© 2018. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2018) 221, jeb185884. doi:10.1242/jeb.185884

RESEARCH ARTICLE

Anti-diuretic activity of a CAPA neuropeptide can compromise *Drosophila* chill tolerance

Heath A. MacMillan*,¶, Basma Nazal‡, Sahr Wali§, Gil Y. Yerushalmi, Lidiya Misyura, Andrew Donini and Jean-Paul Paluzzi

ABSTRACT

For insects, chilling injuries that occur in the absence of freezing are often related to a systemic loss of ion and water balance that leads to extracellular hyperkalemia, cell depolarization and the triggering of apoptotic signalling cascades. The ability of insect ionoregulatory organs (e.g. the Malpighian tubules) to maintain ion balance in the cold has been linked to improved chill tolerance, and many neuroendocrine factors are known to influence ion transport rates of these organs. Injection of micromolar doses of CAPA (an insect neuropeptide) have been previously demonstrated to improve *Drosophila* cold tolerance, but the mechanisms through which it impacts chill tolerance are unclear, and low doses of CAPA have been previously demonstrated to cause anti-diuresis in insects, including dipterans. Here, we provide evidence that low (femtomolar) and high (micromolar) doses of CAPA impair and improve chill tolerance, respectively, via two different effects on Malpighian tubule ion and water transport. While low doses of CAPA are anti-diuretic, reduce tubule K^+ clearance rates and reduce chill tolerance, high doses facilitate K^+ clearance from the haemolymph and increase chill tolerance. By quantifying CAPA peptide levels in the central nervous system, we estimated the maximum achievable hormonal titres of CAPA and found further evidence that CAPA may function as an anti-diuretic hormone in *Drosophila melanogaster*. We provide the first evidence of a neuropeptide that can negatively affect cold tolerance in an insect and further evidence of CAPA functioning as an anti-diuretic peptide in this ubiquitous insect model.

KEY WORDS: Abiotic stress, Insect, Ion homeostasis, Temperature, Neuropeptides

INTRODUCTION

The majority of insects are chill susceptible, meaning that they are injured and killed by exposure to temperatures that slow physiological processes without causing ice formation (Baust and Rojas, 1985; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). There is a growing interest in understanding the biochemical and physiological mechanisms underlying chill susceptibility in ectothermic animals, and several studies have demonstrated that the ability of terrestrial insects to maintain ion and water homeostasis in the cold is closely associated with their chill tolerance (Des Marteaux and Sinclair, 2016; Findsen et al., 2013; Koštál et al., 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2015d).

In particular, the ability to maintain low extracellular $[K^+]$ appears to be critical to chill tolerance (Overgaard and MacMillan, 2017). Chilling slows the activity of membrane-bound ATPases (such as Na^+/K^+-ATPase), leading to rapid membrane depolarization through a reduction in the electrogenic contribution of these primary active transporters to membrane potential (Andersen et al., 2017a; Djamgoz, 1987; MacMillan et al., 2014; Rheuben, 1972). Over minutes to hours, suppressed ion transport enables the net movement of Na^+ and water down their concentration gradients from the haemolymph to the gut. The resulting loss of haemolymph volume, combined with concurrent leak of K^+ down its concentration gradient from tissues to the haemolymph, can cause progressive haemolymph hyperkalemia (Andersen et al., 2017b; Koštál et al., 2006; MacMillan and Sinclair, 2011b; Overgaard and MacMillan, 2017). As the K^+ gradient is a critical determinant of cell membrane potentials, this loss of K^+ balance leads to further cell depolarization, and the combined depolarizing effects of cold and hyperkalemia lead to cell death, probably through triggering of apoptotic signalling cascades (MacMillan et al., 2015c; Yi et al., 2007).

Although chilling can disrupt ion and water balance, leading to organismal injury and death, there is wide variation in chill tolerance among and within insect species, and flies of the genus *Drosophila* are a common and useful model for understanding the mechanisms underlying chill tolerance adaptation and phenotypic plasticity. For example, *Drosophila* species can widely vary in cold tolerance when reared under common-garden conditions; those species that come from more poleward environments are more chill tolerant (Kellermann et al., 2012; MacMillan et al., 2015a) and better maintain K^+ balance in the cold (Andersen et al., 2017c; MacMillan et al., 2015c). Similarly, *Drosophila melanogaster* acclimated to moderately low temperatures (10–15°C) during larval development or as adults are more tolerant of extreme chilling (at 0°C) and better maintain low haemolymph K^+ during cold stress (Colinet and Hoffmann, 2012; MacMillan et al., 2015b).

Given the above evidence of a role for ion homeostasis in insect chill susceptibility and chill tolerance, there has been recent interest in how the organs responsible for the maintenance of osmotic balance may drive cold tolerance adaptation (Andersen et al., 2017c; Des Marteaux et al., 2018; MacMillan et al., 2015d; Terhzaz et al., 2015; Yerushalmi et al., 2018). Conveniently, the physiology of osmotic balance in *D. melanogaster* has been under active investigation for decades. The Malpighian tubules of insects (including *Drosophila*) are a single-cell-thick tubular epithelium, where active transport of ions by V-type H^+-ATPase and Na^+/K^+-ATPase drives the concomitant movement of ions (primarily Na^+/K^+ and Cl^-) and water into the lumen of the

Department of Biology, York University, Toronto, ON, Canada M3J 1P3. *Present address: Department of Biology, Carleton University, Ottawa, ON, Canada K1S 5B6. ‡Present address: Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada N1G 2W1. §Present address: DeGroote School of Business, McMaster University, Hamilton, ON, Canada L8S 4L8.

¶Author for correspondence (heath.macmillan@carleton.ca)

H.A.M., 0000-0001-7598-3273

Received 1 June 2018; Accepted 3 August 2018

Journal of Experimental Biology (2018) 221, jeb185884. doi:10.1242/jeb.185884

tubule to produce an isosmotic primary urine (Dow et al., 1998; O'Donnell, 2008). This primary urine flows into the gut lumen at the midgut–hindgut junction, and active transport in the ileum and rectum allows for the reabsorption of ions and water into the haemocoel and production of a hyperosmotic excreta (Hanrahan and Phillips, 1983; Phillips et al., 1987). The Malpighian tubules of cold-adapted and acclimated *Drosophila* better defend rates of fluid and K^+ secretion at low temperatures; a modification that would help prevent against hyperkalemia (Andersen et al., 2017c; MacMillan et al., 2015d; Yerushalmi et al., 2018). Chill-tolerant drosophilids also reduce rectal K^+ reabsorption during cold stress (preventing hyperkalemia) while those that are chill susceptible have higher rates of K^+ reabsorption in the cold (which would contribute to hyperkalemia) (Andersen et al., 2017c). Similarly, cold-acclimated *D. melanogaster* have reduced rates of K^+ reabsorption at the rectum relative to warm-acclimated flies (Yerushalmi et al., 2018). Although the impacts of cold acclimation on the transport of other ions remain to be examined in *Drosophila*, a recent and thorough analysis with locusts (*Locusta migratoria*) demonstrated that cold acclimation led to increased rates of rectal Na^+, Cl^- and water reabsorption without increasing K^+ reabsorption (Gerber and Overgaard, 2018). Critically, all of these changes suggest that insect cold tolerance is intimately tied to the ability of the Malpighian tubules and rectum to maintain function at low temperature in a manner that favours low extracellular [K^+]. All of the above studies, however, were conducted without the influence of neuroendocrine factors that are known to precisely regulate insect renal function *in vivo*.

