^+ ATPase activity was inhibited with ouabain (1 mmol l^{-1}, N=5); (2) V-type H^+ ATPase activity was inhibited with bafilomycin A_1 (10 µmol l^{-1} in 2% DMSO, N=5) [a parallel experiment tested the effect of saline with 2% DMSO on hindgut reabsorption to ensure that this was not an effect of the DMSO (N=3)]; (3) general Cl^- transport was inhibited with 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS, 100 µmol l^{-1}, N=4) (all chemicals from Sigma-Aldrich, St Louis, MO, USA, except for the bafilomycin A_1 which was from Cayman Chemicals, Ann Arbor, MI, USA). For all treatments, the hindgut was first measured under control conditions after ∼1 h. The preparation was then incubated for 10–15 min with pharmacological agents before the small droplet was changed to assess the effects of pharmacological blockade over a subsequent hour (during this time, the preparation was also exposed to the pharmacological agents).

We also investigated the possibility of active transport regulation by stimulating cAMP pathways. To do so, 1 mmol l^{-1} of the cAMP stimulator 8-bromoadenosine 3′,5′-cyclic monophosphate (Sigma-Aldrich) was added to the buffer (both droplets) to stimulate cAMP pathways in the Malpighian tubules and reabsorption at the hindgut (N=8). Lastly, an experiment was performed in which the concentration of Na^+ in the saline was lowered from the initial 137 mmol l^{-1} to 65 mmol l^{-1} (N=6). For these experiments, it was not possible to change the buffer in which the gut was suspended and they are therefore not compared with the control situation of the same preparations. Instead, the absolute transport rates are compared to the average of the other controls performed at room temperature (22–23°C).

The effects of adaptation and thermal acclimation on hindgut reabsorption capacity

A main objective of this study was to investigate and compare the osmoregulatory capacity at benign and low temperature in three *Drosophila* species with marked differences in cold tolerance (N=7 per species and temperature). Likewise, *D. melanogaster* acclimated to 15, 19 and 23°C (N=7–9 per acclimation temperature and test temperature) were compared to investigate how acclimation influenced hindgut ion and water flux. For all these experiments, ion and water flux was measured at room temperature (24°C) first, after which temperature of the preparation was lowered to 3°C by circulating a cooled 1:1 mixture of water and ethylene glycol through the water-jacketed stage, in which the preparation was kept. Once at 3°C, the procedure of preparing the assay was repeated to obtain repeated measurements of fluid and ion reabsorption. As expected, the transport rates were much lower at low temperature and consequently, measurements took longer to achieve a reasonable change in droplet volume and content (∼3–5 h at 3°C compared with ∼1 h at 24°C).

Data analysis

To estimate the effect of temperature on the transport of ions and water, the temperature coefficients (Q_{10}) were calculated for all three species and for warm- and cold-acclimated *D. melanogaster* as follows:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)},$$

where R_2 is the rate of reabsorption at the highest temperature (T_2, 24°C) and R_1 is the rate of reabsorption at the lowest temperature (T_1, 3°C).

To examine if temperature influenced the selectivity of ion transport (Na^+ transport relative to K^+ transport), the temperature effect ratio was calculated as follows:

$$\text{Temperature effect ratio} = \frac{\Delta Na^+ \text{ reabsorption}}{\Delta K^+ \text{ reabsorption}},$$

where the ΔNa^+ reabsorption and ΔK^+ reabsorption represent the absolute changes in Na^+ and K^+ reabsorption induced by exposure to 3°C relative to 24°C, respectively.

Statistical analyses

All statistical analyses were performed in R 3.6.1 software (https://www.r-project.org/). The stability of fluid and ion reabsorption over time was analyzed with a linear mixed effect model by using the lme function in the nlme package for R (with time as a continuous variable and the fly as a random factor) followed by Dunnett's *post hoc* tests to determine when rates of reabsorption deviated from control values. The effects of pharmacological interventions (NaCN inhibition, cAMP stimulation, inhibition with ouabain, bafilomycin A_1, and DIDS, along with the effect of DMSO) on rates of reabsorption were analyzed using paired t-tests. The effect of lowering the Na^+ concentration in the saline was tested using an unpaired Student's t-test to compare the 'low [Na^+]' reabsorption rate with the average of reabsorption rate from all other control measurements at room temperature that had been performed in 'high [Na^+]' saline.

The effects of species/acclimation and temperature on fluid and ion (Na^+ and K^+) reabsorption rates were analyzed using linear mixed effect models where species (*D. birchii*, *D. melanogaster* or *D. montana*) or acclimation temperature (15, 19 or 23°C) were included in the model as fixed factors along with temperature, while the individual fly was included as a random factor. Interspecific differences in Q_{10} values and temperature effect ratios were analyzed using separate one-way ANOVAs and followed by Tukey's HSD *post hoc* tests. It was, however, not possible to calculate Q_{10} for Na^+ reabsorption of *D. birchii* (because of negative rates of reabsorption at low temperature) and the difference between *D. montana* and *D. melanogaster* was therefore analyzed using a Student's t-test. A similar set of analyses were performed for the

Journal of Experimental Biology (2020) 223, jeb213934. doi:10.1242/jeb.213934

acclimation experiments. In all analyses, the critical level of significance was 0.05, and all values presented are means±s.e.m., unless indicated otherwise. Full details of all analyses can be found in Tables S2 and S3.

RESULTS

Experimental evaluation of hindgut assay

To examine the temporal stability of reabsorption rates in our novel hindgut assay, we collected samples every hour for 4 h from hindguts of *D. melanogaster* operating at room temperature (Fig. 2, see Table S3 for statistical analyses). Fluid reabsorption showed a slow decline over time [P=0.002; fluid reabsorption=1.0 (±0.1) nl min^{-1}−0.1 (±0.0) nl min^{-1} h^{-1}], but remained stable for 3 h before the decline became substantial. A similar temporal decline was also found for K^+ reabsorption [P=0.019; K^+ reabsorption= 17.79±1.80 pmol min^{-1}−1.7 (±0.6) pmol min^{-1} h^{-1}) and Na^+ reabsorption (P<0.001; Na^+ reabsorption=70.3 (±6.9) pmol min^{-1} −10.9 (±2.1) pmol min^{-1} h^{-1}]. In the remaining experiments evaluating the effects of various pharmacological interventions, we obtained a control measurement during the first hour followed by a measurement under 'treatment conditions' during the following hour. The relative changes in activity associated with pharmacological intervention can therefore be compared with the small (and non-significant) changes observed between 0 h and 1 h in the control for time (Fig. 2).

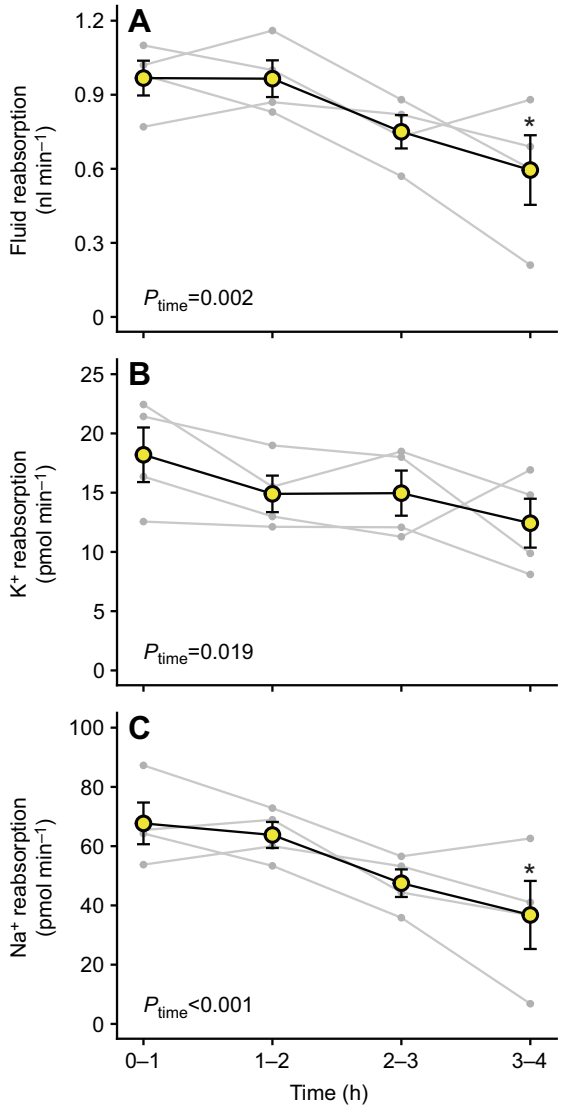

Fig. 2. Stability over time of reabsorption of fluid and ions in the *D. melanogaster* hindgut. Hindgut reabsorption of (A) fluid, (B) K^+ and (C) Na^+ measured over 1 h intervals for a total of 4 h. The assay degraded slowly over time [linear regressions: fluid reabsorption=1.0(±0.1) nl min^{-1} −0.1 (±0.0) nl min^{-1} h^{-1}; K^+ reabsorption=17.7 (±1.8) pmol min^{-1}−1.7 (±0.6) pmol min^{-1} h^{-1}; Na^+ reabsorption=70.3 (±6.9) pmol min^{-1}−10.9 (±2.1) pmol min^{-1} h^{-1}] but was stable for the first ∼2 h and only declined significantly after the third hour of the experiment [asterisks indicate when the time point differed from control values (0–1 h) based on Dunnett's *post hoc* tests].

