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RESEARCH ARTICLE

Cold tolerance is linked to osmoregulatory function of the hindgut in *Locusta migratoria*

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ABSTRACT

There is growing evidence that maintenance of ion and water balance determines cold tolerance in many insects. The hindgut of terrestrial insects is critical for maintaining organismal homeostasis as it regulates solute and water balance of the haemolymph. Here, we used *ex vivo* everted gut sacs of *Locusta migratoria* to examine the effects of temperature (0–30°C), thermal acclimation, hypoxia, and ionic and osmotic forces on bulk water and ion (Na^+, K^+ and Cl^-) movement across the rectal epithelium. These findings were related to simultaneous *in vivo* measurements of water and ion balance in locusts exposed to similar temperatures. As predicted, we observed a critical inhibition of net water and ion reabsorption at low temperature that was proportional to the *in vivo* loss of water and ion homeostasis. Further, cold-acclimated locusts, which are known to defend ion and water balance at low temperature, were characterised by improved reabsorptive capacity at low temperature. These findings strongly support the hypothesis that transport mechanisms in the hindgut at low temperature are essential for cold tolerance. The loss of osmoregulatory capacity at low temperature was primarily caused by reduced active transport, while rectal paracellular permeability to fluorescein isothiocyanate dextran was unchanged at 0 and 30°C. During cold exposure, water reabsorption was independent of major cation gradients across the epithelia, while a reduction in mucosal Cl^- availability and an increase in mucosal osmolality markedly depressed water reabsorption. These findings are discussed in the context of existing knowledge and with suggestions for future physiological studies on cold acclimation and adaptation in insects.

KEY WORDS: Hypoxia, Insect, Ion flux, Paracellular permeability, Rectal sac, Water reabsorption

INTRODUCTION

Insects are the largest animal group in terms of both species richness and biomass, and they play important roles in nearly all ecosystems (Chown and Nicolson, 2004; Harrison et al., 2012). The enormous success of insects is closely linked to the physiological adaptations that have allowed specific species to tolerate specific sets of environmental conditions (Beyenbach, 2016; Chown and Terblanche, 2006; Edney, 1977). Such physiological adaptations are, for example, seen in the studies that connect cold tolerance to osmoregulatory capacity (Andersen et al., 2017a; Des Marteaux et al., 2017, 2018; MacMillan et al., 2015a, 2017; Terhzaz et al., 2015). The capacity to preserve ion and water homeostasis at low temperature is therefore crucial for the thermal tolerance of insects.

Most insect species are chill susceptible, meaning they succumb to low temperatures because of loss of physiological capacity at temperatures above that causing ice formation in their body fluids (Bale, 1996; Nedved, 2000; Overgaard and MacMillan, 2017; Sinclair et al., 2015). One problem for chill-susceptible insects is the loss of water and Na^+ from the haemolymph, which are moved to the gut or other tissues (Des Marteaux and Sinclair, 2016; Koštál et al., 2004; MacMillan and Sinclair, 2011a; MacMillan et al., 2015b; Olsson et al., 2016). This reduction in haemolymph volume causes an increase in the concentration of extracellular K^+ ($[K^+]_{ext}$) that induces cell depolarisation and mortality (Andersen et al., 2017b; Koštál et al., 2004, 2007; MacMillan et al., 2014, 2015c). Accordingly, several studies have demonstrated a correlation between improved osmoregulatory function and cold tolerance in insects that are cold acclimated or cold adapted (Andersen et al., 2017a,b; Coello Alvarado et al., 2015; Findsen et al., 2013; MacMillan et al., 2015a). Furthermore, recent studies have shown specifically how cold acclimation/adaptation preserves ion balance through increased osmoregulatory capacity and/or reduced ion and water leak from the gut (Andersen et al., 2017a; Des Marteaux et al., 2018; MacMillan et al., 2015d; Yerushalmi et al., 2018).

In most terrestrial insects, the regulation of water and ion homeostasis is achieved via the Malpighian tubules and the hindgut (Chapman, 2013; Edney, 1977). Orthopterans are popular comparative models to study thermal tolerance in insects as many facets of their thermal biology have been studied (Andersen et al., 2017b; Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; Findsen et al., 2013; MacMillan and Sinclair, 2011a; Robertson et al., 2017). Moreover, they are a suitable group because the basic processes for ion and water transport can be measured readily (Hanrahan et al., 1984; Phillips and Audsley, 1995; Phillips et al., 1988, 1996; Robertson et al., 2014). In herbivorous insects such as the locust, the Malpighian tubules secrete a KCl-rich primary urine to the hindgut, where selective reabsorption of solutes and water occurs to maintain ion and water balance (Harrison et al., 2012). The rectal region of the hindgut is a major reabsorptive part of this circuit (Irvine et al., 1988; Phillips et al., 1996, 1998). Rectal reabsorption is powered by energy-demanding pumps (e.g. basal Na^+/K^+-ATPase, apical V-type H^+-ATPase and possibly an electrogenic Cl^- pump) that set up an electrochemical gradient to transport ions against local chemical gradients (Audsley et al., 2013; Phillips, 1981; Phillips et al., 1988, 1996). These active transport processes set a local osmotic gradient that drives water reabsorption and therefore link water transport to active ion transport (Goh and Phillips, 1978). Active transport processes are temperature sensitive but the impact of temperature on bulk water and ion movement in insect excretory systems and membrane barrier function (e.g. change in membrane permeability) has been poorly studied (but see references above). Furthermore, it

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List of abbreviations

FD4	fluorescein isothiocyanate dextran with a molecular mass of 4000 Da
J_{Cl}	net chloride flux
J_{ion}	net flux of any specific ion measured
J_K	net potassium flux
J_{Na}	net sodium flux
J_v	net fluid flux
SA	surface area

is largely unknown how osmotic and ionic forces influence movement of water and ion transport at low temperature.

The working hypothesis for the present study is that low temperature impairs ATP-powered transport of ions to a degree where homeostatic regulation is impaired (MacMillan and Sinclair, 2011b; Overgaard and MacMillan, 2017). This is seen both as an insufficient capacity to actively counterbalance the passive leak of ions down their chemical gradients and as an inability to facilitate active water reabsorption to maintain haemolymph volume. Based on this working hypothesis, we set out to explore whether the well-known ionic disturbance observed *in vivo* during cold exposure is closely tied to thermal inhibition of the reabsorptive capacity of the hindgut. We examined this by measuring net rectal water (J_v) and ion (J_{ion}) flux over a range of experimental temperatures (0–30°C) using everted gut sac preparation. These measurements were then related to *in vivo* measurements of haemolymph volume and ion concentration at the same range of experimental temperatures (0–30°C). To investigate this relationship in further detail, we examined whether cold-acclimated locusts, which are known to better defend ion homeostasis at low temperature and have improved cold tolerance (Andersen et al., 2017b), were characterised by sustained rectal water and ion reabsorption (J_v and J_{ion}) rates at low temperatures compared with warm-acclimated locusts. Additionally, as net transport is determined by both active transport and passive leak, we characterised the active component of rectal transport and rectal epithelial paracellular permeability at high and low temperature using severe hypoxia as an inhibitory tool for oxidative ATP production and fluorescein isothiocyanate dextran (FD4) clearance as a marker of paracellular permeability, respectively. Finally, we investigated in more detail how ionic and osmotic gradients challenge the reabsorptive capacity of the hindgut to better understand the putative role of osmotic and ionic forces in insect cold tolerance.

MATERIALS AND METHODS

Experimental animals and experimental protocol

Insects were reared as described by Andersen et al. (2017b). Briefly, fourth to fifth instar nymphs of *Locusta migratoria* (Linnaeus 1758) were obtained from a commercial supplier (Peter Andersen Aps, Fredericia, Denmark) and maintained at 25°C under a 12 h light:12 h dark light cycle. During light hours, locusts had access to a heating lamp, allowing behavioural thermoregulation up to >45°C. Locusts were fed commercial wheat bran and fresh wheat sprouts and had access to water *ad libitum*. All experiments were carried out on adult *L. migratoria* of both sexes, 1–3 weeks past their final moult. Prior to experiments, locusts were placed at constant 30±1°C for 3 days without food but with water available (unless otherwise stated), and are referred to as warm acclimated.

Two sets of experiments were performed: the first measured the effects of chronic exposure to a range of temperatures on *in vivo* ion and water balance; the second used *ex vivo* everted rectal sac preparations to assess how bulk movement of water and major ions across rectal epithelia was affected by temperature, severe hypoxia and changes in mucosal osmolality and ion concentration.