Ion and water balance are under tight neuroendocrine control in insects; rates of transport in the Malpighian tubules and hindgut are independently controlled by a variety of factors (Coast, 2007). Many neuropeptides, for example, are produced in neurosecretory cells in the central nervous system and released into the haemolymph, where they bind to receptors, initiating signalling cascades that alter rates of ion and water secretion or absorption (Coast, 2007). Diuretic factors, such as the corticotropin-releasing factor-related peptide, stimulate fluid secretion by the Malpighian tubules, while anti-diuretic factors can slow rates of primary urine production by the tubules or lead to enhanced reabsorption across the hindgut (Coast et al., 2002). Several factors have been demonstrated to induce diuresis in insects, whereas the number of reported anti-diuretic factors is much more limited, despite widespread appreciation that the ability to slow rates of diuresis is likely to be critical to insect survival under a wide variety of abiotic conditions (Paluzzi, 2012).

The first member of the CAPA peptide family was originally identified and found to be cardioacceleratory in *Manduca sexta* (Huesmann et al., 1995), and genes encoding these peptides were later discovered in *D. melanogaster*, and called *capability* (*capa*) (Davies et al., 1995; Kean et al., 2002). CAPA peptides have been shown to have either diuretic or anti-diuretic effects on tubule function, depending on the specific assay conditions and the species under study (Davies et al., 2013; Ionescu and Donini, 2012; Paluzzi, 2012; Pollock et al., 2004; Rodan et al., 2012). In *D. melanogaster* in particular, CAPA peptides are generally thought to be diuretic, and act through increased nitric oxide, cGMP and Ca^{2+} levels in the principal cells of the tubules (Davies et al., 1995; Davies et al., 2013; Kean et al., 2002). It has also been demonstrated, however, that CAPA can instead have anti-diuretic and anti-kaluretic effects on the tubules of wild-type *D. melanogaster* (Rodan et al., 2012). In addition, there is evidence that in the mosquito *Aedes aegypti* (another dipteran), CAPA peptides are anti-diuretic, act through cGMP, and counteract the actions of diuretic hormones such as 5-HT and mosquito natriuretic peptide (i.e. a DH_{31}-related peptide) (Ionescu and Donini, 2012; Sajadi et al., 2018). Critically, these anti-diuretic effects observed in *A. aegypti* occur at very low (e.g. femtomolar) concentrations of CAPA peptides, while higher supraphysiological doses (e.g. 100 µmol l^{-1}) were instead found to have modest diuretic effects (Ionescu and Donini, 2012). Considering these recent findings from a relatively closely related insect led us to reconsider the question of whether low concentrations of CAPA peptide are indeed capable of causing anti-diuresis in *D. melanogaster*, as observed in other insects (Coast et al., 2010; Paluzzi et al., 2008; Sajadi et al., 2018).

Whether CAPA peptides are diuretic or anti-diuretic is of great importance to understanding neuropeptide control of salt balance, but it is also critical background knowledge if we are to understand the mechanisms through which abiotic stressors, such as desiccation or cold stress, impact organismal fitness. Given their role in osmotic balance, and the importance of osmotic balance to both desiccation and chill tolerance, members of the CAPA peptide family have already been tested in such a context (Terhzaz et al., 2015). In *Drosophila*, cold stress leads to upregulation of *capa* mRNA; injections (µmol l^{-1}) of *Manse*-CAP2b achieving a micromolar titre speed up recovery from chill coma, and targeted knockdown of the *capa* gene slows chill coma recovery (Terhzaz et al., 2015). The physiological means by which CAPA has these effects on chill tolerance, however, remain unknown.

Here, we test the hypothesis that CAPA peptide exerts differential activity on chill tolerance in *D. melanogaster* through dose-dependent effects of this neuropeptide on Malpighian tubule ion and water secretion. We predicted that very low (e.g. fmol l^{-1}) doses of CAPA would be anti-diuretic (inhibit fluid secretion by the Malpighian tubules), which would impair K^+ clearance from the haemolymph and thereby impair chill tolerance. We further predicted that higher doses (µmol l^{-1}) of CAPA would be diuretic, which would improve chill tolerance through increased K^+ clearance by the tubules. We follow this mechanistic analysis by addressing a simple question: what levels of CAPA peptides are likely to occur in the haemolymph of a free-living adult fly?

MATERIALS AND METHODS

Animal husbandry

The population of *Drosophila melanogaster* Meigen 1830 used in this study was derived from isofemale lines collected in southwestern Ontario, Canada in 2007 (Marshall and Sinclair, 2010). All flies were reared at 25°C (14 h:10 h light:dark cycle) in 200 ml bottles containing 50 ml Bloomington *Drosophila* medium (Lakovaara, 1969). Groups of ∼80 adult flies were given access to fresh food for 2–3 h to oviposit before being removed to ensure rearing densities of ∼100 eggs per bottle. Females were collected under brief CO_2 anaesthesia (<2 min exposure to CO_2) upon final ecdysis and transferred to vials containing 7 ml of the same medium at a density of 20 flies per vial, where they were left to mature for 7 days, in part to avoid effects of anaesthesia on chill tolerance (Nilson et al., 2006). All experiments were thus conducted on 7-day-old virgin female flies.

Short-term effects of CAPA on chill coma recovery

To examine the effects of CAPA injection on chill coma recovery time (CCRT), we conducted a dose–response experiment. Individual female flies (N=9–14 flies per treatment group) were transferred (without anaesthesia) to 4 ml glass screw top vials that were submerged in an ice-water slurry (0°C), which rapidly induced

chill coma as it is below the critical thermal minimum temperature for this population of *D. melanogaster* (MacMillan et al., 2017). Flies were left at 0°C for either 30 min or 2 h, whereupon they were individually removed from the ice-water and placed on a Plasticine surface on top of a double-walled glass plate held at 0°C. A mixture of ethylene glycol and water was circulated through the glass dish from a refrigerated circulating bath (MX7LL, VWR International, Mississauga, Canada) to keep the flies in chill coma during injection.

Solutions containing an insect (*A. aegypti*) CAPA neuropeptide (*Aedae*CAPA2: pQGLVPFPRV-NH_2), were prepared in order to achieve a final post-injection concentration of 10^{-15}, 10^{-12}, 10^{-9} and 10^{-6} mol l^{-1} based on an ∼80 nl haemolymph volume (Folk et al., 2001). CAPA peptide solutions were made up in a 1:1 mixture of *Drosophila* saline (117 mmol l^{-1} NaCl, 20 mmol l^{-1} KCl, 8.5 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} glutamine, 20 mmol l^{-1} glucose, 4.3 mmol l^{-1} NaH_2PO_4, $NaHCO_3$, 15 mmol l^{-1} MOPS, pH 7.0) and Schneider's insect medium (Sigma-Aldrich, Oakville, ON, Canada). Injections (18.4 nl) were administered into the haemolymph at the base of the left wing (mesopleurum) using a pulled glass microcapillary connected to a Nanoject system (Drummond Scientific, Broomall, PA, USA). Control flies were placed on the cooled plate but received no injection, whereas sham-injected flies were injected with saline that did not contain any CAPA peptide. Flies were subsequently returned to their vials and re-submerged in the ice-water at 0°C. All flies spent a total of 3 h at 0°C before being removed to record chill coma recovery time, and thus the groups injected at 30 min and 2 h differed only in the time during the cold stress at which the injection was given.