Overall, we found that control preparations were performing at similar rates across the different experiments (see Table 1): fluid reabsorption rate was 1.0±0.1 nl min^{-1} (mean±s.d., range: 0.8–1.2), K^+ reabsorption rate was 17.2±1.5 pmol min^{-1} (range 14.9–20.3) and Na^+ reabsorption rate was 69.3±6.4 pmol min^{-1} (range 58.5–77.8).

The effects of pharmacological stimulation/inhibition of ion transport are also reported in Table 1 (see Table S2 for statistical analyses). Hindgut reabsorption is clearly dependent on aerobic ATP production since exposure to 1 mmol l^{-1} NaCN caused a 60± 9% (mean±s.e.m.) reduction in fluid reabsorption rate and a 67±8% and 72±10% reduction in K^+ and Na^+ reabsorption rates, respectively. The ability to reabsorb ions and fluid is, however, also dependent on the provision of fluid and ions originating from the fore- and midgut (gut content) as well as the Malpighian tubules (primary urine). Thus, when we blocked the upstream supply of ions and fluid by adding 1 mmol l^{-1} NaCN to the large droplet, fluid reabsorption was reduced by 69±7%, K^+ reabsorption by 62±4%, while Na^+ reabsorption was reduced by 75±10%. Accordingly, a blockade of both the upstream osmoregulatory organs and the hindgut with 1 mmol l^{-1} also resulted in a severe inhibition of fluid (−83±6%), K^+ (−78±2%) and Na^+ (−97±5%) reabsorption (Table 1).

We also briefly examined the role of some major ion transporters and channels suspected to be involved in hindgut reabsorption of herbivorous insects. We first blocked Na^+/K^+ ATPase activity with 1 mmol l^{-1} ouabain. This resulted in a 52±14% decrease in fluid reabsorption. K^+ reabsorption remained relatively unchanged (2± 19% decrease) while Na^+ reabsorption showed a non-significant drop of 38±12%. Application of the V-type H^+ ATPase blocker bafilomycin A_1 (10 µmol l^{-1}) resulted in a 45±5% reduction of fluid reabsorption. K^+ reabsorption was slightly, and non-significantly reduced (17±17% decrease) while Na^+ reabsorption decreased by 60± 6%. The addition of bafilomycin A_1 required the use of a 2% DMSO concentration in the saline; however, a parallel set of experiments showed that 2% DMSO alone had neither major, nor significant effects on reabsorption rates (Table 1). DIDS (100 µmol l^{-1}) was used to block all Cl^--related transporters and channels in the hindgut and resulted in an almost complete abolishment of all transport; fluid reabsorption was reduced by 84±4%, K^+ reabsorption by 78±7%, and Na^+ reabsorption by 92±3. To examine if stimulation of cAMP pathways altered hindgut reabsorption, the cAMP stimulator 8-bromo cAMP was added (1 mmol l^{-1}), resulting in a 47±10% increase in fluid reabsorption associated, a doubling of K^+ reabsorption (102± 28% increase) and a 68±19% increase in Na^+ reabsorption. Finally, we examined the effects of lowering the Na^+ concentration in the fluid surrounding both the upstream osmoregulatory organs and the hindgut (from 137 to 65 mmol l^{-1}). This elicited a small and non-significant reduction in fluid reabsorption (19±10% decrease). K^+ reabsorption remained unchanged (0±7% decrease) while Na^+ reabsorption was reduced by 45±7%.

Journal of Experimental Biology (2020) 223, jeb213934. doi:10.1242/jeb.213934

Table 1. Results of pharmacological interventions on the reabsorption of fluid and ions in the hindgut of *Drosophila melanogaster*

Treatment	Application	Variable	Control	Treatment	Change (%)	*N*	*P*-value
1 mmol l^{-1} NaCN	Hindgut	**Fluid**	**1.0±0.0**	**0.4±0.1**	**−60±9**	3	**0.018**
		K^+	16.2±3.5	5.2±1.8	−61±8		0.055
		Na^+	**58.5±10.2**	**17.8±8.7**	**−72±10**		**0.014**
1 mmol l^{-1} NaCN	Malpighian tubules, fore- and midgut	**Fluid**	**1.2±0.2**	**0.4±0.1**	**−69±7**	3	**0.005**
		K^+	**20.3±5.3**	**8.1±2.6**	**−62±4**		**0.048**
		Na^+	**77.8±17.5**	**23.1±12.2**	**−75±10**		**0.014**
1 mmol l^{-1} NaCN	Hindgut, Malpighian tubules, fore- and midgut	**Fluid**	**1.0±0.1**	**0.2±0.1**	**−83±6**	5	**0.001**
		K^+	**17.3±3.2**	**3.9±1.0**	**−78±2**		**0.004**
		Na^+	**71.1±11.2**	**3.3±4.3**	**−97±5**		**0.002**
1 mmol l^{-1} ouabain	Hindgut	**Fluid**	**0.9±0.1**	**0.4±0.1**	−52±14	5	**0.029**
		K^+	16.4±3.4	14.0±2.9	−2±19		0.265
		Na^+	73.0±7.3	43.4±8.0	−38±12		0.058
10 µmol l^{-1} bafilomycin A_1 (2% DMSO)	Hindgut	**Fluid**	**1.1±0.1**	**0.6±0.0**	**−45±5**	5	**0.004**
		K^+	18.2±2.4	14.3±2.5	−17±17		0.350
		Na^+	**70.8±8.3**	**26.9±3.4**	**−60±6**		**0.006**
2% DMSO	Hindgut	Fluid	1.0±0.1	0.9±0.1	−6±2	3	0.070
		K^+	14.9±1.4	16.4±1.4	+10±3		0.081
		Na^+	72.7±6.5	64.6±3.0	−10±6		0.237
100 µmol l^{-1} DIDS	Hindgut	**Fluid**	**0.9±0.1**	**0.1±0.0**	**−84±4**	4	**0.003**
		K^+	**17.6±0.6**	**3.9±1.1**	**−78±7**		**0.002**
		Na^+	**71.9±4.7**	**5.6±2.1**	**−92±3**		**0.001**
1 mmol l^{-1} 8-bromo-cAMP	Hindgut, Malpighian tubules, fore- and midgut	**Fluid**	**0.8±0.1**	**1.1±0.2**	**+47±10**	8	**0.002**
		K^+	**16.8±1.6**	**32.2±3.7**	**+102±28**		**0.002**
		Na^+	**59.5±5.1**	**97.1±8.8**	**+68±19**		**0.002**
65 mmol l^{-1} Na^+ in saline	Hindgut, Malpighian tubules, fore- and midgut	Fluid	1.0±0.0*	0.8±0.1	−19±10	6	0.091
		K^+	17.2±0.1*	17.2±0.1	−0±7		0.984
		Na^+	**68.0±3.0***	**37.6±5.0**	**−45±7**		**<0.001**

Control measurements were followed by an experiment in which the hindgut, the upstream foregut and midgut and Malpighian tubules, or both systems were exposed to a pharmacological agent to test the involvement of active transport and specific transporters in determining hindgut fluid and ion reabsorption. Fluid reabsorption is presented as nl min^{-1} while ion reabsorption (K^+ and Na^+) is in pmol min^{-1}. Reabsorption rates presented in bold showed a significant effect of the intervention; for full statistical analyses see Table S2.

*The control values for reabsorption in the experiments involving lowered [Na^+] in the saline consist of 36 measurements performed in the other experiments under the same conditions (i.e. control measurements for all other experiments presented in this table and the control measurements from the time control).