Effects of temperature on *in vivo* ion and water balance

The ionic and osmotic consequences of low temperature exposure were determined by measuring haemolymph Na^+, K^+ and Cl^- concentration and extractable haemolymph volume in warm-acclimated locusts after 2 days at 30±1, 20±1, 10±1, 5±0.5 or 0°C (N=7 per group).

For measurements of haemolymph ion concentration, haemolymph was collected in a capillary tube from the cervical membrane or from the hindlegs. A 5 µl sample of haemolymph was transferred to an Eppendorf tube containing 2 ml 100 ppm lithium buffer (Sherwood Scientific Ltd, Cambridge, UK) for measurement of [Na^+] and [K^+] by flame photometry (Flame Photometer, Model 420, Sherwood Scientific Ltd). A 1 µl sample of haemolymph was used for measurement of [Cl^-] by colorimetric assay following the manufacturer's protocol (MAK023 chloride assay kit, Sigma-Aldrich, Steinem, Germany). All measurements of ion concentrations were referenced to standards of known concentration.

Haemolymph volume was approximated in a separate set of animals. Here, locusts were weighed to the nearest 0.001 mg on a balance (Sartorius R200D, Göttingen, Germany) after which haemolymph was sampled from the cervical membrane as described above. The animal was then cut open and residual haemolymph was removed by quickly blotting the tissue with filter paper. Locusts were then reweighed and haemolymph volume was calculated from the mass difference (assuming a specific gravity of 1) and expressed as a percentage of initial wet mass. This simplistic method gave comparable volumes when compared with an inulin method previously used in our lab to estimate haemolymph volume in *L. migratoria* (O'Sullivan et al., 2016).

Ex vivo experimental series: determination of rectal reabsorption capacity using everted rectal sacs

The hindgut is a major osmoregulatory organ in insects and here we undertook a series of experiments to examine whether organismal water and ion balance is related to net water (J_v) and ion flux (J_{ion}) across rectal epithelia. Specifically, we used *ex vivo* everted rectal sacs to examine the effects of temperature, thermal acclimation, severe hypoxia and changes in mucosal osmolality and ion concentration on J_v and J_{ion}.

Preparation of everted rectal sacs

Everted rectal sac preparation and incubation followed a protocol modified from Hanrahan et al. (1984). A lateral incision was made in the abdomen and the hindgut was kept moist with a standard serosal saline (see composition of salines in Table 1) while the trachea and connective tissue were removed under a dissection microscope. A heat-flared polyethylene tube (PE90, 0.86 mm i.d., 1.27 mm o.d.) was inserted and tied to the anterior margin of the rectal pads with a double silk ligature. The rectum was gently everted by withdrawal of the PE90 tube until the posterior margin of the rectal pad emerged and the preparation was then placed in a standard mucosal saline (Table 1). Standard serosal saline was injected through the PE90 tube to rinse the rectum thoroughly before the posterior margin of the rectal pads was tied closed with a second double silk ligature. Any fluid remaining in the sac was then removed through the PE90 tube using a 25 µl blunt Hamilton syringe (Hamilton®, Gastight®, no. 1702). To approximate ionic

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Table 1. Ion composition of salines used in the present study

	Standard condition (control)		Modified mucosal side			
	Serosal side	Mucosal side	↑[Na^+] and ↓[K^+]	↓[Cl^-]	↑Osmolality	↑↑Osmolality
Na^+	**120**	**45**	**120**	45	45	45
Cl^-	110	110	**110**	**50**	110	110
K^+	**6**	**90**	**6**	90	90	90
PO_4^{3-}	6	6	6	6	6	6
HCO_3^-	2	2	2	3	2	2
Ca^{2+}	2	2	2	3	2	2
Mg^{2+}	4	4	4	4	4	4
SO_4^{2-}	–	–	15	–	–	–
Proline	15	38	15	38	38	38
Glycine	14	4	14	4	4	4
Trehalose	16	–	16	–	–	–
Glucose	17	17	17	17	17	17
Osmolality[1]	330	330	330	330	**630**	**1030**

Concentrations are given in mmol l^{-1} and the final osmolality is given in mOsm kg^{-1}.

[1]Sucrose was added to adjust the osmolality of the different solutions.

The standard mucosal and serosal salines were formulated to resemble Malpighian tubule fluid and haemocoel fluid, respectively, and are defined as the 'standard condition' and represent the reference group for all comparisons. The composition of salines is based on the Ringer solution of Mordue (1969) with modifications from Hanrahan et al. (1984), widely used to bathe locust everted rectum sacs in early studies. The mucosal saline used to bathe the everted rectal sacs was replaced by serosal saline on the mucosal side or modified mucosal saline for experiments manipulating osmotic and ionic gradients across the rectal wall. The pH of all salines was adjusted to 7.0 with the addition of NaOH. Differences in ion concentration and osmolality between saline solutions are highlighted in bold.

conditions *in vivo*, sacs were filled with a known volume of standard serosal saline (haemocoel side) and suspended in a bath containing 50 ml standard mucosal saline (rectal lumen side). This condition is referred to as the 'standard condition'. The baths were surrounded by a water jacket system to control the experimental temperature and the mucosal saline was continuously bubbled with O_2.

Pilot experiments demonstrated that everted rectal sac preparations remained stable for up to 6 h (data not shown). Additionally, after termination of pilot experiments, everted rectal sacs were placed in a mucosal saline containing 10 mmol l^{-1} amaranth to test for possible leakage (the epithelium is impermeable to amaranth and any leak would indicate structural damage in the preparation; Phillips and Dockrill, 1968). No dye was observed on the haemocoel side, attesting to the stability and quality of the preparation.

Measurements of net water flux

Net fluid flux (J_v) was measured gravimetrically. Before each experimental period, everted rectal sacs were blotted on medical wipes and filled with a known volume (typically around 2 µl) of standard serosal saline. The precise volume injected into the sacs was determined by weighing the sacs to the nearest 0.0001 mg before (i.e. empty sac mass; m_e) and after filling (i.e. initial sac mass; m_i) using a microbalance (Sartorius MSA6.6S-0CE-DM). After a set time (t) under the desired experimental conditions (typically 1 h), sacs were blotted and re-weighed to determine the final sac mass (m_f). The haemocoel fluid (final absorbate) was subsequently sampled for later analysis using a blunt Hamilton syringe. The sacs were then rinsed with standard serosal saline and prepared for a new incubation hour as described above. Therefore, every sac preparation was used as its own control and incubated for up to 5 h. As changes in total sac mass could be due to a change in both tissue and haemocoel volume, comparison of empty sac mass measured at the start and end of each incubation period (m_{e1} and m_{e2}, respectively) could be used to report any changes in tissue volume (typically <1%, due to tissue swelling and change of hydration state of the tissue). Hence, net change in haemocoel volume was determined by subtracting the change in tissue volume from the measured final sac mass. At the end of the experimental series, rectal sacs were cut open by a longitudinal incision and the gross surface area (SA) of the exposed epithelial surface was determined by placing a clear acetate sheet onto the epithelium and tracing its outline. The acetate sheet was then cut along the outline and the epithelial surface area was estimated from the mass of the sheet compared with the mass of a sheet of known area. Then, measured individual rectal SAs were converted to a standard rectum size of 0.631 cm^2 instead of conversion into the standard unit of 1.00 cm^2 for analysis. This allowed direct comparison with previous studies where rectal SA of the sac was not determined and net fluxes are reported as per rectum (Goh and Phillips, 1978). The standard rectum size selected of 0.631 cm^2 was determined from the gross SA of 50 recta and is comparable to the SA for locust rectum found in the literature (Hanrahan and Phillips, 1984; Irvine et al., 1988).

Net fluid transport (J_v) was calculated as previously described by Whittamore et al. (2016) with some modification (i.e. correction for tissue volume changes and use of an everted preparation):

$$J_v = \frac{(m_f - m_i) - (m_{e1} - m_{e2})/SA}{t}, \quad (1)$$

where m_f is the final sac mass (mg), m_i is the initial sac mass (mg), m_{e1} is the initial empty sac mass (mg), m_{e2} is the final empty sac mass (mg), SA is the surface area of the sac (cm^2) and t is the duration of incubation (h). J_v was then expressed as µl h^{-1} per rectum, where rectum size was 0.631 cm^2. Positive values for J_v indicate net fluid absorption from the rectal lumen towards the haemolymph, while negative values indicate net fluid secretion.