CCRT was measured as previously described (MacMillan et al., 2015b). Briefly, flies, in their glass vials, were removed from the cold and placed on a laboratory bench lined with paper at 23°C. The flies were observed in their vials without being disturbed, and the time taken for each fly to right itself and stand on all six legs was recorded.

Effects of CAPA peptide injection on chill coma recovery after prolonged chilling

To determine whether CAPA injection similarly impacted chill coma recovery after chronic chilling, we measured chill coma recovery following 16 h exposure to 0°C (N=18–19 flies per treatment group). As in the previous experiment, *D. melanogaster* females were individually separated into 4 ml glass vials that were submerged in an ice-water slurry (0°C). After 15 h at 0°C, flies were injected with 18.4 nl of either the sham or CAPA peptide (to achieve final concentrations of 10^{-6} mol l^{-1} and 10^{-15} mol l^{-1}, the lowest and highest doses used in the previous short-term chill experiment). Control animals were handled as described above but received no injection. The flies were then returned to their vials and placed in the ice-water slurry for a further 1 h (total exposure 16 h at 0°C), at which point the flies were removed from the ice bath and transferred to room temperature to measure CCRT as above.

Effects of CAPA peptide injection on survival following cold stress

To examine whether CAPA peptide injection influences survival following prolonged chilling, we injected flies early in a 16 h cold stress and recorded survival outcomes (N=50 flies per treatment group). As above, individual flies were placed into 4 ml glass vials and submerged in an ice-water mixture (0°C). Flies were injected after 1 h at 0°C with either the saline alone (i.e. sham), or saline containing CAPA peptide to achieve a final titre of 10^{-6} mol l^{-1} or 10^{-15} mol l^{-1} in the haemolymph. The flies were then returned to their vials, and held at 0°C for a further 15 h (16 h at 0°C in total) upon which they were removed from the cold and transferred to 40 ml plastic vials containing 7 ml of fresh food medium in groups of 10. The flies were then held under their rearing conditions (25°C) for 24 h, before being visually inspected with minimal disturbance to determine survival, which was scored for each fly on a 5-point scale: 1=dead (no movement), 2=moving but unable to stand, 3=standing but not climbing, 4=climbing, 5=flying.

Effects of CAPA and cGMP on Malpighian tubule function

We measured the effects of low (10^{-15} mol l^{-1}) and high (10^{-6} mol l^{-1}) doses of CAPA as well as low (10^{-8} mol l^{-1}) and high (10^{-3} mol l^{-1}) levels of cGMP on Malpighian tubule fluid and ion secretion rates using Ramsay assays combined with the ion-selective electrode technique, as previously described (MacMillan et al., 2015d). Flies (CAPA: N=25–32 per treatment group; cGMP: N=12–13 per treatment group) were dissected under *Drosophila* saline to carefully isolate the anterior pair of Malpighian tubules, which were separated from the gut at the ureter. The pair of tubules connected at the ureter were transferred to a Sylgard-lined Petri dish with ∼4-mm-deep wells that were set 0.5 cm apart. The dish was filled with hydrated paraffin oil to prevent sample evaporation. A 20 µl droplet of a 1:1 mixture of Schneider's insect medium and *Drosophila* saline was added to each well. This mixture contained either no neuropeptide or no cGMP (i.e. control), 10^{-6} mol l^{-1} or 10^{-15} mol l^{-1} CAPA, or 10^{-8} mol l^{-1} or 10^{-3} mol l^{-1} 8-bromo cGMP (Sigma-Aldrich), a membrane-permeable analogue of cGMP with greater resistance to phosphodiesterases compared with its parent compound. One pair of tubules was placed in the drop of bathing medium and the proximal end of one tubule was pulled out of the drop and wrapped around a minuten pin. As the tubule remaining in the bathing solution actively secretes fluid, a droplet forms at the ureter. After 30 min, the droplet was detached from the ureter, lifted off of the Sylgard surface and its diameter was measured with an eyepiece micrometer. Droplet volume was calculated from the diameter of the secreted droplet.

Concentrations of ions in the primary urine secreted by the Malpighian tubules were measured using the ion-selective microelectrode technique (Rheault and O'Donnell, 2004). Ion-selective microelectrodes were pulled from glass capillaries (TW150-4; World Precision Instruments, Sarasota, FL, USA) using a P-97 Flaming Brown micropipette puller (Sutter Instruments, San Rafael, CA, USA) to produce a probe with a short shank and wide angle with a ∼5 µm tip diameter. Micropipettes were then silanised at 300°C with *N*,*N*-dimethyltrimethylsilylamine (Fluka, Buchs, Switzerland). For K^+ measurements, the micropipette was back-filled with 100 mmol l^{-1} KCl and front-filled with K^+ ionophore (potassium ionophore I cocktail B; Fluka). For Na^+ measurements, the micropipette was back-filled with 100 mmol l^{-1} NaCl and front-filled with Na^+ ionophore (sodium ionophore II cocktail A; Fluka). Ion-selective microelectrodes were dipped in a solution of polyvinylchloride in tetrahydrofuran to prevent the ionophore from leaking out of the microelectrode upon contact with the paraffin oil (Rheault and O'Donnell, 2004). The circuit was completed with a reference electrode, pulled from glass capillaries (IB200F-4, World Precision Instruments), and back-filled with 500 mmol l^{-1} KCl. Both electrodes were connected to an amplifier (ML 165 pH Amp), which was connected to PowerLab 4/30 data acquisition system (AD Instruments, Colorado Springs, CO, USA). Data were recorded using Labchart 6 Pro software (AD Instruments).

Ion concentrations (mmol l^{-1}) in the secreted droplets were calculated using the following equation (Donini et al., 2008):

$$[X] = [C] \times 10^{\Delta V/S}, \quad (1)$$

where $[X]$ is the concentration of the secreted fluid droplet, $[C]$ is the ion concentration of one of the standards, ΔV is the voltage difference between the secreted fluid droplet and the voltage measured in the same standard, S is the difference in voltage between two standard solutions (which cover a 10-fold difference in ion concentration). Rates of ion secretion from the tubule were then calculated from droplet concentrations and fluid secretion rates.