Interspecific differences in hindgut transport during cold exposure

Hindgut fluid and ion reabsorption was measured at warm (24°C) and low (3°C) temperature in three *Drosophila* species characterized by high (*D. montana*), intermediate (*D. melanogaster*) and low (*D. birchii*) tolerance to cold. Fluid reabsorption rates were similar at the warm temperature in all species (~0.9 nl min^{-1}; P=0.716) (Fig. 3A). As hypothesized, fluid reabsorption decreased in all species during cold exposure (P<0.001), and this effect tended to be larger in the most cold-sensitive species such that fluid reabsorption rate was decreased to 0.06±0.02 nl min^{-1} in *D. birchii*, 0.15±0.03 nl min^{-1} in *D. melanogaster* and 0.22±0.04 in *D. montana* (P=0.279). This tendency was more obvious (and statistically significant, P=0.004) when the response to lowered temperature was analyzed from the species-specific Q_{10} values, which ranged from 4.5±0.8 for *D. birchii* to 2.6±0.3 for *D. melanogaster* and 1.9±0.2 for *D. montana* (Fig. 3B).

The K^+ reabsorption rate differed between the three species at 24°C with the highest rate (29.3±2.6 pmol min^{-1}) in the cold-tolerant *D. montana*, an intermediate rate (16.9±1.9 pmol min^{-1}) in *D. melanogaster* and the lowest rate (8.0±1.5 pmol min^{-1}) in the cold-sensitive *D. birchii* (P<0.001) (Fig. 3C). Cooling to 3°C reduced K^+ reabsorption in all species (P<0.001), but did so in a species-specific manner (P<0.001) such that it was decreased to ~1–4 pmol min^{-1} in all species at 3°C. These differences were, however, not reflected in their respective Q_{10} values, where *D. montana* (3.5±0.6) tended to have a higher Q_{10} than *D. melanogaster* (2.3±0.2), but was relatively similar to that of *D. birchii* (2.9±0.6) (Fig. 3D).

Rates of Na^+ reabsorption varied considerable between species at warm temperature (Fig. 3E) with values of 76.0±6.9, 70.5±6.2 and 56.0±8.1 pmol min^{-1} for *D. montana*, *D. melanogaster* and *D. birchii*, respectively (P=0.001). Cold exposure decreased Na^+ reabsorption in all species (P<0.001), and the absolute reduction was similar across the three species (no interaction, P=0.552) such that the highest Na^+ reabsorption was found in *D. montana* (25.4±5.6 pmol min^{-1}), followed by *D. melanogaster* (12.1±2.6 pmol min^{-1}), while *D. birchii* switched to net Na^+ secretion (−7.4±4.1 pmol min^{-1}) (Fig. 3E). The negative rate of reabsorption found for *D. birchii* prevents a calculation of a Q_{10}; however, when comparing the two other species, we found a non-significant tendency (P=0.096) for a lower Q_{10} in the cold-tolerant *D. montana* (1.9±0.2) compared with that of *D. melanogaster* (2.6±0.2) (Fig. 3F).

The different proportional effects of temperature on Na^+ and K^+ reabsorption were also reflected in large and significant differences in the temperature effect ratio of Na^+ versus K^+ reabsorption (P<0.001). Thus, *D. birchii* was characterized by a much larger reduction in Na^+ reabsorption relative to the reduction in K^+ reabsorption (temperature effect ratio of 11.7±1.9) than *D. montana* (1.9±0.5) with intermediate values for *D. melanogaster* (5.1±1.4) (Fig. 5A). In other words, the cold-sensitive species (*D. birchii*) reduces Na^+ reabsorption rate ~12-fold more than it reduces K^+ reabsorption rate while the cold-tolerant species (*D. montana*) reduces Na^+ reabsorption rate only 2-fold compared with the reduction in K^+ reabsorption rate.

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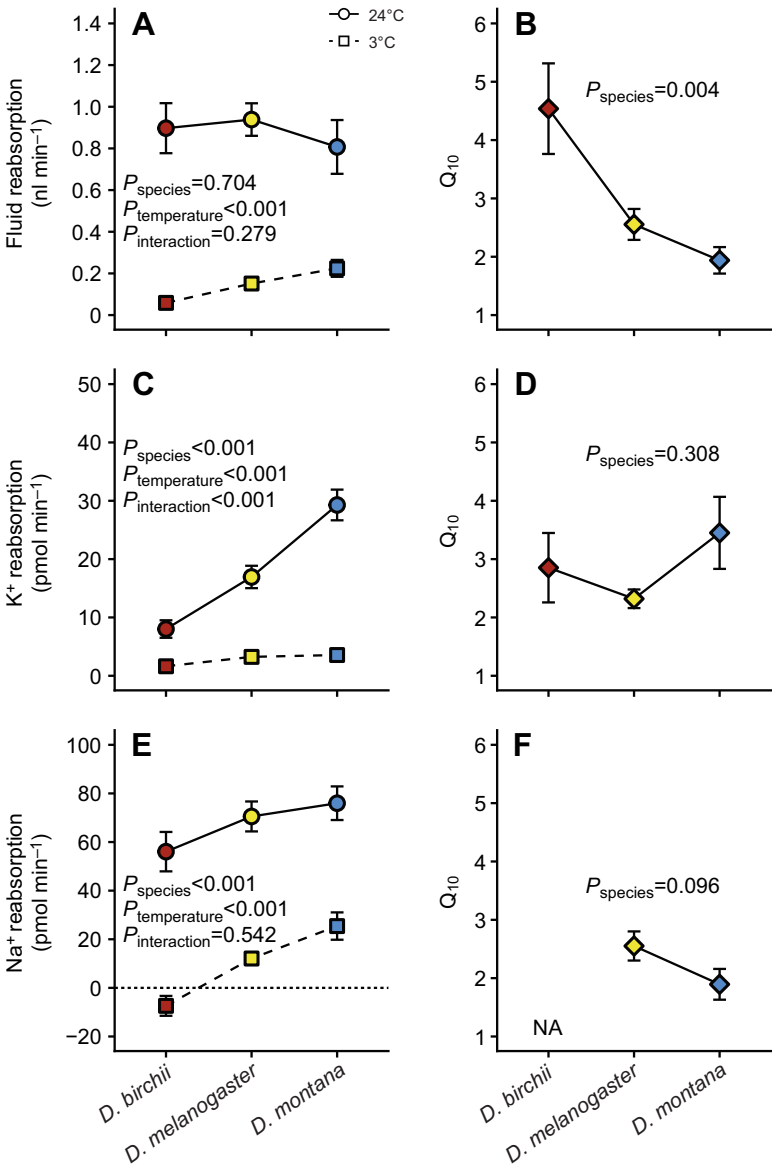

Fig. 3. The effect of temperature on the fluid and ion reabsorption at the hindgut of three different *Drosophila* species. Hindgut reabsorption (left column) was measured at 24°C (circles) and 3°C (squares) and from these results the representative Q_{10} values were calculated (right column) for fluid (A,B), K^+ (C,D) and Na^+ (E,F). The horizontal dashed line in C indicates zero reabsorption, and negative values indicate the transition to net secretion. *N*=7 per species and temperature combination, and error bars not shown are covered by the symbols. For Na^+ reabsorption, the switch to Na^+ secretion prevented calculation of Q_{10} for *D. birchii* (NA).

Intraspecific differences in hindgut transport during cold exposure

In a parallel set of experiments, we tested how acclimation of *D. melanogaster* to warm (23°C), intermediate (19°C) and cool (15°C) temperature affected hindgut reabsorption. Overall, the effects of cold acclimation (Fig. 4) were very similar to those found for cold adaptation (Fig. 3). There was a significant difference in fluid reabsorption rate between acclimation groups (P=0.050), which were largely driven by differences measured at the warm temperature (Fig. 4A). Thus, fluid reabsorption rate in warm-acclimated *D. melanogaster* (1.2±0.1 nl min^{-1}) was higher than that of controls (19°C acclimated; 0.9±0.1 nl min^{-1}) and cold-acclimated flies (0.8±0.1 nl min^{-1}). Exposure to cold drastically lowered these rates (P<0.001) in all acclimation groups, but the magnitude of this reduction was dependent on the acclimation treatment (P<0.001). Cold-acclimated flies maintained a higher rate of fluid reabsorption at 3°C (0.24±0.03 nl min^{-1}) compared with control flies (0.15±0.03 nl min^{-1}), which in turn had higher reabsorption rates than their warm-acclimated conspecifics (0.11 ±0.01 nl min^{-1}). These differences also manifest in the derived Q_{10} values (P<0.001), which ranged from 3.4±0.3 in warm-acclimated *D. melanogaster* to 2.6±0.3 in controls and 1.8±0.1 in the cold-acclimated conspecifics (Fig. 4B).