Measurement of net ion flux

Net ion flux (J_{ion}) was calculated for each incubation period from the change in volume and specific ion (Na^+, Cl^-, K^+) concentrations of the haemocoel fluid as follows:

$$J_{ion} = \frac{(V_f \times C_f) - (V_i \times C_i)/SA}{t}, \quad (2)$$

where V_f is the final haemocoel volume (µl), C_f is the final haemocoel concentration of the targeted ion (mmol l^{-1}), V_i is the

initial haemocoel volume (µl), C_i is the initial haemocoel concentration of the targeted ion (mmol l^{-1}), SA is the surface area of the sac (cm^2) and t is the duration of incubation (h). J_{ion} was then expressed as µmol h^{-1} per rectum, where the rectum size was 0.631 cm^2. Ion concentrations of the haemocoel fluid were determined as described above for *in vivo* experiments using flame photometry (Na^+ and K^+) and a colorimetric assay (Cl^-).

Effects of temperature, thermal acclimation, severe hypoxia, osmolality and mucosal ion concentration on net rectal water and ion flux

The basic preparation of the everted rectal sac described above was used for a range of experiments in which the experimental conditions (temperature, oxygen level and saline composition) were manipulated. J_v and J_{ion} measured under each experimental condition were compared with J_v and J_{ion} measured under standard conditions (i.e. standard salines at 30°C with constant O_2 bubbling; see Table 1). Hence, typical experiments were initiated and/or terminated by baseline measurements under standard conditions. The precise experimental conditions of each series are specified below.

Series 1: thermal dependence of net water and ion flux

The temperature dependence of J_v and J_{ion} across the rectal wall of *L. migratoria* was evaluated by incubation of everted rectal sacs under standard conditions for 2×1 h at 30°C and then for 2×1 h at one of the tested temperatures: 20, 10, 5 or 0°C (N=6 per group).

Series 2: effect of thermal acclimation on net water and ion flux

The effect of thermal acclimation on J_v and J_{ion} across the rectum wall was investigated on warm- and cold-acclimated locusts. Locusts were acclimated for 5 days at either 30±1°C (N=5) or 11±1°C (N=5; a temperature at which cold tolerance is enhanced at the organismal level; see Andersen et al., 2017b). Following the acclimation period, J_v and J_{ion} were determined by incubation of everted rectal sacs in standard mucosal saline at their acclimation temperatures for 2×1 h followed by 1 h at 0°C and finishing with 1 h at the acclimation temperature of the comparison group. To test the reversibility of the acclimation response and plasticity/sensitivity of the tissues to temperature changes, the order of the incubation periods was inverted in a separate set of acclimated animals (N=5, per group) but incubation at 0°C was omitted. Thereby, everted rectal sacs from cold-acclimated locusts were incubated 2×1 h at 30°C prior to incubation for 2×1 h at their acclimation temperature and vice versa.

Series 3: metabolic dependence of net water and ion flux

The metabolic dependence of net water and ion flux at high and low temperatures was examined using O_2 depletion as an inhibitory tool for oxidative phosphorylation. Severe hypoxia was induced by replacing the regular constant O_2 bubbling with constant N_2 bubbling into the bath and bubbling of the serosal saline with N_2 prior to injection into everted rectal sacs. To limit gas exchange, the water surface was covered with expanded polystyrene and wrapped in aluminium foil. Everted rectal sacs were incubated under standard conditions for 2×1 h (i.e. 30°C and constant O_2) followed by 2×1 h at either 30°C+N_2 (N=5) or 0°C+N_2 (N=6) before returning to standard conditions for 1 h. J_v and J_{ion} measured under anoxic conditions were compared with J_v and J_{ion} observed under standard conditions and hypothermia.

Series 4: temperature dependence of rectal paracellular permeability

The temperature dependence of rectal paracellular permeability was determined by measuring rectal clearance of the paracellular permeability probe fluorescein isothiocyanate dextran (FD4, molecular mass 4000 Da) as previously described (Bao et al., 2014; Yang et al., 2002; Zhang et al., 2009) with some modifications. Briefly, everted rectal sacs (N=5) were suspended in standard mucosal saline containing 2.0 mg ml^{-1} FD4 (mucosal side) for 2×1 h at 30°C followed by 2×1 h at 0°C. Between each incubation hour, haemocoel fluid (i.e. final absorbate) was removed, and sacs were rinsed with fresh standard serosal saline before refilling with standard serosal saline (without FD4). The concentration of FD4 was determined in the haemocoel fluid and rinsing fluid using a fluorescence plate reader (Victor^3 1420-041, Multilabel Plate Counter, PerkinElmer®, Waltham, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Rectal permeability was expressed as the mucosal-to-serosal clearance of FD4:

$$\text{FD4 clearance} = \frac{\text{FD4}_{ser} \times V_f}{\text{FD4}_{muc} \times t \times \text{SA}}, \quad (3)$$

where, $FD4_{ser}$ is the concentration of FD4 in haemocoel fluid (i.e. serosal side) measured at the end of the incubation period (ng $µl^{-1}$), $FD4_{muc}$ is the initial concentration of FD4 in the mucosal saline (ng $µl^{-1}$), V_f is the final volume of haemocoel fluid in the sacs (µl), SA is the surface area of the sac (cm^2) and t is the duration of incubation (h). FD4 clearance was then expressed as nl h^{-1} per rectum, where rectum size was 0.631 cm^2.

Series 5: dependence of net water and ion flux on osmotic and ionic gradients at high and low temperature

The dependence of J_v and J_{ion} on osmotic and ionic gradients was investigated at 30 and 0°C. J_v and J_{ion} of all everted rectal sacs was assessed under standard conditions and under one of the tested ionic and osmotic conditions. To determine the dependence of J_{ion} on mucosal [Na^+], [K^+] and [Cl^-], the composition of the bathing salines was manipulated to reduce or increase mucosal [Na^+], [K^+] and [Cl^-], thereby manipulating ionic gradients (see Table 1 for saline compositions). Everted rectal sacs were always filled with the standard serosal saline. To test the influence of ionic gradients and mucosal [ion] on J_v and J_{ion}, sacs were then placed either in a serosal saline to cancel the typical cation gradients and increase mucosal [Na^+] (N=7) or in a modified mucosal saline with reduced mucosal [Cl^-] to create a Cl^- gradient (N=5). Sacs were incubated under these experimental conditions for 2×1 h at 30°C followed by 2×1 h at 0°C before returning to standard conditions for 1 h (i.e. in standard mucosal saline with low [Na^+], high [K^+] and high [Cl^-]).

To test the influence of osmotic gradients, the mucosal side was made hyperosmotic by adding sucrose to the standard mucosal saline, thereby creating an osmotic gradient of 300 mOsm kg^{-1} (N=6) or of 700 mOsm kg^{-1} (N=5) across the rectal wall (Table 1). Everted rectal sacs were incubated for 2×1 h under standard conditions before an epithelial osmotic gradient was created and the sacs incubated for 2×1 h at 30°C followed by 1 h at 0°C. The osmolality of the saline solutions was determined and adjusted on a vapour pressure osmometer (Micro-Osmometer model 3320, Advanced Instruments, MA, USA). For each experiment, J_v and J_{ion} measured were compared with J_v and J_{ion} observed under standard conditions (i.e. standard mucosal saline with low [Na^+], high [K^+] and high [Cl^-]). In the experiment involving osmotic gradient, the size of the absorbate collected prevented us to determine J_{ion} (haemocoel volume <2 µl).

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Data analysis

All data are presented as means±s.e.m. and *N* indicates the number of locusts used. Data collected from the same everted rectal sac under a given experimental condition were pooled for graphical representations. All statistical comparisons were performed using GraphPad Prism v5.02 software (La Jolla, CA, USA) and $P<0.05$ was considered significant. Data were analysed using either one-way ANOVA followed by Tukey's *post hoc* multiple comparison test or repeated-measures two-way ANOVA, as specified in the figure legends. When necessary, data were log-transformed to meet the parametric assumptions of ANOVA.