Quantification of CAPA peptides in the *Drosophila melanogaster* haemolymph and nervous system

Haemolymph was extracted from flies (N=60 flies per sample) as previously described (MacMillan and Hughson, 2014), pooled into methanol:acetic acid:water (90:9:1) and frozen for later processing. The thoracicoabdominal ganglion was dissected from adult female *D. melanogaster* using forceps under *Drosophila* saline in the view of a dissecting microscope. Each dissection took approximately 2 min and ganglia were transferred to a microcentrifuge tube containing methanol:acetic acid:water (90:9:1) held on ice as they were individually dissected to produce three biological replicates, each containing 20 ganglia, which were stored at −80°C for later analysis. Peptidergic extracts were then isolated by sonicating ganglionic samples on ice for two consecutive 5 s pulses using an XL 2000 Ultrasonic Processor (Qsonica LL, Newtown, CT, USA). Ganglionic and haemolymph homogenates were then centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatants were transferred to new microcentrifuge tubes, dried in a Jouan RC10 series vacuum concentrator (Jouan, Winchester, VA, USA) and reconstituted in 0.4% trifluoroacetic acid (TFA). Samples were then applied to C18 Sep-Pak cartridges (Waters Associates, Mississauga, ON, Canada) following sequential washing and equilibration with 10 ml of acetonitrile (ACN), 5 ml of 50% ACN, 0.5% acetic acid (HAcO), and finally 5 ml 0.1% TFA. To ensure complete binding of peptidergic extracts, samples were passed through the Sep-Pak cartridge at least three times. Once the samples were loaded, the cartridges were first washed/desalted with 5 ml 0.1% TFA and subsequently with 5 ml 0.5% HAcO to remove TFA. Samples were then eluted with 2 ml each of 0.5% HAcO, 10%, 20%, 30%, 40% and 50% ACN all containing 0.5% HAcO. The eluants were dried in a vacuum concentrator as above and samples were then stored at −20°C for later quantification analysis using an enzyme-linked immunosorbent assay (ELISA).

A CAPA peptide-specific ELISA was developed based on an earlier report describing a crustacean cardioactive peptide (CCAP)-specific ELISA used in the stick insect *Baculum extradentatum* (Lange and Patel, 2005). A rabbit anti-CAPA affinity-purified polyclonal antibody (a generous gift from Prof. Ian Orchard, University of Toronto Mississauga, ON, Canada) was diluted 1:1000 in carbonate buffer (15 mmol l^{-1} Na_2CO_3, 35 mmol l^{-1} $NaHCO_3$, pH 9.4) and 100 µl of this antibody solution was applied into each well of a high-binding 96-well ELISA plate (Sarstedt, Montreal, QC, Canada) and incubated overnight at 4°C. The following day, wells of the ELISA plate were washed three times with 250 µl wash buffer (346 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 5.1 mmol l^{-1} NaH_2PO_4, 0.5% Tween-20) and after the final wash, each well was loaded with 250 µl block solution [phosphate buffered saline containing 0.5% (w/v) each of skimmed milk powder and protease-free bovine serum albumin] and incubated for 1.5–2 h at room temperature (RT). During the blocking step, standards and unknowns were prepared as follows: commercially synthesized *D. melanogaster* CAPA2 (*Drome*CAPA2: ASGLVAFPRV-NH_2) peptide and an N-terminally biotinylated *Drome*CAPA2 (biotin–CAPA2) peptide analogue (GenScript, Piscataway, NJ, USA) were prepared in block solution. Serial dilutions of synthetic *Drome*CAPA2 ranging from 2.5 fmol/100 µl to 50 pmol/100 µl were loaded in triplicate onto the ELISA plate along with block solution alone applied to wells with and without antibody coating (for maximum signal and blank/background controls, respectively). Once standards were loaded, aliquots of the dried ganglionic fractions or haemolymph samples from above resuspended in block solution were loaded (100 µl/well) in triplicate onto the ELISA plate. The standards and unknowns were incubated at RT for 1.5 h and then 100 fmol of biotin–*Drome*CAPA2 was dispensed to each well already containing the standards or unknown samples and the plate was incubated overnight at 4°C on a bidirectional rocking platform. The next day, contents in the wells were discarded and the plate was washed four times with wash buffer (250 µl/well). After the last wash was discarded, wells were loaded with 100 µl Avidin–HRP conjugate (Bio-Rad, Mississauga, ON, Canada) diluted 1:2000 in block buffer and incubated for 1.5 h at RT. Following this incubation, contents in the wells were discarded and the plate was washed three times with wash buffer (250 µl/well). After the final wash solution was discarded, each well was loaded with 3,3′,5,5′-tetramethylbenzidine (TMB) liquid horseradish peroxidase substrate and incubated for approximately 10 min at RT to allow blue end product development. Without discarding the TMB solution, each well then received 100 µl of 2 N HCl to stop the reaction and the absorbance was then measured at 450 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Data analysis

All data analysis was completed in the R environment for statistical computing, version 3.4.3 (http://www.r-project.org). Chill coma recovery time following 3 h at 0°C was compared among treatment groups and injection times using a two-way ANOVA. Chill coma recovery times following prolonged chilling were not normally distributed (Shapiro–Wilk test; W=0.94, P=0.002), so a Kruskal–Wallis test was used to test for effects of injection treatment (10^{-15} mol l^{-1} and 10^{-6} mol l^{-1} CAPA) on chill coma recovery following 16 h at 0°C. Similarly, a Kruskal–Wallis test was used to test for effects of injection treatment on locomotor function and survival scores among flies following 16 h at 0°C. In both cases, pairwise comparisons were completed with a Wilcoxon rank sum test. Effects of CAPA peptide or cGMP concentration on Malpighian tubule fluid, Na^+ and K^+ secretion rates, and $[Na^+]$ and $[K^+]$ in the secreted fluid were tested using ANOVA followed by Tukey's HSD (if data were normally distributed) or were conducted on Kruskal–Wallis tests, followed by Wilcoxon rank sum tests (if the data were not normally distributed). In all cases, *post hoc* analyses were corrected for multiple comparisons (Benjamini and Hochberg, 1995). All values reported in the results are mean±s.e.m. unless otherwise stated.

RESULTS

Short-term effects of CAPA on chill coma recovery

The effects of CAPA injection during a short (3 h) chilling stress on CCRT were strongly dose-dependent (Fig. 1), and sham-injected flies recovered from chill coma slightly later than those given no injection (two-way ANOVA: F=123.4, P<0.001). Flies injected with the lowest dose of CAPA peptide (10^{-15} mol l^{-1}) recovered