K^+ reabsorption (Fig. 4C) was influenced by acclimation temperature, (P=0.002) with the highest rates measured in the cold-acclimated flies (21.1±2.2 pmol min^{-1}), intermediate rates measured in control flies (16.9±1.9 pmol min^{-1}) and the lowest rates measured in warm-acclimated flies (11.1±1.2 pmol min^{-1}). These rates were markedly reduced by exposure to low temperature (P<0.001), but the degree of suppression depended on acclimation temperature (P=0.003) such that cold-acclimated flies were better able to maintain rates of K^+ reabsorption (6.5± 0.5 pmol min^{-1}) compared with both control and warm-acclimated flies (3.2±0.7 pmol min^{-1} and 3.9±0.4 pmol min^{-1}, respectively). These patterns resulted in Q_{10} being highest in control flies (2.3±0.2), whereas it was similar in the cold- and warm-acclimated conspecifics (1.7±0.0 and 1.7±0.1, respectively) (Fig. 4D).

Rates of Na^+ reabsorption were generally independent of acclimation temperature (P=0.995), but there was a highly

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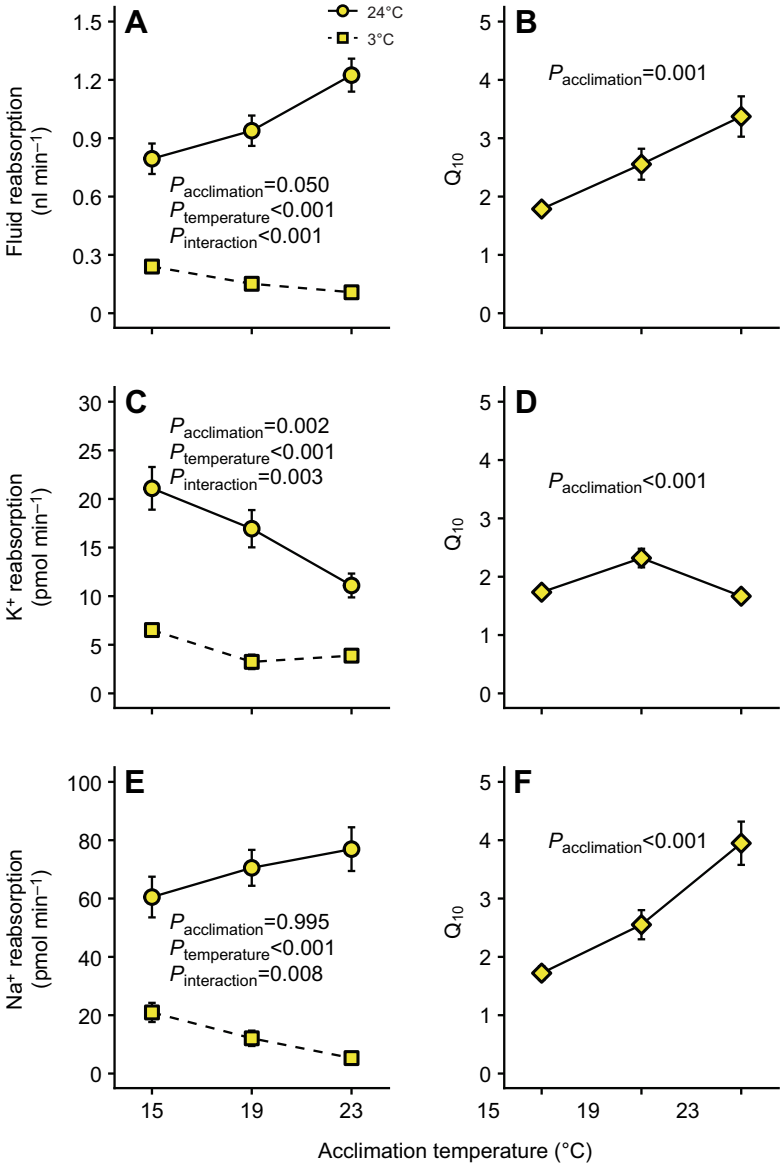

Fig. 4. The effect of cold exposure on hindgut reabsorption in acclimated *D. melanogaster*. Reabsorption as the hindgut (left column) was measured at 24°C (circles) and 3°C (squares) in acclimated *D. melanogaster* and representative Q_{10} values were calculated (right column) for fluid (A,B), K^+ (C,D) and Na^+ (E,F) reabsorption. *N*=9, 7 and 9 per acclimation temperature and temperature combination, and error bars not shown are covered by the symbols.

significant interaction between acclimation group and the response to cooling (Fig. 4E). Thus, Na^+ reabsorption was markedly reduced by exposure to 3°C ($P<0.001$), but this reduction was dependent on acclimation temperature ($P=0.008$) such that cold-acclimated flies had the highest rate of Na^+ reabsorption at 3°C (21.0±3.3 pmol min^{-1}) while control flies reabsorbed 12.1±2.6 pmol min^{-1} and warm-acclimated flies only managed to reabsorb 5.3±1.1 pmol min^{-1}. The derived Q_{10} value therefore also showed significant differences between acclimation treatments ($P<0.001$) with the lowest values found in cold-tolerant, cold-acclimated flies (1.7±0.1), the highest in warm-acclimated flies (3.9±0.4) and an intermediate Q_{10} in control flies (2.6±0.2) (Fig. 4F).

To evaluate the cold-induced reduction in Na^+ reabsorption relative to the cold induced reduction in K^+ reabsorption we calculated the temperature effect ratios of ΔNa^+ reabsorption/ΔK^+ reabsorption for the three acclimation groups. Here, we found a clear effect of acclimation (Fig. 5B, $P=0.004$) such that the cold-acclimated flies had the lowest ratio (2.8±0.4), control flies an intermediate ratio (5.1±1.4), and warm-acclimated flies the highest ratio (12.4±2.8). Accordingly, warm acclimated flies have a much larger reduction in Na^+ reabsorption rate than cold-acclimated flies when this is calculated proportional to their reduction in K^+ reabsorption rate.

DISCUSSION

A novel assay to study epithelial function in the hindgut of *Drosophila*

Maintenance of extracellular volume and ion composition is essential to survival in all animals (Bernard, 1872; Beyenbach, 2016; Edney, 1977; Harrison et al., 2012), and in insects, it is primarily the Malpighian tubules and the gut that are responsible for maintaining osmotic and ionic balance of the hemolymph (Edney, 1977; Phillips, 1970). Studies of bulk ion and water transport in small insects (<10 mg) has hitherto focused on Malpighian tubules because of the relative ease in preparing and measuring net secretion in this system (using the Ramsay assay; see Dow et al., 1994 and Rheault and O'Donnell, 2004). Here, we present a novel experimental assay which allows *in vitro* measurements of bulk fluid and ion movement across the hindgut epithelia in *Drosophila*. Fluid reabsorption of the hindgut remained relatively stable over time (~2 h, Fig. 2), which allows for repeated measurements.

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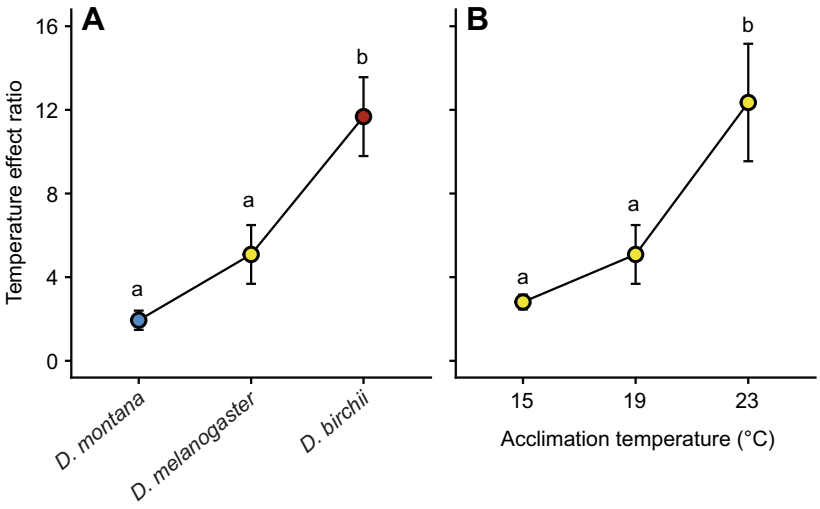

Fig. 5. The temperature effect ratio between K^+ and Na^+ reabsorption. The temperature effect ratio is presented here as the absolute effect of cold exposure on Na^+ reabsorption relative to the effect on K^+ reabsorption in (A) three *Drosophila* species and (B) acclimated *D. melanogaster*. *N*=7 per species and 9, 7 and 9 per acclimation temperature for *D. melanogaster* (note that the data for *D. melanogaster* are shown in both A and B as these flies were acclimated to 19°C for the interspecific experiments). Groups not sharing letters are statistically different based on Tukey's HSD *post hoc* tests.