RESULTS

Effects of temperature on *in vivo* water and ion balance and *ex vivo* net water and ion flux

Haemolymph volume and ionic composition were progressively altered with decreasing temperature (Fig. 1A,B), particularly below 5°C. Warm-acclimated locusts kept for 2 days at 5 and 0°C had significantly reduced volume of haemolymph compared with warm-acclimated locusts placed at 30°C (Fig. 1A). In addition, at 0°C, haemolymph [Na^+] and [Cl^-] decreased by ∼25% whereas [K^+] doubled (Fig. 1B). [Cl^-] was only significantly reduced at 0°C whereas [Na^+] and [K^+] were also significantly reduced at 5°C. Measurement of bulk water (J_v) and specific ion (i.e. Na^+, K^+ and Cl^-) movement (J_{ion}) across everted rectal sacs demonstrated a net reabsorption of both water and ions from the rectal lumen to the haemocoel side at both high and low temperature. The mean±s.e.m. values of J_v and J_{ion} at 30, 20, 10, 5 and 0°C are presented in Table 2 and Fig. 1C,D. Net reabsorption of water and ions across the rectum wall was gradually reduced with decreasing temperature (Fig. 1C,D and Table 2). J_{Na} was severely depressed at 10°C, where only 30% of the flux at 30°C was preserved. J_v, J_K and J_{Cl} reached their maximal inhibition at 5°C, where flux was reduced to 23%, 24% and 32% of that at 30°C, respectively.

Effect of thermal acclimation on net water and ion flux

In warm-acclimated locusts, J_v and J_{ion} measured for any ion were significantly decreased at 10 and 0°C. Cold acclimation slightly, though significantly, enhanced J_v across the rectal wall at 10°C but not at 0°C (Fig. 2A). J_{Na} and J_{Cl} across the rectal wall of cold-acclimated locusts at 10°C were similar to J_{Na} and J_{Cl} measured across cold- and warm-acclimated recta at 30°C (Fig. 2B,C). J_K was not significantly different between warm- and cold-acclimated locust at any temperature tested (Fig. 2D). At 0°C, J_v and J_{ion} were not significantly different between warm- and cold-acclimated locusts. The benefit of the cold-acclimation treatment was lost following acute exposure to 30°C (see Table S1).

Metabolic dependence of net water and ion flux

Replacement of constant O_2 bubbling with N_2 bubbling had a significant effect on rectal J_v and J_{ion} at high temperature but no

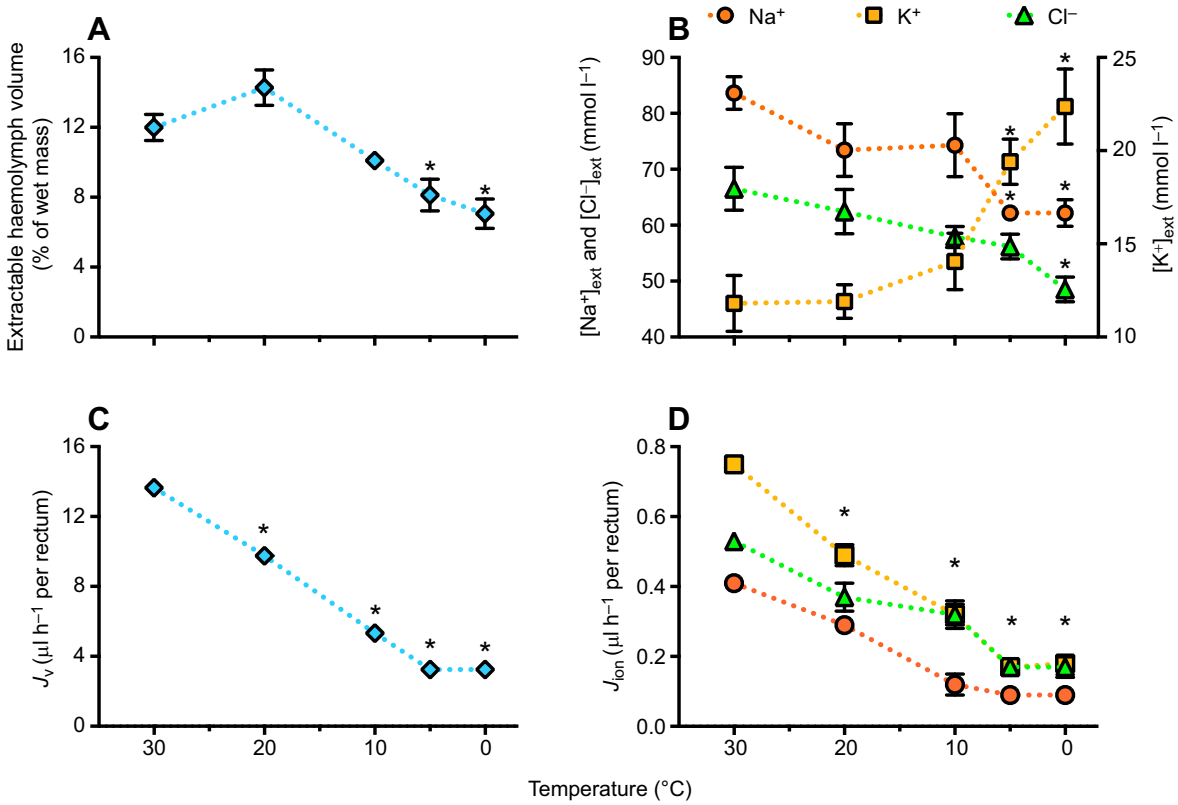

Fig. 1. Effect of temperature on organismal homeostasis and rectal osmoregulatory capacity in *Locusta migratoria*. (A) Extractable volume and (B) ionic (Na^+, K^+, Cl^-) composition of haemolymph (extracellular ion concentration) in warm-acclimated locusts exposed for 2 days to 30, 20, 10, 5 and 0°C (*N*=7 per group). (C) Net water and (D) ion (Na^+, K^+, Cl^-) flux across the rectal wall of warm-acclimated locusts at 30, 20, 10, 5 and 0°C (*N*=6 per group). Data in C and D were standardised to a rectum size of 0.631 cm^2, determined from the gross surface area of 50 recta (see Materials and methods). Data were analysed with one-way ANOVA followed by Tukey's multiple comparison test ($P<0.05$); values significantly different from control values (i.e. at 30°C) are indicated by an asterisk (see Table 2 for more details). Positive values for net water flux (J_v) and ion flux (J_{ion}) indicate net reabsorption from the rectal lumen towards the haemolymph. For some points, the error bars are shorter than the height of the symbol and are therefore not visible.

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Table 2. Net water and ion flux measured across the rectum wall of *Locusta migratoria* at 30, 20, 10, 5 and 0°C

	J_v		J_{Na}		J_K		J_{Cl}	
Temperature (°C)	Net flux	% Control	Net flux	% Control	Net flux	% Control	Net flux	% Control
30	14.1±0.2[a]	–	0.41±0.01[a]	–	0.75±0.02[a]	–	0.53±0.02[a]	–
20	9.8±0.3[b]	72	0.29±0.02[b]	71	0.49±0.03[b]	65	0.37±0.04[b]	70
10	5.3±0.1[c]	39	0.12±0.03[c]	29	0.32±0.03[c]	43	0.32±0.04[c]	61
5	3.3±0.1[d]	24	0.09±0.02[c]	22	0.17±0.02[d]	23	0.17±0.02[d]	32
0	3.2±0.2[d]	24	0.09±0.02[c]	22	0.18±0.02[d]	24	0.17±0.03[d]	32

Net water flux (J_v) is expressed as μl h^{-1} per rectum and net ion flux (J_{ion}) as μmol h^{-1} per rectum with a rectum size of 0.631 cm^2; these values are accompanied by the relative reductions (%) compared with controls. Positive values for J_v and J_{ion} indicate net reabsorption from the rectal lumen towards the haemolymph. *N*=6 per group; different letters indicate means that are significantly different (*P*<0.05) based on one-way ANOVA followed by Tukey's multiple comparison test.

significant effect at low temperature (Fig. 3). Severe hypoxia drastically reduced rectal J_v and J_{ion} by ∼40% (for J_K) and 60% (for J_v, J_{Na} and J_{Cl}) at high temperature but did not significantly alter J_v and J_{ion} at low temperature.

Temperature dependence of rectal paracellular permeability

The clearance of the paracellular permeability probe FD4 was not significantly different at 30°C and after 2 h at 0°C (Fig. 4A). The concentration of FD4 measured in the absorbate was ∼3-fold higher at 0°C compared with that measured at 30°C (Fig. 4B). Nevertheless, the final volume in the absorbate was proportionally (i.e. ∼3.5-fold) lower as a result of slowed water reabsorption (Fig. 4C), so the FD4 content (volume×concentration) was not different in total absorbate collected at 30 or 0°C (8.7±2.4 ng versus 10.7±3.1 ng per rectal sac, respectively).