Journal of Experimental Biology (2018) 221, jeb185884. doi:10.1242/jeb.185884

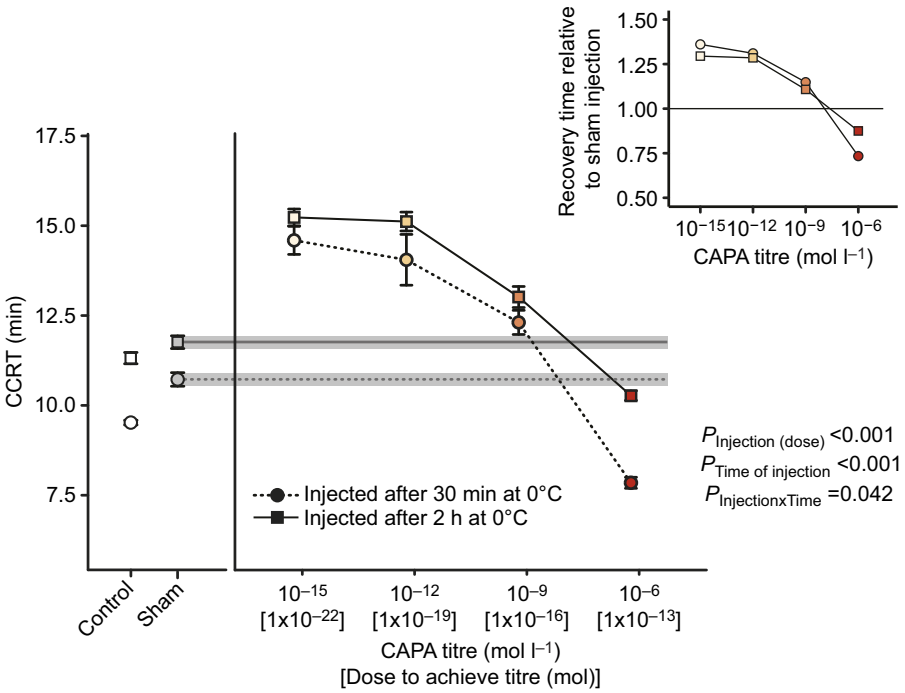

Fig. 1. Dose-responsive effects of CAPA injection on chill coma recovery time of adult female *Drosophila melanogaster*. All flies were held individually at 0°C for a total of 3 h, and were injected either 30 min (circles with dashed line) or 2 h (squares with solid line) into the cold stress. Flies were injected with a dose of CAPA peptide in saline (doses shown along the *x*-axis) to achieve an effective titre of 10^{-15}, 10^{-12}, 10^{-9}, 10^{-6} mol l^{-1} CAPA in the haemolymph (estimated effective titres). Sham flies were given an injection of saline only (without CAPA) whereas control flies were not injected. *N*=9–14 flies per treatment group. Inset shows recovery time relative to sham injection for each titre. There was a significant effect of injection dose and time, as well as a significant interaction between time and dose, on time to recovery according to a two-way ANOVA.

from chill coma 3.65 min (or 33%) more slowly than sham-injected flies, whereas those injected with the highest dose (10^{-6} mol l^{-1}) recovered 1.94 min (or 20%) faster than sham-injected flies, on average. Flies injected with CAPA peptide 30 min into the cold stress period recovered more quickly than those injected 2 h into the cold stress period (F=51.6, P<0.001), regardless of the dose applied, but the same trend was observed in sham and even control flies (that received no injection), indicating that this effect of timing is likely an artefact and that the timing of CAPA injection has little effect on CCRT (Fig. 1, insert). The dose of CAPA applied and the timing of injection did, however, significantly interact to impact CCRT (two-way ANOVA: F=2.4, P=0.042). This modest interaction appears to be driven by a somewhat larger effect of early injection on flies given the highest dose (10^{-6} mol l^{-1}) of CAPA (Fig. 1).

Effects of CAPA peptide injection after prolonged chilling on chill coma recovery and survival

CAPA peptide injection also had significant effects on chill coma recovery following prolonged cold exposure (Fig. 2A; Kruskal–Wallis test: H=22.2, P<0.001). Flies given a sham injection of saline before recovering from 16 h at 0°C did not significantly differ in CCRT from control flies given no injection (P=0.447). Flies that were injected with a low dose (10^{-15} mol l^{-1}) of CAPA peptide recovered significantly more slowly than control (P=0.010) and sham-injected flies (P=0.021). By contrast, flies injected with a high dose (10^{-6} mol l^{-1}) of CAPA recovered more quickly from chill coma than both control flies (P=0.047) and sham-injected flies (P=0.010). A separate set of flies injected in the same manner (15 h into a 16 h exposure to 0°C) were observed 24 h after injection. CAPA injection significantly affected the incidence of chilling injury and death following this prolonged cold exposure (Fig. 2B; Kruskal–Wallis test: H=22.9, P<0.001). Flies injected with a low dose (10^{-15} mol l^{-1}) of CAPA peptide had lower survival scores than those given a sham injection (P=0.011), while those given a high dose (10^{-6} mol l^{-1}) were more likely to be uninjured 24 h after removal from the cold (P=0.019). The median survival score for a fly injected with a low dose (10^{-15} mol l^{-1}) of CAPA peptide was 2 (a fly that was moving but unable to stand) while that of a fly injected with a high dose (10^{-6} mol l^{-1}) was above 4 (a fly that can climb with coordination and potentially fly).

Effects of CAPA on Malpighian tubule function

To test whether the observed effects of CAPA peptide on cold tolerance were driven by effects on Malpighian tubule function we directly measured tubule fluid and ion secretion rates using Ramsay assays. The dose of CAPA applied to tubules significantly altered rates of primary urine production (Fig. 3A; H=8.3, P=0.016). Specifically, tubules treated with 10^{-15} mol l^{-1} CAPA had ∼28% lower secretion rates relative to both control (saline only; P=0.032) and a higher dose (10^{-6} mol l^{-1}) of CAPA peptide (P=0.024). The dose of CAPA applied did not affect the [Na^+] of the secreted fluid (Fig. 3B; H=2.1, P=0.348), but strongly affected [K^+] in the secreted fluid (Fig. 3C; H=18.0, P<0.001). Tubules bathed in 10^{-6} mol l^{-1} CAPA peptide produced fluid with significantly higher [K^+] than both control tubules (42% higher, P<0.001) and those bathed in the lower dose of CAPA peptide (34% higher, P=0.005), while fluid from tubules bathed in 10^{-15} mol l^{-1} CAPA and control tubules did not differ in [K^+] (P=0.849). Using fluid secretion rates and measured ion concentrations, we quantified rates of ion secretion by the tubules during the assay. The dose of CAPA peptide applied had significant effects on the secretion of both Na^+ (Fig. 3D; H=8.5, P=0.014) and K^+ ions (Fig. 3E; H=10.1, P=0.006). In the case of Na^+, tubules bathed in the lower dose (10^{-15} mol l^{-1}) of CAPA peptide secreted less Na^+ than control tubules (P=0.021) or those bathed in 10^{-6} mol l^{-1} CAPA (P=0.043). Tubules bathed in 10^{-15} mol l^{-1} CAPA secreted significantly less K^+ than those bathed in 10^{-6} mol l^{-1} CAPA (P=0.004), and K^+ secretion from control tubules was intermediate between the two CAPA treatments (P>0.05 in both cases). Using rates of Na^+ and K^+ secretion, we calculated the ratio of these two ions secreted from the tubules (Na^+:K^+ ratio), and this ratio was significantly impacted by the dose of CAPA peptide administered (Fig. 3F; H=9.4, P=0.009). Malpighian tubules exposed to 10^{-6} mol l^{-1} CAPA had a significantly lower Na^+:K^+ ratio than control tubules (P=0.008), and tubules exposed to