Furthermore, we found the predicted effects of metabolic blockade and pharmacological intervention (Table 1) and exposure to low temperature (general reduction in transport with Q_{10} of ∼2–5, Figs 3 and 4) on the rates of reabsorption.

By comparing the absolute rates of reabsorption from this study with measurements of primary urine secretion from *D. melanogaster* (from MacMillan et al., 2015a) we found that the Malpighian tubules secrete ∼3.4 times more fluid, ∼28.5 times more K^+ and ∼6% more Na^+ than is being reabsorbed. Some of these differences could relate to the methodological differences between the Ramsay assay and the hindgut assay presented here: in the Ramsay assay, it is often difficult to include the lower, reabsorptive segment of the tubule (see O'Donnell and Maddrell, 1995) and there is no fluid pressure to be overcome at the end of the secreting tubule, meaning that secretion is probably overestimated. In the hindgut assay, the Malpighian tubules may work against a fluid pressure inside the gut; the primary urine passes through the reabsorptive tubule segment before passing into the ileum, where it is mixed with gut content coming from the midgut, altering the amount and content of substrate (e.g. gut content and primary urine) to reabsorb from. Reabsorption in the midgut is also likely to change the substrate available for reabsorption. Lastly, we did not quantify the amount of fluid and ions leaving the system as fecal droplets which could also explain why rates of secretion at the Malpighian tubules and absorption at the hindgut are not aligned. Sporadic measurements of the ion concentration of the fecal droplets of control *D. melanogaster* did, however, reveal a high K^+ concentration (95.6±6.5 mmol l^{-1}, *N*=14) and a moderate Na^+ concentration (48.2±3.5 mmol l^{-1}, *N*=14). Unfortunately, we did not measure the volume of these droplets, but this could at least partially account for the discrepancy in the reported transport rates.

The dynamic interplay between secretion and reabsorption is also demonstrated from the studies in which we inhibit ATP-dependent transport at the Malpighian tubules with NaCN. This treatment reduced both fluid and cation reabsorption at the hindgut to an extent similar to that of NaCN poisoning of the hindgut itself (Table 1). This effect is probably not caused by CN^- ions being transported by the fluid flow because NaCN blockade at both ends of the system caused even higher reductions in fluid reabsorption, but it demonstrates logically that hindgut reabsorption is dependent on the provision from the osmoregulatory organs upstream. This was also seen from the experiment in which we decreased buffer Na^+ concentration. This treatment leads to a reduction in the primary urine Na^+ concentration (Beyenbach, 2019; MacMillan et al., 2015a), and accordingly, we found here that Na^+ reabsorption is also reduced (Table 1).

The mechanisms underlying ion and water reabsorption in the insect hindgut have been most studied in locusts (primarily by Phillips and co-workers, see Black et al., 1987; Hanrahan and Phillips, 1983; Phillips, 1981; Phillips et al., 1987; Phillips et al., 1996). Using locusts, they proposed the involvement of basolateral and intermembrane (i.e. within the scalariform complex) Na^+/K^+ ATPase activity driving particularly Na^+ and fluid reabsorption, apical V-type H^+ ATPase activity coupled to H^+ and Cl^- recycling driving Cl^- and secondarily K^+ reabsorption, and the involvement of an unidentified apical Cl^- ATPase reabsorbing Cl^- combined with basolateral Cl^- channels and K^+ following secondarily through channels. The pharmacological blockades used in this study suggest at least some similarity between the locust model and the mechanisms involved in *Drosophila* hindgut reabsorption: (1) inhibition of Na^+/K^+ ATPase activity with ouabain greatly reduced Na^+ and fluid reabsorption; (2) blockade of V-type H^+ ATPase activity with bafilomycin A_1 reduced fluid and Na^+ reabsorption suggesting a role for H^+ recycling and likely also the apical membrane potential in reabsorption of fluid and Na^+; (3) blockade of Cl^- channels and transporters with DIDS almost completely abolished transport, suggesting that all cation transport is either linked directly to Cl^- transport or at least reliant on the movement of Cl^- to maintain charge neutrality. Both Na^+/K^+ ATPase and V-type H^+ ATPase are highly expressed and enriched in the *Drosophila* hindgut and rectal pads (FlyAtlas; see Chintapalli et al. 2007; Chintapalli et al., 2013; Leader et al., 2018) and are therefore strong candidates for involvement in maintenance of ion and water homeostasis, and so are several other ion transporters and channels such as Na^+-K^+-2Cl^- cotransporters, inwardly rectifying K^+ channels and a range of Cl^- channels and anion exchangers.

Furthermore, we demonstrate that at least some of these processes could be modulated by cAMP, as addition of the cAMP-stimulator 8-bromo cAMP greatly increased reabsorption of both fluid and cations, particularly K^+. Increased intracellular concentrations of cAMP are a typical response to several forms of neuroendocrine stimulation (Coast et al., 2002; Dow et al., 2018; Phillips and Audsley, 1995) and these findings therefore indicate a potential for humoral regulation of hindgut reabsorption, although a multitude of cellular mechanisms are modulated via this pathway. Nonetheless, we found evidence that at least some of the transporters involved in

hindgut reabsorption in *Drosophila* are shared with locusts (see Phillips et al., 1987), but we stress that the mechanisms involved in this transport are complex and likely involve a range of channels, transporters and neuroendocrine factors (with more being identified; e.g. Luan et al., 2015), which could now be studied in further detail using the novel assay presented here.

The hindgut assay requires relatively modest experimental equipment (a microscope, a camera, a micromanipulator and a steady hand) and the simple experimental approach of this assay will allow researchers to address many questions regarding epithelial function of a model insect (*Drosophila*) and probably also other small insects such as mosquitos. The hindgut assay is more time-consuming than the Ramsay assay, but has several advantages compared with the SIET assay currently used to study hindgut ion flux. For example, it is possible to measure bulk transport of all ions, whereas measurements of Na^+ transport are difficult using the SIET system as it requires a non-physiologically low saline Na^+ concentration (Naikkhwah and O'Donnell, 2012). Furthermore, our hindgut assay allows for measurement of bulk fluid transport. The development and optimization of the Ramsay assay paved the way for a multitude of mechanistic and comparative studies of excretory epithelia and we hope this hindgut assay will inspire future research on the role of reabsorptive epithelial function (Beyenbach et al., 2010; Dow and Davies, 2003; Dow et al., 2018; Halberg et al., 2015; Wheeler and Coast, 1990).

Cold-adapted species preserve osmoregulatory capacity at low temperatures

Exposure to stressful cold causes chill-susceptible insect species to lose ion balance characterized by hemolymph hyperkalemia, leading to tissue injury and death (Bayley et al., 2018; Overgaard and MacMillan, 2017). Accordingly, several studies have indicated a role of sustained osmoregulatory function at low temperature in mitigating this debilitating hemolymph hyperkalemia (Andersen et al., 2017c; Des Marteaux et al., 2017; Gerber and Overgaard, 2018; MacMillan et al., 2015a; Yi and Lee, 2005). In the present study, we examined three chill-susceptible *Drosophila* species that are known to differ markedly in their cold tolerance. We have previously used this comparative model system and the difference in cold tolerance is exemplified by the highly chill-tolerant *D. montana* having a markedly lower LT_{50}, a much quicker recovery following a standard cold exposure and a much lower temperature threshold for the transition into chill coma than the moderately chill-tolerant *D. melanogaster* and the chill-sensitive *D. birchii* (Andersen et al., 2015; Andersen and Overgaard, 2019).