Dependence of net water and ion flux on osmolality and mucosal ion concentration at high and low temperature

The influence of mucosal ion concentration on J_v and J_{ion} is shown in Table 3 and Fig. 5A,B. To test the influence of cation gradients on bulk water and ion movement, the standard mucosal saline (i.e. standard condition) used to bathe everted rectal sacs was replaced by serosal saline to cancel the cation gradients and test the effect of increased [Na^+] and reduced [K^+] on the mucosal side. Furthermore, we performed a different set of experiments with modified mucosal saline to test the effect of reduced mucosal [Cl^-] on J_v and J_{ion}.

J_v was not affected by modification of ionic gradients and the corresponding change in mucosal Na^+, K^+ and Cl^- availability at 30°C (Fig. 5A and Table 3). At 0°C, J_v was halved when mucosal [Cl^-] was reduced (Fig. 5A and Table 3) whereas the change in

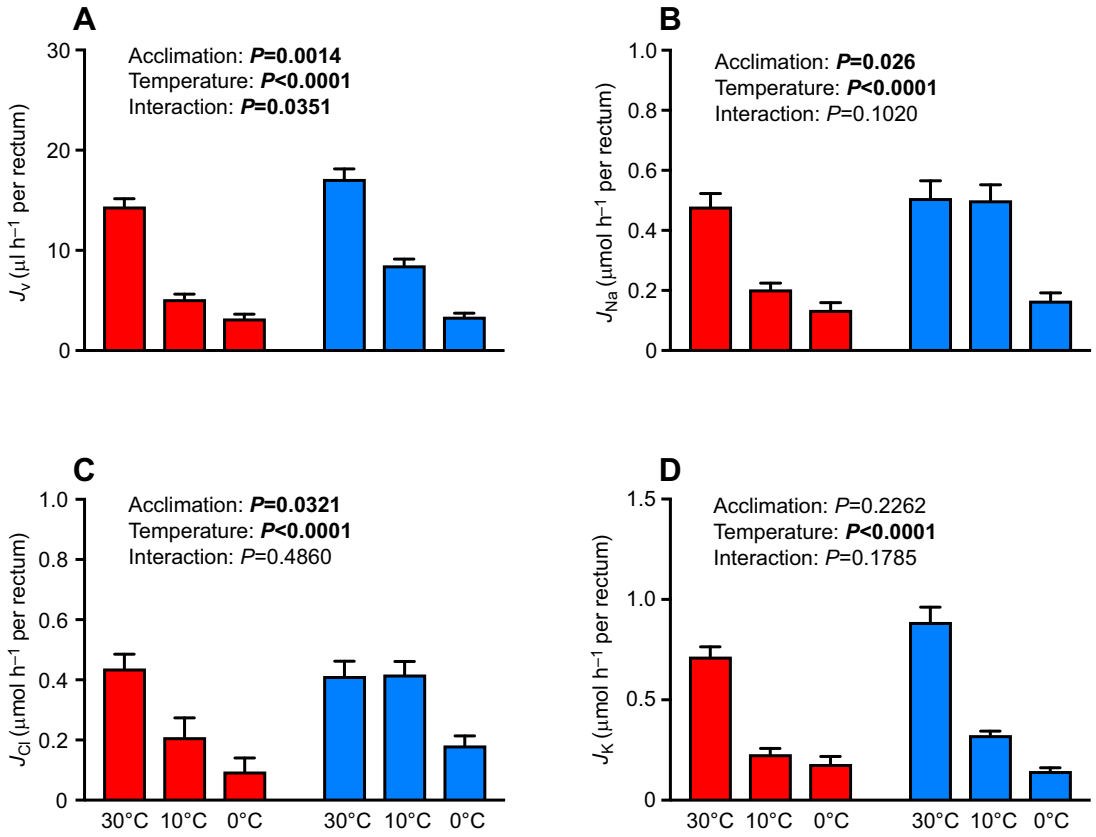

Fig. 2. Effect of thermal acclimation on rectal osmoregulatory capacity. (A) Net water (J_v) and (B–D) ion (J_{ion}) flux across the rectum wall at 30, 10 and 0°C in locusts acclimated for 5 days at either 30±1°C (warm acclimated; red) or 11±1°C (cold acclimated; blue). Data in A–D were standardised to a rectum size of 0.631 cm^2. *N*=7 per group; data were analysed with repeated-measures two-way ANOVA (*P*<0.05). Positive values for J_v and J_{ion} indicate net reabsorption from the rectal lumen towards the haemolymph.

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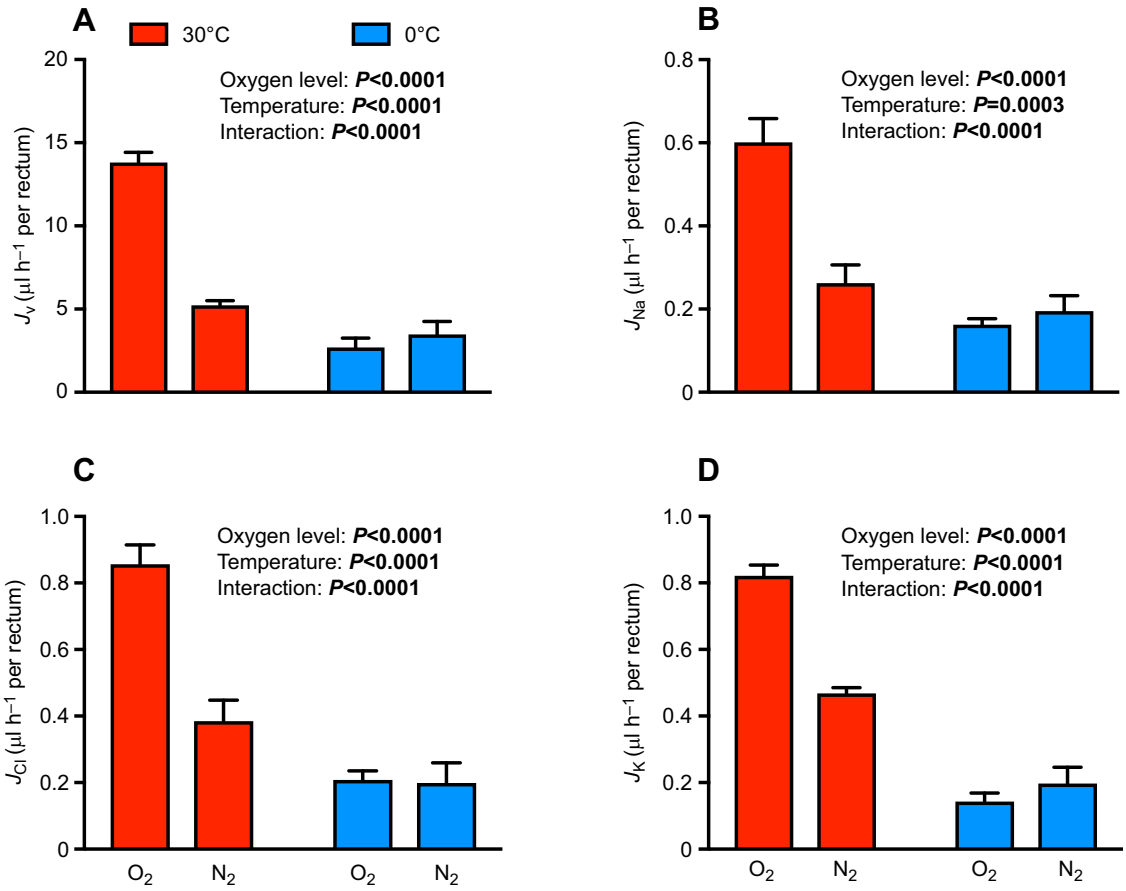

Fig. 3. Effect of hypoxia and hypothermia on rectal osmoregulatory capacity. (A) Net water (J_v) and (B–D) ion (J_{ion}) flux across the rectum wall at 30 and 0°C with either constant O_2 (normoxia) or N_2 (severe hypoxia) bubbling in warm-acclimated locusts. Data in A–D were standardised to a rectum size of 0.631 cm^2. *N*=5 per group; data were analysed with repeated-measures two-way ANOVA (*P*<0.05). Positive values for J_v and J_{ion} indicate net reabsorption from the rectal lumen towards the haemolymph.

mucosal cation concentration had no effect. In addition, J_v was sensitive to changes in mucosal osmolality. At 30°C, an osmotic gradient of 300 mOsm kg^{-1} from the lumen significantly reduced J_v (3.7±0.8 µl h^{-1} per rectum) compared with that under the standard condition where no osmotic gradient was present (J_v=14.76±0.3 µl h^{-1} per rectum). Increasing the osmotic gradient to 700 mOsm kg^{-1} ultimately prevented water reabsorption and reversed the direction of water movement: J_v was negative (−0.5±0.4 µl h^{-1} per rectum), indicating net water secretion from the haemocoel side to the rectal lumen (Fig. 5B). At 0°C, the reduction in J_v induced by the osmotic gradients was proportional to the reduction observed in the absence of osmotic gradients (i.e. ∼4-fold; Fig. 5B).