Journal of Experimental Biology (2018) 221, jeb185884. doi:10.1242/jeb.185884

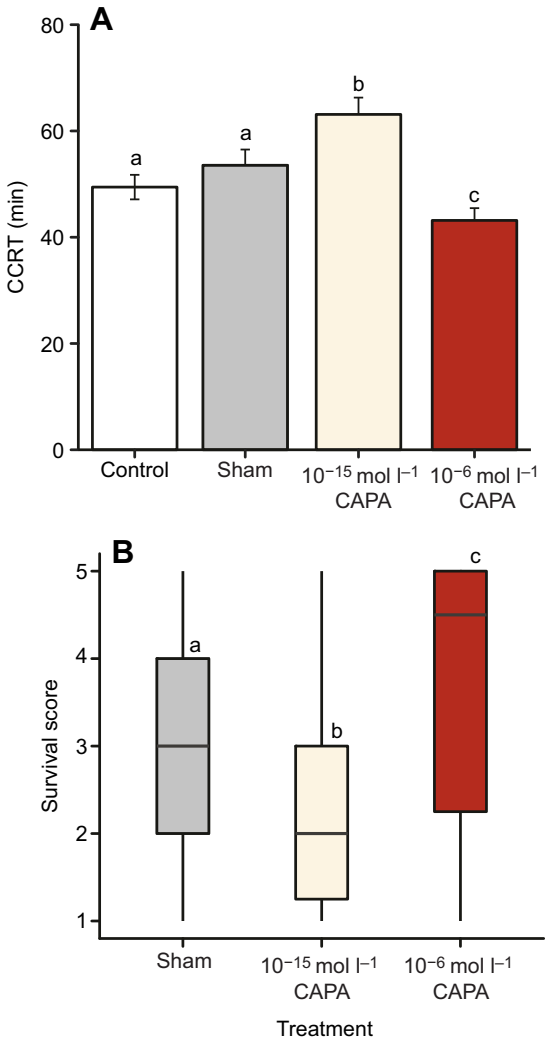

Fig. 2. Dose-dependent effects of CAPA peptide injection on chill coma recovery time and survival following prolonged cold stress in adult female *D. melanogaster*. (A) Mean±s.e.m. chill coma recovery time of flies exposed to 0°C for 16 h. *N*=18–19 flies per treatment group. (B) Box plot of survival scores of flies following 16 h at 0°C. *N*=50 flies per treatment group. Survival was scored on a five point scale, with a score of 5 being a fly that is able to stand, walk in a coordinated manner, and initiate flight, and 1 being a fly showing no signs of life (see Materials and Methods for details). The central horizontal line indicates the median value, the box represents the inter-quartile range, and vertical lines denote the full range of the data. In both experiments, flies were injected 15 h into a cold stress of 16 h at 0°C. Flies were injected with saline containing 1×10^{-22} or 1×10^{-13} mol CAPA peptide to achieve an effective circulating titre of 10^{-15} or 10^{-6} mol l^{-1} in the haemolymph, were given a sham injection of saline only or received no injection (control). Bars or boxes that share a letter within a panel do not differ significantly, according to Kruskal–Wallis tests (see text for details).

10^{-15} mol l^{-1} CAPA did not differ from either the control (P=0.306) or the 10^{-6} mol l^{-1} CAPA peptide treatment (P=0.072).

Effects of cGMP on Malpighian tubule function

We tested for effect of two doses of 8-bromo-cGMP on the function of *Drosophila* tubules. The dose of cGMP applied significantly impacted rates of primary urine production (Fig. 4A; F=3.8, P=0.030). Tubules bathed in 10 nmol l^{-1} (10^{-8} mol l^{-1}) cGMP had reduced rates of secretion relative to control tubules (P=0.034), whereas tubules exposed to 1 mmol l^{-1} cGMP (10^{-3} mol l^{-1}) had similar rates of secretion to control tubules (P=0.893). We found that the dose of cGMP did not impact [Na^+] (Fig. 4B; F=0.4, P=0.673) or [K^+] in the secreted fluid (Fig. 4C; F=2.4, P=0.103). The dose of cGMP applied significantly impacted Na^+ secretion rates (Fig. 4D; F=4.3, P=0.021) and tended (nearly significantly) to impact K^+ secretion rates (Fig. 4E; F=3.2, P=0.051). *Post hoc* analyses revealed that tubules exposed to a low dose of cGMP (10^{-8} mol l^{-1}) secreted significantly less Na^+ (P=0.027) and K^+ (P=0.041) than control tubules, while those exposed to the higher dose of cGMP (10^{-3} mol l^{-1}) did not differ from the control rates of secretion of either ion (Na^+: P=0.948; K^+: P=0.316). Exposure to cGMP significantly impacted the ratio of Na^+ and K^+ secreted by the Malpighian tubules (H=7.6, P=0.022), but none of the *post hoc* pairwise comparisons revealed significant differences among the groups (Fig. 4F; P>0.05 in all cases).

CAPA peptide quantification in the central nervous system of *Drosophila melanogaster*

We developed a sensitive ELISA for the quantification of *D. melanogaster* CAPA peptides with a linear range of 25 fmol to 25 pmol, which spans three orders of magnitude (Fig. 5A). To ensure specificity, we tested cross-reactivity of some structurally related insect peptides, including pyrokinins [*A. aegypti* CAPA-PK1: AGNSGANSGMWFGPRL-NH_2 (Predel et al., 2010)], sNPF [*A. aegypti* sNPF-$1_{(4\text{-}11)}$: SPSLRLRF-NH_2 (Predel et al., 2010)] and FMRFa [*Rhodnius prolixus* FMRFa: GNDNFMRF-NH_2 (Sedra and Lange, 2014)], but no cross-reactivity was observed when up to 5 pmol of these peptides were tested (data not shown). Using this ELISA, we were unable to quantify any CAPA peptide in haemolymph extracts from pools of 60 adult females (data not shown). Considering the sensitivity of the *D. melanogaster* CAPA peptide ELISA developed here with reliable detection down to as low as 25 fmol, this result indicates that the amount of circulating CAPA peptide in the fly haemolymph (~80 nl) is below 1.25 fmol (equating to an effective titre of less than 15 nmol l^{-1}). CAPA material in the nervous system of adult female *D. melanogaster* was quantified using the CAPA peptide ELISA and showed that the average thoracicoabdominal ganglionic extract from a single fly contains ~41 fmol of CAPA-like peptides (Fig. 5B), which eluted from the C18 Sep-Pak column in the 20% and 30% solvent fractions. Synthetic *Drome*CAPA2 peptide processed identically using a C18 Sep-Pak cartridge demonstrated a similar elution profile to the CAPA peptide material in the ganglionic extract. Considering the average CAPA peptidergic material determined per fly along with the previously determined adult female *D. melanogaster* haemolymph volume of ~80 nl (Folk et al., 2001), the maximum achievable haemolymph titre if all CAPA material is simultaneously released from the nervous system would be ~512 nmol l^{-1}.

DISCUSSION

This study is the first to report contrasting dose-dependent effects of CAPA peptides on fluid and ion secretion by the Malpighian tubules of *Drosophila melanogaster*, and the first to describe negative effects of a neuropeptide on the chill tolerance of any insect. These results support previous findings related to both high and low doses of CAPA peptides, and raise additional questions about the role of CAPA neuropeptides in insects *in vivo*. Similarly to prior evidence (Terhzaz et al., 2015), we found that a micromolar dose of CAPA peptide led to faster recovery from chill coma in *D. melanogaster*. We also report, for the first time, a significant effect of CAPA administration on survival following prolonged chilling (Fig. 2B).