Consistent with our hypothesis, we found dramatic interspecific differences in hindgut responses to low temperature. All species reduced ion and fluid reabsorption when exposed to cold but the reductions in reabsorption of fluid and specific ions differed among species. The most cold-tolerant species (*D. montana*) was able to defend Na^+ and fluid reabsorption compared with the reduction in K^+ reabsorption, while the more cold-sensitive congeners (e.g. *D. birchii*) reduced Na^+ and fluid reabsorption more compared with K^+ reabsorption rates (see Fig. 3 and Fig. 5A). These adaptive responses all act to prevent the characteristic hyperkalemic condition that causes chill injury. Specifically, preservation of Na^+ reabsorption helps to prevent loss of Na^+ balance which, along with the maintained rate of fluid reabsorption, will secure hemolymph volume. Hyperkalemia in insects is often attributed to the reduction of hemolymph volume that concentrates K^+ remaining in the extracellular fluid (MacMillan and Sinclair, 2011; Olsson et al., 2016). Preservation of fluid reabsorption of the cold-adapted species exposed to cold is therefore important for normokalemia, and this is further aided by a relative reduction in K^+ reabsorption. These results are corroborated by previous studies of osmoregulation in the same *Drosophila* species. For example, Andersen et al. (2017c) used the SIET method to demonstrate that cold-tolerant *Drosophila* species (e.g. *D. montana*) lower K^+ reabsorption more than their cold sensitive congeners. Thus, the assay is able to detect clear species-specific differences in response to temperature and the findings in the present study support those reported previously.

The interspecific differences we observe in the hindgut response to cold are more or less opposite to the effects observed for changes in secretory function in the Malpighian tubules previously (MacMillan et al., 2015a) and the responses therefore reinforce each other. Specifically, for the cold-tolerant *D. montana*, it is observed that K^+ secretion is relatively higher in the Malpighian tubules and relatively lower in the hindgut in response to cold. Similarly, fluid and particularly Na^+ secretion are reduced in cold-exposed Malpighian tubules of *D. montana* and both fluid and Na^+ reabsorption are defended better. Thus, the thermal responses of both secretion and reabsorption of K^+, Na^+, and fluid act to alleviate hemolymph hyperkalemia in chill-tolerant insects.

We observed a reversal of Na^+ flux from Na^+ reabsorption to functional Na^+ secretion in *D. birchii* (Fig. 3E). Leak of Na^+ down electrochemical gradients from the hemolymph into the gut was first proposed as a cause of reduced hemolymph volume by MacMillan and Sinclair (2011), who studied changes in volume and composition of hemolymph during cold exposure in the fall field cricket (*Gryllus pennsylvanicus*). They found that Na^+ and fluid leaked towards the gut, and since then, several other reports have implicated similar disturbances in other insects (Des Marteaux and Sinclair, 2016; Gerber and Overgaard, 2018; Koštál et al., 2007; Koštál et al., 2004; Koštál et al., 2006) including *Drosophila* (MacMillan et al., 2015a,b, 2016). Additionally, leak assays have been used to investigate the passive movement of Na^+ over the gut in insects and these studies have generally found that cold-sensitive *Drosophila* are more susceptible to paracellular transepithelial leak at low temperature (Andersen et al., 2017c; MacMillan et al., 2017). Despite these observations, this is the first study to present direct measurements of cold-induced leak of ions down their electrochemical gradients across a transporting epithelium in a chill-susceptible insect.

Thermal acclimation mitigates loss of osmoregulatory function in the cold

Differences in cold tolerance are also found within species (e.g. intraspecific variation), and insects are highly plastic in their lower thermal limits if given time to acclimate or acclimatize to cold (Lee, 2012; Mellanby, 1954; Overgaard et al., 2011; Sinclair et al., 2003), *D. melanogaster* included (Colinet and Hoffmann, 2012; MacMillan et al., 2017). It is therefore not surprising that cold-acclimated or winter-acclimatized *Drosophila* are more chill tolerant and are better able to protect their hemolymph volume and ion balance (MacMillan et al., 2015b, 2016). Accordingly, recent studies have found that cold-acclimated insects are better able to maintain osmoregulatory function when exposed to stressful cold (Gerber and Overgaard, 2018; Yerushalmi et al., 2018; Yi and Lee, 2005). These studies have demonstrated plasticity in the function of Malpighian tubules and the gut, but prior to this study, no measurements of bulk fluid and ion movements have been made for the hindgut of small insects like *Drosophila*.

Here, we used *D. melanogaster* acclimated to three temperatures known to alter cold tolerance (Bubliy et al., 2002; MacMillan et al., 2015d; Schou et al., 2017). Similar to the experiments on

interspecific variation (see Fig. 3), we found differences between acclimation groups in their hindgut reabsorption capacity at low temperature (Fig. 4). Exposure to low temperature reduced reabsorption regardless of acclimation temperature, but as with the chill-tolerant *D. montana*, cold-acclimated *D. melanogaster* were better at maintaining fluid transport rates (Fig. 4A,B) and also exhibited a larger reduction in K^+ reabsorption (Fig. 4C,D), and an improved maintenance of Na^+ reabsorption (Fig. 4E,F). These findings corroborate previous findings made in an orthopteran (Gerber and Overgaard, 2018) and SIET measurements made on the hindgut of acclimated *D. melanogaster* (Yerushalmi et al., 2018). Combined with measurements of ion and fluid secretion by the Malpighian tubules of acclimated *D. melanogaster* (Yerushalmi et al., 2018), the overall result is highly similar to that described between species: improved cold tolerance is achieved through modulations to the osmoregulatory systems that act to protect hemolymph volume and Na^+ concentration and alleviate hemolymph hyperkalemia.

Future directions

Overall, there is now mounting evidence that improved chill tolerance in *Drosophila* is tightly linked to the ability to maintain osmoregulatory function at low temperature in both sides of the osmoregulatory system: the combined actions of the Malpighian tubules and the hindgut act in synchrony to prevent loss of hemolymph volume and hyperkalemia. While the present study identifies clear inter- and intraspecific differences in ion and fluid transport and their response to cold, it does not reveal the physiological mechanism behind these differences.

Recent studies have suggested that neuroendocrine regulation plays a central role in modulating cold tolerance via actions on secretory mechanisms (Terhzaz et al., 2015; MacMillan et al., 2018). However, both the current and previous studies have demonstrated differences in osmoregulatory capacity using *in vitro* assays that are devoid of neuroendocrine input. The cAMP stimulation experiments conducted in this study indicate, at the very least, that there is a potential for the presence of regulatory mechanisms acting via cAMP pathways; it does not, however, provide insight into the nature of these and additional research is needed to investigate this in detail. Nonetheless, it is clear that at least some of the differences found in relation to acclimation and adaptation must relate to more permanent physiological adjustments. Such adjustments could include changes in protein expression or changes in membrane phospholipid composition which could affect the active transport capacity as well as the passive conductance of the epithelia.

The pharmacological interventions used to target specific ion transporters in this study revealed several key candidates for involvement in fluid and ion reabsorption in the *Drosophila* hindgut, namely Na^+/K^+ ATPase activity, the V-type H^+ ATPase activity and Cl^- transporters/channels, but more research, specifically immunohistochemical localization and enzyme activity assays, is needed before this can be verified. Other highly enriched transporters and channels include a Na^+-K^+-2Cl^- cotransporter and inwardly-rectifying K^+ channels (Chintapalli et al., 2013; Leader et al., 2018). Both have specific blockers (bumetanide and Ba^{2+}, respectively), are essential for Malpighian tubule function (see Ianowski and O'Donnell (2004) and their enriched expression in the hindgut could indicate key roles in maintaining ion and water reabsorption. Thus, future research could be directed towards unraveling: (1) the exact details of the physiological mechanism driving hindgut reabsorption; (2) the dynamic interplay between secretion at the Malpighian tubules and reabsorption at the hindgut; (3) the putative humoral/neuroendocrine regulation of hindgut reabsorption; and (4) the intricacies of how osmoregulatory function is recruited in *Drosophila* during stressful challenges such as dehydration, extreme temperatures, or diet and salt stress.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Data availability

All data collected for this study have been deposited in the Dryad Data Repository (Andersen and Overgaard, 2020): dryad.vmcvdncpr.