J_{ion} was modified by a change in mucosal ion concentration. Mucosal [Na^+] and [K^+] highly influenced J_{Na} and J_K across the rectal wall, independently of the temperature (Table 3). At both 30 and 0°C, the change in J_{Na} and J_K was proportional to the change in mucosal [Na^+] and [K^+]. Specifically, a 2.5-fold change in mucosal [Na^+] induced a ∼2-fold change in net Na^+ flux, while an 8-fold change in mucosal [K^+] availability induced a similar ∼8.5-fold change in net K^+ flux (Table 3). Interestingly, the overall J_{cation} (sum of J_{Na} and J_K) was unchanged. J_{Na} and J_K were only altered by changes in mucosal [Cl^-] at 0°C. In contrast, J_{Cl} was unaltered by mucosal [K^+] and [Na^+] at both 30 and 0°C. Furthermore, J_{Cl} sensitivity to mucosal [Cl^-] was temperature dependent. Hence, a 2.5-fold reduction in mucosal [Cl^-] did

Table 3. Influence of mucosal [ion] on net water and ion flux across the rectal wall of *L. migratoria* at high (30°C) and low (0°C) temperature

	J_v		J_{Na}		J_K		J_{Cl}	
Mucosal [ion]	30°C	0°C	30°C	0°C	30°C	0°C	30°C	0°C
Standard condition	14.76±0.32^a	3.24±0.16	0.47±0.02^a	0.09±0.02	0.78±0.07^a	0.21±0.02	0.61±0.04^a	0.17±0.03
↓[Cl^-]	14.68±0.61^a	1.50±0.12*	0.50±0.03^a	0.02±0.02*	0.58±0.03^a	0.06±0.01*	0.50±0.06^a	0.01±0.03*
↑[Na^+] – ↓[K^+]	13.06±0.45^a	3.50±0.29	1.12±0.05^b	0.33±0.06	0.09±0.01^b	0.02±0.004	0.65±0.09^a	0.18±0.04

Net water flux (J_v) is expressed as µl h^{-1} per rectum and net ion flux (J_{ion}) as µmol h^{-1} per rectum with a rectum size of 0.631 cm^2. Positive values for J_v and J_{ion} indicate net reabsorption from the rectal lumen towards the haemolymph. *N*=5–7 per group; different letters indicate means that are significantly different from the standard condition at 30°C (first row). The percentage change in J_v and J_{ion} between 30 and 0°C under the same experimental condition was calculated and compared with the percentage change observed under standard conditions; an asterisk indicates a significant difference (*P*<0.05) based on one-way ANOVA followed by Tukey's multiple comparison test.

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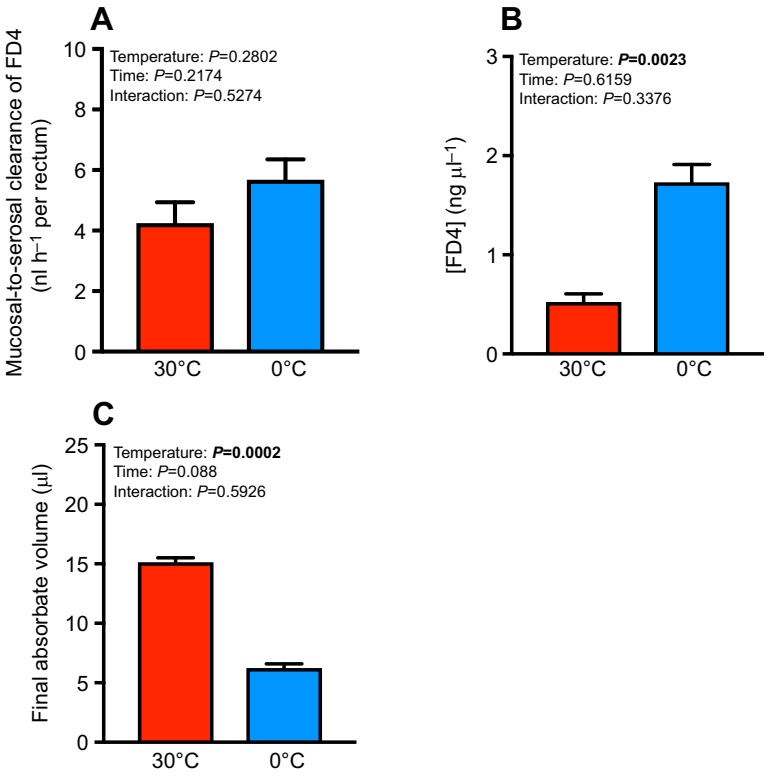

Fig. 4. Temperature dependence of rectal paracellular permeability. (A) Mucosal-to-serosal clearance of FD4 across the rectal wall of warm-acclimated locusts was determined from (B) the concentration of FD4 in the absorbate and (C) the fluid volume of the final absorbate at 30 and 0°C. Data in A were standardised to a rectum size of 0.631 cm^2. *N*=5 per group; data were analysed with repeated-measures two-way ANOVA (*P*<0.05).

not significantly influence J_{Cl} at 30°C but drastically reduced it at 0°C (Table 3). The influence of osmotic gradient on J_{ion} could not be determined because of the reduced volume of absorbate collected, preventing the determination of ion concentration in the final absorbate.

DISCUSSION

Maintenance of water and ion balance is critical for cold tolerance of chill-susceptible insects (Koštál et al., 2004; Overgaard and MacMillan, 2017). Here, we have provided direct evidence that osmoregulatory capacity of the hindgut is linked to cold tolerance in *L. migratoria*. Firstly, we showed that the capacity of the hindgut to reabsorb water and major ions at low temperatures is closely linked to the animal's capacity to maintain organismal water and ion homeostasis under these conditions. Secondly, we showed that warm- and cold-acclimated locusts, known to differ in their cold tolerance (Andersen et al., 2017b), differ in their hindgut osmoregulatory capacity in a corresponding manner. Finally, this study investigated the major physiological factors that determine hindgut osmoregulatory capacity during cold exposure. We showed that active transport is reduced at low temperature while rectal paracellular permeability is unchanged during acute cold exposure. We further showed that water reabsorption is severely depressed by severe hypoxia (probably as a result of suppressed active transport) and influenced by osmotic gradients and the transepithelial Cl^- gradient whereas it is independent of the strength of specific cation gradients across the epithelium (Na^+ and K^+ gradients).

Effects of temperature on *in vivo* water and ion balance and *ex vivo* water and ion flux

Consistent with earlier observations in *L. migratoria* and other orthopterans, we found that water and cation balance of the *L. migratoria* changes during chronic exposure to low temperature (Andersen et al., 2017b; Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; Findsen et al., 2013; MacMillan and Sinclair, 2011a). Haemolymph volume decreased along with $[Na^+]_{ext}$ whereas $[K^+]_{ext}$ more than doubled at temperatures below 5°C. Transport of Cl^- has been implicated as an important component of insect osmoregulation (Audsley et al., 2013; Peach and Phillips, 1991; Phillips et al., 1996) and here we observed a significant decrease in $[Cl^-]_{ext}$ that was proportional to the decrease in $[Na^+]_{ext}$ during chronic cold exposure. A previous study using *Gryllus* crickets showed that the reduction in haemolymph volume was associated with a proportional increase in gut water volume, suggesting a strong interaction between the haemolymph and the gut (MacMillan and Sinclair, 2011a). The changes we observed in haemolymph water and ion balance occurred at considerably higher temperatures than those leading to chill coma in *L. migratoria* (around 0±0.5°C as reported by Findsen et al., 2014). Our observations are in agreement with recent studies which suggest that cold-induced loss of movement in *L. migratoria* is caused primarily by neuronal dysfunction (Robertson et al., 2017) whereas loss of extracellular ion homeostasis is associated with the chill injury that develops during chronic cold exposure (Overgaard and MacMillan, 2017).