Notably, the effects of CAPA injection on chill CCRT were similar whether flies were injected early or late in the cold stress

Journal of Experimental Biology (2018) 221, jeb185884. doi:10.1242/jeb.185884

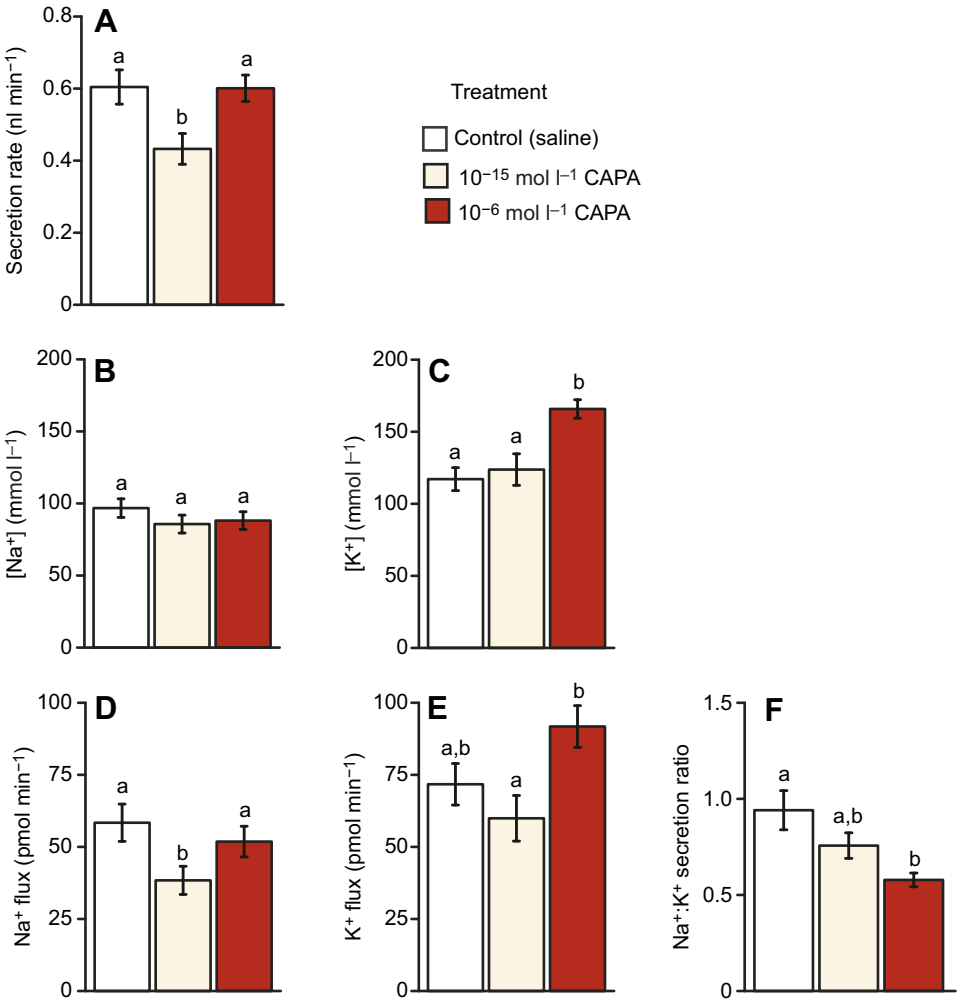

Fig. 3. Effects of CAPA peptide on *in vitro* fluid and cation (Na^+ and K^+) secretion rates of unstimulated Malpighian tubules of adult female *D. melanogaster*. 1 fmol l^{-1} (10^{-15} mol l^{-1}) or 1 µmol l^{-1} (10^{-6} mol l^{-1}) CAPA peptide was applied to otherwise unstimulated tubules. Data shown are rates of fluid secretion by the tubules (A) as well as concentrations of Na^+ (B) and K^+ (C) measured in the secreted droplets, rates of Na^+ (D) and K^+ (E) flux expressed independently of water flux, and the ratio of Na^+:K^+ (F) in the secreted fluid. In all cases, values presented are means±s.e.m. Bars that share a letter within a panel do not differ significantly. *N*=25–32 tubules per treatment group.

(see Fig. 1, insert). Recovery from chill coma has been suggested to be dependent on the degree to which an insect has lost ion balance in the cold (dependent on the temperature and duration of cold stress), as well as the rate of ion and water homeostasis recovery following rewarming (MacMillan et al., 2012; Overgaard and MacMillan, 2017). If this is the case, our current result implies that the effects of CAPA peptide on ion and water balance are minimal during the cold stress and that the titre of CAPA in the haemolymph is instead primarily influencing rates of ion transport (and thus the recovery of ion balance) upon rewarming. Given these results, and the knowledge that CAPA receptors are located exclusively in the Malpighian tubule principal cells of *D. melanogaster* (Terhzaz et al., 2012), we specifically considered the effects of CAPA peptide on Malpighian tubule function at room temperature, which is most relevant to its effects on chill coma recovery. It is possible that the peptide simply cannot bind at low temperatures or otherwise does not alter tubule function in the cold. Alternatively, this result may simply support the observation that rates of transport are strongly suppressed during chilling [~20-fold between 25°C and 0°C in the same population of flies used in the present study (Yerushalmi et al., 2018)], and as such, stimulation or further suppression is unlikely to have any measurable effect. To address these possibilities, careful analysis of the effects of temperature on neuropeptide signalling and renal function across a range of temperatures will be required, as the vast majority of neuropeptide effects in ectotherms have been documented at or near room temperature (De Haes et al., 2015; Nässel and Winther, 2010).

Injection of CAPA peptide was previously demonstrated to have no effect on the survival of *D. melanogaster* following 1 h at −6°C (Terhzaz et al., 2015). Our approach in the present study differed in that the flies were instead subjected to a chronic exposure to a less extreme temperature (16 h at 0°C). Here, flies injected with a low dose of CAPA (10^{-15} mol l^{-1}) 30 min before they were removed from the cold suffered greater chilling injury, while those injected with a high dose (10^{-6} mol l^{-1}) were significantly less injured than control flies 24 h following the cold stress. Injuries suffered from chilling in the absence of ice formation are often conceptually divided into direct chilling injury (resulting from severe acute cold stress) and indirect chilling injury (resulting from chronic, but milder cold stress). These two forms of injury have also been suggested to be associated with different underlying mechanisms; while direct chilling injury is thought to be a consequence of irreversible membrane phase changes and protein denaturation, indirect chilling injury is instead attributed to a more gradual loss of ion and water balance or oxidative stress (Koštál et al., 2006; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013). Our results support the notion that direct and indirect chilling injury are influenced by independent physiological mechanisms, and that neuropeptide effects on ion and water balance may mitigate or exacerbate indirect chilling injury while having little effect on direct chilling injury. Regardless of the mechanisms at play, the