Supplementary information

Supplementary information available online at
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References

Andersen, M. K. and Overgaard, J. (2019). The central nervous system and muscular system play different roles for chill coma onset and recovery in insects. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **233**, 10-16. doi:10.1016/j.cbpa.2019.03.015

Andersen, M. K. and Overgaard, J. (2020). Data from: Maintenance of hindgut reabsorption during cold exposure is a key adaptation for *Drosophila* cold tolerance. *Dryad Dataset* doi:10.5061/dryad.vmcvdncpr

Andersen, J. L., Manenti, T., Sørensen, J. G., MacMillan, H. A., Loeschcke, V. and Overgaard, J. (2015). How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. *Funct. Ecol.* **29**, 55-65. doi:10.1111/1365-2435.12310

Andersen, M. K., Folkersen, R., MacMillan, H. A. and Overgaard, J. (2017a). Cold acclimation improves chill tolerance in the migratory locust through preservation of ion balance and membrane potential. *J. Exp. Biol.* **220**, 487-496. doi:10.1242/jeb.150813

Andersen, M. K., Jensen, S. O. and Overgaard, J. (2017b). Physiological correlates of chill susceptibility in Lepidoptera. *J. Insect Physiol.* **98**, 317-326. doi:10.1016/j.jinsphys.2017.02.002

Andersen, M. K., MacMillan, H. A., Donini, A. and Overgaard, J. (2017c). Cold tolerance of Drosophila species is tightly linked to epithelial K^+ transport capacity of the Malpighian tubules and rectal pads. *J. Exp. Biol.* **220**, 4261-4269. doi:10.1242/jeb.168518

Bayley, J. S., Winther, C. B., Andersen, M. K., Grønkjær, C., Nielsen, O. B., Pedersen, T. H. and Overgaard, J. (2018). Cold exposure causes cell death by depolarization-mediated Ca^{2+} overload in a chill-susceptible insect. *Proc. Natl Acad. Sci. USA* **115**, E9737-E9744. doi:10.1073/pnas.1813532115

Bernard, C. (1872). *De la Physiologie générale*: Hachette.

Beyenbach, K. W. (2016). The plasticity of extracellular fluid homeostasis in insects. *J. Exp. Biol.* **219**, 2596-2607. doi:10.1242/jeb.129650

Beyenbach, K. W. (2019). Voltages and resistances of the anterior Malpighian tubule of *Drosophila melanogaster*. *J. Exp. Biol.* **222**, jeb201574. doi:10.1242/jeb.201574

Beyenbach, K. W. and Piermarini, P. M. (2008). Osmotic and ionic regulation in insects. In *Osmotic and Ionic Regulation: Cells and Animals*, pp. 231-293.

Beyenbach, K. W. and Piermarini, P. M. (2011). Transcellular and paracellular pathways of transepithelial fluid secretion in Malpighian (renal) tubules of the yellow fever mosquito *Aedes aegypti*. *Acta Physiol.* **202**, 387-407. doi:10.1111/j.1748-1716.2010.02195.x

Beyenbach, K. W., Skaer, H. and Dow, J. A. T. (2010). The developmental, molecular, and transport biology of Malpighian tubules. *Annu. Rev. Entomol.* **55**, 351-374. doi:10.1146/annurev-ento-112408-085512

Black, K., Meredith, J., Thomson, B., Phillips, J. and Dietz, T. (1987). Mechanisms and properties of sodium transport in locust rectum. *Can. J. Zool.* **65**, 3084-3092. doi:10.1139/z87-468
Bubliy, O. A., Riihimaa, A., Norry, F. M. and Loeschcke, V. (2002). Variation in resistance and acclimation to low-temperature stress among three geographical strains of *Drosophila melanogaster*. *J. Therm. Biol.* **27**, 337-344. doi:10.1016/S0306-4565(01)00098-5
Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007). Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39**, 715. doi:10.1038/ng2049
Chintapalli, V. R., Wang, J., Herzyk, P., Davies, S. A. and Dow, J. A. T. (2013). Data-mining the FlyAtlas online resource to identify core functional motifs across transporting epithelia. *BMC Genomics* **14**, 518. doi:10.1186/1471-2164-14-518
Coast, G. M., Orchard, I., Phillips, J. E. and Schooley, D. A. (2002). Insect diuretic and antidiuretic hormones. *Adv. Insect. Physiol.* **29**, 279-409. doi:10.1016/S0065-2806(02)29004-9
Colinet, H. and Hoffmann, A. A. (2012). Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. *Funct. Ecol.* **26**, 84-93. doi:10.1111/j.1365-2435.2011.01898.x
Davies, S.-A., Cabrero, P., Marley, R., Corrales, G. M., Ghimire, S., Dornan, A. J. and Dow, J. A. (2019). Epithelial function in the *Drosophila* malpighian tubule: an *in vivo* renal model. In *Kidney Organogenesis* (ed. S. Vainio), pp. 203-221: Springer.
Des Marteaux, L. E. and Sinclair, B. J. (2016). Ion and water balance in *Gryllus* crickets during the first twelve hours of cold exposure. *J. Insect Physiol.* **89**, 19-27. doi:10.1016/j.jinsphys.2016.03.007
Des Marteaux, L. E., Khazraeenia, S., Yerushalmi, G. Y., Donini, A., Li, N. G. and Sinclair, B. J. (2017). The effect of cold acclimation on active ion transport in cricket ionoregulatory tissues. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **216**, 28-33. doi:10.1016/j.cbpa.2017.11.005
Donini, A. and O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. *J. Exp. Biol.* **208**, 603-610. doi:10.1242/jeb.01422
Dow, J. A. and Davies, S. A. (2003). Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev.* **83**, 687-729. doi:10.1152/physrev.00035.2002
Dow, J., Maddrell, S., Görtz, A., Skaer, N., Brogan, S. and Kaiser, K. (1994). The malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* **197**, 421-428.
Dow, J. A. T., Halberg, K. A., Terhzaz, S. and Davies, S. A. (2018). *Drosophila* as a model for neuroendocrine control of renal homeostasis. In *Model Animals in Neuroendocrinology: From Worm to Mouse to Man* (ed. M. Ludvig and G. Levkowitz), pp. 81-100.
D'Silva, N. M., Donini, A. and O'Donnell, M. J. (2017). The roles of V-type H^+-ATPase and Na^+/K^+-ATPase in energizing K^+ and H^+ transport in larval *Drosophila* gut epithelia. *J. Insect Physiol.* **98**, 284-290. doi:10.1016/j.jinsphys.2017.01.019
Edney, E. B. (1977). Excretion and osmoregulation. In *Water Balance in Land Arthropods* (ed. D. S. Farner, W. S. Hoar, B. Hoelldobler, H. Langer and M. Lindauer), pp. 96-171: Springer.
Gerber, L. and Overgaard, J. (2018). Cold tolerance is linked to osmoregulatory function of the hindgut in *Locusta migratoria*. *J. Exp. Biol.* **221**, jeb173930. doi:10.1242/jeb.173930
Gerencser, G. A. and Zhang, J. (2003). Chloride ATPase pumps in nature: do they exist? *Biol. Rev.* **78**, 197-218. doi:10.1017/S146479310200605X
Gupta, B. L., Wall, B. J., Oschman, J. L. and Hall, T. A. (1980). Direct microprobe evidence of local concentration gradients and recycling of electrolytes during fluid absorption in the rectal papillae of *Calliphora*. *J. Exp. Biol.* **88**, 21-48.
Halberg, K. A., Terhzaz, S., Cabrero, P., Davies, S. A. and Dow, J. A. T. (2015). Tracing the evolutionary origins of insect renal function. *Nat. Commun.* **6**, 6800. doi:10.1038/ncomms7800
Hanrahan, J. and Phillips, J. (1983). Cellular mechanisms and control of KCl absorption in insect hindgut. *J. Exp. Biol.* **106**, 71-89.
Hanrahan, J., Meredith, J., Phillips, J. and Brandys, D. (1984). Methods for the study of transport and control in insect hindgut. In *Measurement of Ion Transport and Metabolic Rate in Insects* (ed. T. J. Bradley and T. A. Miller), pp. 19-67: Springer.
Harrison, J. F., Woods, H. A. and Roberts, S. P. (2012). *Ecological and Environmental Physiology of Insects*: Oxford University Press.
Ianowski, J. P. and O'Donnell, M. J. (2004). Basolateral ion transport mechanisms during fluid secretion by *Drosophila* Malpighian tubules: Na^+ recycling, Na^+: K^+: $2Cl^-$ cotransport and Cl^- conductance. *J. Exp. Biol.* **207**, 2599-2609. doi:10.1242/jeb.01058
Koštál, V., Vambera, J. and Bastl, J. (2004). On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. *J. Exp. Biol.* **207**, 1509-1521. doi:10.1242/jeb.00923
Koštál, V., Yanagimoto, M. and Bastl, J. (2006). Chilling-injury and disturbance of ion homeostasis in the coxal muscle of the tropical cockroach (*Nauphoeta cinerea*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **143**, 171-179. doi:10.1016/j.cbpb.2005.11.005
Koštál, V., Renault, D., Mehrabianová, A. and Bastl, J. (2007). Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: role of ion homeostasis. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147**, 231-238. doi:10.1016/j.cbpa.2006.12.033
Leader, D. P., Krause, S. A., Pandit, A., Davies, S. A. and Dow, J. A. T. (2018). FlyAtlas 2: a new version of the *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Res.* **46**, D809-D815. doi:10.1093/nar/gkx976
Lee, R. (2012). *Insects at Low Temperature*: Springer Science & Business Media.
Luan, Z., Quigley, C. and Li, H.-S. (2015). The putative Na^+/Cl^--dependent neurotransmitter/osmolyte transporter inebriated in the *Drosophila* hindgut is essential for the maintenance of systemic water homeostasis. *Sci. Rep.* **5**, 7993. doi:10.1038/srep07993
MacMillan, H. A. and Sinclair, B. J. (2011). The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. *J. Exp. Biol.* **214**, 726-734. doi:10.1242/jeb.051540
MacMillan, H. A., Andersen, J. L., Davies, S. A. and Overgaard, J. (2015a). The capacity to maintain ion and water homeostasis underlies interspecific variation in *Drosophila* cold tolerance. *Sci. Rep.* **5**, 18607. doi:10.1038/srep18607
MacMillan, H. A., Andersen, J. L., Loeschcke, V. and Overgaard, J. (2015b). Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **308**, R823-R831. doi:10.1152/ajpregu.00465.2014
MacMillan, H. A., Baatrup, E. and Overgaard, J. (2015c). Concurrent effects of cold and hyperkalaemia cause insect chilling injury. *Proc. R. Soc. B* 282, 20151483. doi:10.1098/rspb.2015.1483
MacMillan, H. A., Ferguson, L. V., Nicolai, A., Donini, A., Staples, J. F. and Sinclair, B. J. (2015d). Parallel ionoregulatory adjustments underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance. *J. Exp. Biol.* **218**, 423-432. doi:10.1242/jeb.115790
MacMillan, H. A., Schou, M. F., Kristensen, T. N. and Overgaard, J. (2016). Preservation of potassium balance is strongly associated with insect cold tolerance in the field: a seasonal study of *Drosophila subobscura*. *Biol. Lett.* **12**, 20160123. doi:10.1098/rsbl.2016.0123
MacMillan, H. A., Yerushalmi, G. Y., Jonusaite, S., Kelly, S. P. and Donini, A. (2017). Thermal acclimation mitigates cold-induced paracellular leak from the *Drosophila* gut. *Sci. Rep.* **7**, 8807. doi:10.1038/s41598-017-08926-7
MacMillan, H. A., Nazal, B., Wali, S., Yerushalmi, G. Y., Misyura, L., Donini, A. and Paluzzi, J.-P. (2018). Anti-diuretic activity of a CAPA neuropeptide can compromise *Drosophila* chill tolerance. *J. Exp. Biol.* **221**, jeb185884. doi:10.1242/jeb.185884
Mellanby, K. (1954). Acclimatization and the thermal death point in insects. *Nature* **173**, 582-583. doi:10.1038/173582b0
Naikkhwah, W. and O'Donnell, M. J. (2012). Phenotypic plasticity in response to dietary salt stress: Na^+ and K^+ transport by the gut of *Drosophila melanogaster* larvae. *J. Exp. Biol.* **215**, 461-470. doi:10.1242/jeb.064048
O'Donnell, M. J. and Maddrell, S. (1995). Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female *Drosophila*. *J. Exp. Biol.* **198**, 1647-1653.
O'Donnell, M. J., Ianowski, J. P., Linton, S. M. and Rheault, M. R. (2003). Inorganic and organic anion transport by insect renal epithelia. *Biochim. Biophys. Acta Biomembr.* **1618**, 194-206. doi:10.1016/j.bbamem.2003.07.003
Olsson, T., MacMillan, H. A., Nyberg, N., Stærk, D., Malmendal, A. and Overgaard, J. (2016). Hemolymph metabolites and osmolality are tightly linked to cold tolerance of *Drosophila* species: a comparative study. *J. Exp. Biol.* **219**, 2504-2513. doi:10.1242/jeb.140152
Overgaard, J. and MacMillan, H. A. (2017). The integrative physiology of insect chill tolerance. *Annu. Rev. Physiol.* **79**, 187-208. doi:10.1146/annurev-physiol-022516-034142
Overgaard, J., Kristensen, T. N., Mitchell, K. A. and Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *Am. Nat.* **178**, S80-S96. doi:10.1086/661780
Phillips, J. E. (1970). Apparent transport of water by insect excretory systems. *Am. Zool.* **10**, 413-436. doi:10.1093/icb/10.3.413
Phillips, J. (1981). Comparative physiology of insect renal function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **241**, R241-R257. doi:10.1152/ajpregu.1981.241.5.R241
Phillips, J. E. and Audsley, N. (1995). Neuropeptide control of ion and fluid transport across locust hindgut. *Am. Zool.* **35**, 503-514. doi:10.1093/icb/35.6.503
Phillips, J. E., Hanrahan, J., Chamberlin, M. and Thomson, B. (1987). Mechanisms and control of reabsorption in insect hindgut. *Adv. Insect Physiol.* **19**, 329-422. doi:10.1016/S0065-2806(08)60103-4
Phillips, J. E., Wiens, C., Audsley, N., Jeffs, L., Bilgen, T. and Meredith, J. (1996). Nature and control of chloride transport in insect absorptive epithelia. *J. Exp. Zool. A Ecol. Genet. Physiol.* **275**, 292-299. doi:10.1002/(SICI)1097-010X(19960701)275:4<292::AID-JEZ7>3.0.CO;2-K