An important site for water and ion reabsorption in the gut of orthopterans is the posterior region of the hindgut, i.e. the rectum. An early study in the Pyrenean grasshopper *Oedipoda germanica* suggested only a minor influence of temperature on rectal fluid reabsorption (Houlihan and Sell, 1984), but that study only tested reabsorption at benign temperatures (from 15 to 35°C). Here, using *ex vivo* everted rectal sacs, we investigated whether hypothermia alters water and ion reabsorption across the rectum of locusts in a manner that is proportional to the homeostatic disruption of the haemolymph composition and volume. Overall, these values for J_v and J_{ion} were positive, indicating that the bulk of water and ion movement is dominated by net water and ion reabsorption from the

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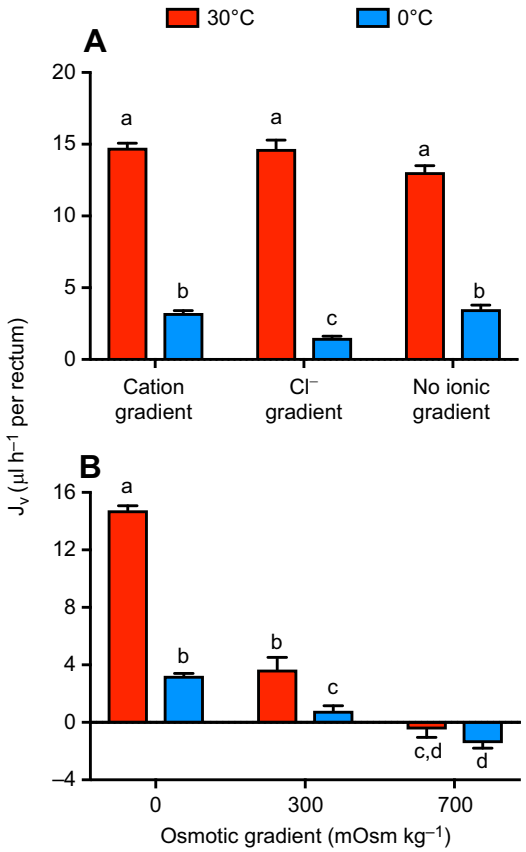

Fig. 5. Dependence of net water flux on osmotic and ionic gradients at high and low temperature. Net water flux (J_v) across the rectal wall of warm-acclimated locusts (A) without an ionic gradient or with cation (Na^+/K^+) or Cl^- gradients or (B) with osmotic gradients of 300 or 700 mOsmol kg^{-1} (set by increasing the osmolality of the mucosal saline, i.e. using the standard serosal saline and a hyperosmotic mucosal saline) at 30 and 0°C. Data in A and B were standardised to a rectum size of 0.631 cm^2. *N*=5–7 per group; data were analysed with one-way ANOVA followed by Tukey's multiple comparison test (different letters indicate a significant difference, $P<0.05$). Positive values for J_v indicate net fluid absorption from the rectal lumen towards the haemolymph while negative values indicate net fluid secretion from the haemocoel towards the rectal lumen.

rectal lumen to the haemocoel side across the rectum of *L. migratoria*. In accordance with our hypothesis, we found that water and ion reabsorption rates were gradually and significantly reduced with decreasing experimental temperature. Thus, the reduction in J_v, J_{Na} and J_{Cl} during low-temperature exposure agrees well with our *in vivo* measurements of $[Na^+]_{ext}$, $[Cl^-]_{ext}$ and haemolymph volume, which were all reduced (Fig. 1 and Table 2). Hence, alterations in haemolymph volume and composition during cold exposure are, at least partly, linked to cold-induced depression of rectal reabsorption capacity.

The drastic increase in *in vivo* $[K^+]_{ext}$ cannot be directly explained by rectal transport capacity as K^+ reabsorption (J_K) decreased at low temperatures while $[K^+]_{ext}$ increased. A recent study found regional differences in K^+ transport rate along the gut of *L. migratoria* and observed the highest reabsorption rate of K^+ at the ileum and the lowest rate at the colon and rectum (Robertson et al., 2014). The increase in $[K^+]_{ext}$ could therefore be tied to a combination of reduced haemolymph volume, passive diffusion/leak of K^+ along its chemical gradient towards the haemolymph compartment and/or decreased active removal of K^+ at other regions of the excretory system, such as the Malpighian tubules. Recent studies comparing cold- and warm-acclimated/adapted *Drosophila* have, for example, shown that differences in epithelial leak, K^+ excretion at the Malpighian tubule and K^+ reabsorption at the gut all contribute to intra- and inter-specific differences in homeostatic capacity during cold exposure in insects (Andersen et al., 2017b; MacMillan et al., 2015c, 2017; Yerushalmi et al., 2018).

Effect of acclimation on net water and ion flux

A number of previous studies in insects, including orthopterans, have shown that cold acclimation improves the ability to maintain ion balance and hence improve cold tolerance (Coello Alvarado et al., 2015; Koštál et al., 2004, 2007; MacMillan et al., 2015b). This is also the case for *L. migratoria*, where cold acclimation improves the ability to preserve and recover $[Na^+]_{ext}$ and $[K^+]_{ext}$ during and following cold exposure, respectively (Andersen et al., 2017b; Findsen et al., 2013). Considering the observed reduction in rectal reabsorption capacity at low temperature, we hypothesised that improved regulation of extracellular ion composition at low temperature is directly linked to increased osmoregulatory capacity of the rectum. Using a similar thermal treatment to that in Andersen et al. (2017b), we showed that the improved homeostatic capacity associated with cold acclimation is linked to a superior capacity for water and ion reabsorption at low temperature. Specifically, acclimation enhanced net water, Na^+ and Cl^- reabsorption (J_v, J_{Na} and J_{Cl}) at low temperature (10°C) in cold-acclimated locusts (Fig. 2). This could be caused by sustained activity of ion motive pumps in cold-acclimated animals or by changes in passive resistance of the epithelia, but future electrophysiological and pharmacological studies are needed to examine this in detail. Interestingly, net K^+ reabsorption was not increased in cold-acclimated locusts, which could be interpreted as an adaptive response as K^+ reabsorption would increase the hyperkalaemia associated with cold exposure (Fig. 2). However, cold- and warm-acclimated locusts showed similar low levels of J_v and J_{ion} at 0°C (the temperature where large differences in *in vivo* homeostatic capacity have been found between acclimation groups). This suggests that homeostatic capacity is also tied to changes in the relative activity of specific ion transporters (i.e. Cl^- and Na^+ reabsorption is increased and K^+ reabsorption is unchanged in cold-acclimated animals). Further, the effects of cold acclimation on J_v, J_{Na} and J_{Cl} appeared to be transient as pre-incubation of recta from cold-acclimated locusts at 30°C prevented the enhancement of J_v, J_{Na} and J_{Cl} observed with recta maintained and exposed to low temperature (Table S1). It is well established that insect osmoregulatory function is plastic and able to respond quickly to changing demands (Beyenbach, 2016; Terhzaz et al., 2015). Our finding that rapid re-warming of the recta removes the functional differences between acclimation groups suggest that this plasticity is linked to a temporary/reversible regulatory mechanism such as protein post-translational phosphorylation, but this suggestion needs to be tested in future studies.

Metabolic dependence of net water and ion flux

To characterize the active and passive components of rectal reabsorption, we used severe hypoxia to inhibit active transport. Severe hypoxia decreased active transport considerably at high temperature but there was no additional inhibition of J_v and J_{ion} at low temperature (Figs 1C and 3). This suggests that all active transport was already depleted by low-temperature exposure. At high temperature, ~60% of water and ion transport was inhibited by severe hypoxia, suggesting that more than half of rectal bulk water and ion movement is dependent on oxidative ATP production,

which is consistent with earlier studies (Chamberlin and Phillips, 1982; Goh and Phillips, 1978; Williams et al., 1978). Nevertheless, ~40% of net water and ion flux remained despite hypothermia and severe hypoxia. Locusts can sustain long periods of severe hypoxia and our observation agrees with the general assumption that water and ion transport processes in locust rectum are partly passive (i.e. passive diffusion along osmotic and ionic gradients). It is also possible that anaerobic ATP production fuelled some of the water and ion movement during severe hypoxia but further studies are needed to investigate this suggestion.