R Core Team. (2019). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. See http://www.R-project.org/.

Ramsay, J. (1954). Active transport of water by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera, Phasmidae). *J. Exp. Biol.* **31**, 104-113.

Rheault, M. R. and O'Donnell, M. J. (2004). Organic cation transport by Malpighian tubules of *Drosophila melanogaster*: application of two novel electrophysiological methods. *J. Exp. Biol.* **207**, 2173-2184. doi:10.1242/jeb.01003

Schindelin, J., Rueden, C. T., Hiner, M. C. and Eliceiri, K. W. (2015). The ImageJ ecosystem: an open platform for biomedical image analysis. *Mol. Reprod. Dev.* **82**, 518-529. doi:10.1002/mrd.22489

Schou, M. F., Mouridsen, M. B., Sørensen, J. G. and Loeschcke, V. (2017). Linear reaction norms of thermal limits in *Drosophila*: predictable plasticity in cold but not in heat tolerance. *Funct. Ecol.* **31**, 934-945. doi:10.1111/1365-2435.12782

Sinclair, B. J., Vernon, P., Klok, C. J. and Chown, S. L. (2003). Insects at low temperatures: an ecological perspective. *Trends Ecol. Evol.* **18**, 257-262. doi:10.1016/S0169-5347(03)00014-4

Terhzaz, S., Teets, N. M., Cabrero, P., Henderson, L., Ritchie, M. G., Nachman, R. J., Dow, J. A. T., Denlinger, D. L. and Davies, S.-A. (2015). Insect capa neuropeptides impact desiccation and cold tolerance. *Proc. Natl Acad. Sci. USA* **112**, 2882-2887. doi:10.1073/pnas.1501518112

Wall, B. J. and Oschman, J. L. (1970). Water and solute uptake by rectal pads of *Periplaneta americana*. *Am. J. Physiol.* **218**, 1208-1215. doi:10.1152/ajplegacy.1970.218.4.1208

Wall, B. and Oschman, J. (1975). Structure and function of the rectum in insects. *Fortschr. Zool.* **23**, 193-222.

Wheeler, C. H. and Coast, G. M. (1990). Assay and characterisation of diuretic factors in insects. *J. Insect Physiol.* **36**, 23-34. doi:10.1016/0022-1910(90)90147-8

Yerushalmi, G. Y., Misyura, L., MacMillan, H. A. and Donini, A. (2018). Functional plasticity of the gut and the Malpighian tubules underlies cold acclimation and mitigates cold-induced hyperkalemia in *Drosophila melanogaster*. *J. Exp. Biol.* **221**, jeb174904. doi:10.1242/jeb.174904

Yi, S.-X. and Lee, R. E. (2005). Changes in gut and Malpighian tubule transport during seasonal acclimatization and freezing in the gall fly *Eurosta solidaginis*. *J. Exp. Biol.* **208**, 1895-1904. doi:10.1242/jeb.01596