Temperature dependence of rectal paracellular permeability

As net transport is influenced by both active transport and passive leak, we assessed whether rectal paracellular permeability changed with temperature. Recent studies have produced molecular and cellular evidence that the passive leak of ions and water is influenced by alteration of the gut barrier function during cold exposure (Andersen et al., 2017b; Des Marteaux et al., 2017; MacMillan et al., 2017). To assess the paracellular permeability of the rectal wall in warm-acclimated locusts during short-term cold exposure, we quantified the mucosal-to-serosal clearance of FD4. FD4 is a widely accepted marker of paracellular permeability (Bao et al., 2014; MacMillan et al., 2017; Wang et al., 2013; Yang et al., 2002; Zhang et al., 2009). In agreement with previous studies on *Drosophila*, we observed a significant increase in FD4 concentration during cold exposure, suggesting an increase in permeability. However, the volume of absorbate was proportionally reduced during cold exposure as a result of the reduction in J_v at low temperature described above. Therefore, we did not observe a significant increase in mucosal-to-serosal clearance of FD4 across the rectal wall, suggesting that the paracellular permeability of the gut is not altered by short-term exposure (2 h) at 0°C in warm-acclimated locusts (Fig. 4). Accordingly, it is possible that some of the change in FD4 concentration (i.e. increased leak) observed in recent studies is also linked to changes in haemolymph volume during cold exposure (Andersen et al., 2017b; MacMillan et al., 2017).

Dependence of net water and ion flux on osmolality and mucosal ion concentration at high and low temperature

Bulk transport of water and ions is dependent on the ionic and osmotic gradients that drive/oppose active transport. However, to our knowledge, the relative importance of specific ionic and osmotic gradients across the rectal wall at low temperatures is largely unknown. To gain insight into the mechanisms of hindgut osmoregulation, we investigated hindgut sensitivity to osmotic gradients and mucosal ion composition. Sustained fluid transport across the rectal wall is primarily associated with active transport of ions from the mucosal to the serosal side of the epithelium (Beyenbach and Piermarini, 2008; Chapman, 2013; Goh and Phillips, 1978; Harrison et al., 2012). Accordingly, we manipulated the mucosal concentration of major ions to determine their influence on J_v and J_{ion} at high and low temperature, as it has been suggested that water balance is influenced by epithelial Na^+ gradients (Des Marteaux and Sinclair, 2016; MacMillan and Sinclair, 2011a; MacMillan et al., 2015b; Olsson et al., 2016). To test this hypothesis, we incubated everted rectal sacs with or without a Na^+ gradient across the rectal wall at high and low temperature. However, we did not observe any influence of Na^+ gradient on J_v at either temperature (Fig. 5A and Table 3). Thus, water reabsorption appears to be mechanistically unrelated to the transepithelial Na^+ gradient. Importantly, this experiment only examines the role of cation gradients for rectal water transport and it is possible that the strength of particular ion gradients influences active or passive flux in other parts of the osmoregulatory system. The sensitivity of J_v to osmotic gradients across the rectal wall has previously been established in studies of the locust *Schistocerca gregaria* (i.e. an osmotic gradient of 650 mOsm kg^{-1} prevented fluid reabsorption at 30°C; Goh and Phillips, 1978). However, the effect of osmotic gradient at low temperature has not been investigated previously. As seen in Fig. 5B and Table 3, J_v was significantly reduced by an osmotic gradient across the rectal wall. An osmotic gradient of 300 mOsm kg^{-1} considerably reduced water reabsorption while an osmotic gradient of 700 mOsm kg^{-1} completely prevented water reabsorption by active transport and led to a net secretion of water (i.e. water movement into the rectal lumen by osmosis).

Considering the impairment of water transport in the face of strong osmotic gradients across the rectal epithelia, we speculate that evacuation of the gut contents before cold exposure could prevent some of the water drift that has been associated with acute cold exposure. Indeed, many insects empty their guts in response to cold exposure (for reviews, see Andreadis and Athanassiou, 2017; Ganji and Moharramipour, 2017; Rozsypal, 2015). This strategy is usually linked to depression of supercooling points but similar responses may also improve homeostatic regulation in chill-sensitive insects that are also sensitive to the effects of low temperature irrespective of freezing.

Regarding the dependence of J_{ion} on specific ionic gradients, J_{Na} and J_K were highly dependent on and proportional to the mucosal availability of Na^+ and K^+, respectively, as shown in Table 3. In contrast, J_{Cl} was maintained when mucosal [Cl^-] was reduced at high temperature. This suggests some flexibility of the active transport systems where total cation transport can be maintained at similar levels using either of the two major cations while anion transport may necessitate a constant flow of Cl^- associated with active transport. Indeed, at low temperature the active component of ion and water transport was suppressed and both J_v and J_{Cl} were drastically reduced (Table 3 and Fig. 5A), in agreement with the assumption that water movement is tied to active transport of Cl^- (Audsley et al., 2013; Hanrahan and Phillips, 1984). In addition, J_{Cl} was not influenced by changes in mucosal [Na^+] and [K^+] tested, which concurs with previous evidence that Cl^- transport is not coupled to Na^+ and K^+ movement (Phillips et al., 1988, 1996).

Conclusions

This study documents the regulation of bulk water and ion transport across the rectum of *L. migratoria* during cold exposure and provides insight into the metabolic, ionic and osmotic control of rectal reabsorption at high and low temperature. We have demonstrated that temperature influences bulk water and ion movement across the rectum of an insect in a manner that can explain the well-known organismal loss of water and ion homeostasis during cold exposure. Further, we showed that cold acclimation improves net water, Cl^- and Na^+ reabsorption at low temperature, which partly explains the previously reported maintenance of homeostasis and increased cold tolerance in cold-acclimated *L. migratoria* (Andersen et al., 2017b). Additionally, we demonstrated that osmotic and Cl^- gradients have a marked influence on reabsorptive capacity while water reabsorption seems to be independent of the strength of Na^+ or K^+ gradients. The present study therefore confirms the link between osmoregulatory capacity and thermal tolerance in insects (Overgaard and MacMillan, 2017). Considering this association, future electrophysiological and pharmacological studies are necessary to

investigate how aspects of cold adaptation and acclimation are tied to osmoregulatory function in insects. These studies could include investigation of specific ion transporters (e.g. Na^+/K^+-ATPase, V-type H^+-ATPase and Cl^- electrogenic pump), iono-regulatory peptides [e.g. chloride transport stimulating hormone (CTSH) and insect transport neuropeptide (ITP) at the hindgut], signalling pathways (e.g. cAMP and cGMP), membrane barrier function and permeability and how these factors are influenced by cold exposure, cold acclimation and cold adaptation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.G., J.O.; Methodology: L.G., J.O.; Validation: L.G., J.O.; Formal analysis: L.G.; Investigation: L.G.; Writing - original draft: L.G.; Writing - review & editing: J.O.; Visualization: L.G.; Supervision: J.O.; Project administration: L.G., J.O.; Funding acquisition: J.O.

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Table S1. Net water and ion flux measured across the rectal wall of cold-acclimated *Locusta migratoria* after acute exposure to high temperature (i.e. 2 h at 30℃) prior to incubation at 10℃.

Acclimation	J_V		J_{Na}		J_K		J_{Cl}	
group	30℃	10℃	30℃	10℃	30℃	10℃	30℃	10℃
Warm acclimated	14,68 ± 0,66***a***	4,73 ± 0,44***b***	0,49 ± 0,03***a***	0,16 ± 0,01***b***	0,83 ± 0,03***a***	0,24 ± 0,02***b***	0,65 ± 0,04***a***	0,31 ± 0,02***b***
Cold acclimated	14,64 ± 0,65***a***	4,36 ± 0,22***b***	0,49 ± 0,04***a***	0,16 ± 0,01***b***	0,70 ± 0,04***a***	0,21 ± 0,01***b***	0,68 ± 0,12***a***	0,32 ± 0,08***b***
RM-2-way ANOVA	Acclimation: P=0.5244 Temperature: **P<0.0001** Interaction: P=0.8949		Acclimation: P=0.8101 Temperature: **P=0.0001** Interaction: P=0.9119		Acclimation: P=0.1271 Temperature: **P<0.0001** Interaction: P=0.7296		Acclimation: P=0.8548 Temperature: **P=0.0231** Interaction: P=0.9100	

In this set of acclimated-locusts, the order of the incubation periods was reversed to test the plasticity and reversibility of the acclimation response. Hence, everted sacs from cold-acclimated locusts were first incubated at 30 ℃ prior to incubation at 10 ℃ (the temperature at which cold-acclimation enhanced J_v and J_{ion}; cf. Fig.2). Net water flux (J_v,) is expressed as µl rectum^{-1} h^{-1} and net ion flux (J_{ion}) as µmol h^{-1} per rectum, where rectum size was standardized to 0.631 cm^2. Positive values for J_v and J_{ion} indicate net reabsorption from the rectal lumen towards the haemolymph. *N*=5 per group and the statistical significance of each factors are presented in the table following repeated-measures two-way ANOVA.