Journal of Experimental Biology (2018) 221, jeb185884. doi:10.1242/jeb.185884

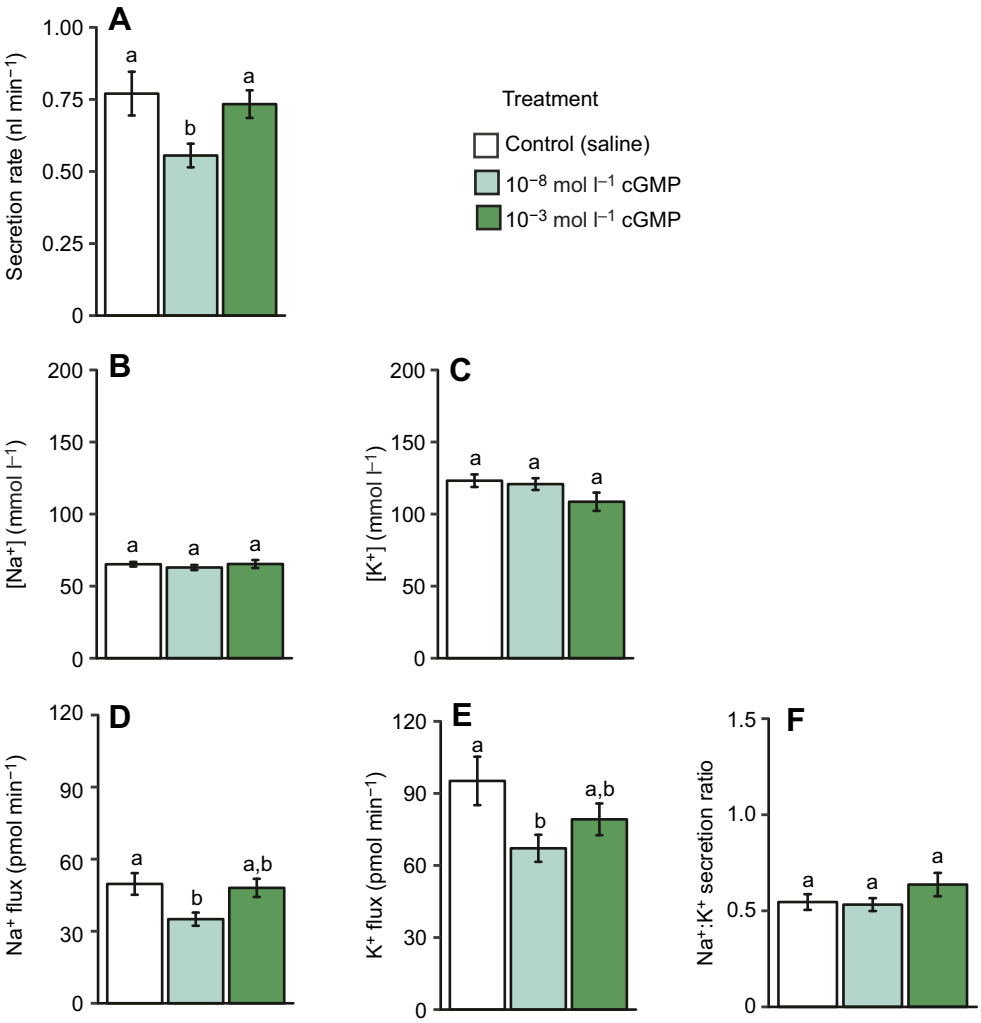

Fig. 4. Effects of cGMP on *in vitro* fluid and cation (Na^+ and K^+) secretion of the Malpighian tubules of adult female *D. melanogaster*. A 10 nmol l^{-1} (10^{-8} mol l^{-1}) or 1 mmol l^{-1} (10^{-3} mol l^{-1}) dose of cGMP was applied to otherwise unstimulated tubules. Data shown are rates of fluid secretion by the tubules (A) as well as concentrations of Na^+ (B) and K^+ (C) measured in the secreted droplets, rates of Na^+ (D) and K^+ (E) ion flux expressed independently of water flux, and the ratio of Na^+:K^+ (F) in the secreted fluid. In all cases, values presented are means±s.e.m. Bars that share a letter within a panel do not differ significantly. *N*=12 or 13 tubules per treatment group.

effects of CAPA we observed on survival following cold stress mirror our observations for CCRT; whereas high doses of CAPA improved chill tolerance in *D. melanogaster*, low doses had the opposite effect.

Receptors for CAPA peptides are found only in the Malpighian tubules of *D. melanogaster* (Terhzaz et al., 2012), so we focused our attention on the effects of low and high doses of CAPA on tubule fluid and ion secretion rates using Ramsay assays. Exposure of tubules to femtomolar (10^{-15} mol l^{-1}) doses of CAPA peptide reduced rates of ion and fluid secretion by the tubules (Fig. 3D,E). Since the initial description of CAPA peptides as modulators of Malpighian tubule secretion rates in *D. melanogaster* (Davies et al., 1995), no studies to our knowledge have tested the effects of this peptide on fluid secretion below concentrations of 1 nmol l^{-1}

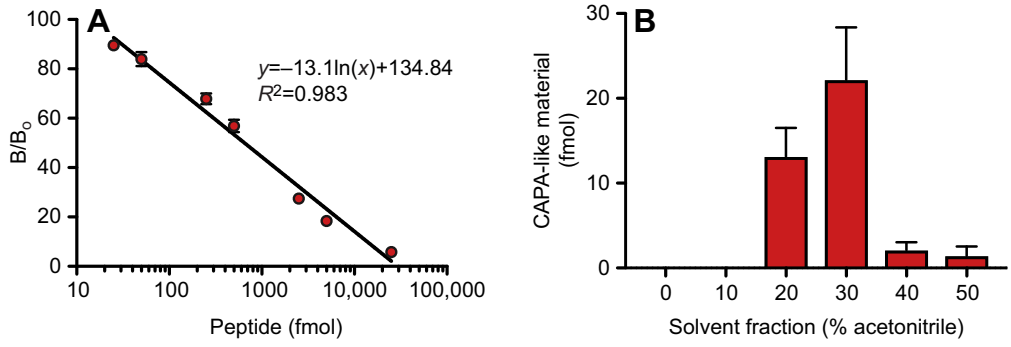

Fig. 5. Results of an ELISA against *Drome*CAPA2 in homogenates of the thoracicoabdominal ganglion of female *D. melanogaster*. (A) Linear regression analysis of standard curve of ELISA generated using *Drome*CAPA2. B/B_o represents standard bound/maximum bound. *N*=3 technical replicates per standard concentration, produced by serial dilution. Error bars that are not visible are obscured by the symbols. (B) Mean±s.e.m. quantity of CAPA-like material found in the thoracicoabdominal ganglion determined from quantification of three biological replicates, each containing 20 ganglia. *N*=3 biological replicates, each containing 20 ganglia.

(10^{-9} mol l^{-1}) in this species. Instead, most have described effects of higher concentrations (typically 10^{-8} to 10^{-6} mol l^{-1}), and this approach has led to the elucidation of the signalling pathway underlying this effect (Davies et al., 1997; Kean et al., 2002; MacPherson et al., 2001; Pollock et al., 2003; Pollock et al., 2004; Rosay et al., 1997). Notably, Rodan and colleagues (Rodan et al., 2012) previously reported anti-diuretic effects of CAPA on the tubules of wild-type *D. melanogaster*. This antidiuretic effect was observed at 10^{-7} mol l^{-1} CAPA, but only after a prolonged exposure of tubules to the peptide (greater than 30 min). Our results support an anti-diuretic role of CAPA, and further suggest that CAPA peptide is particularly anti-diuretic at lower (femtomolar to picomolar) concentrations in *D. melanogaster*. Very similar effects of low concentrations of CAPA peptide have been observed in both larval (Ionescu and Donini, 2012) and adult (Sajadi et al., 2018) mosquitoes (*A. aegypti*). Our results suggest that low concentrations of CAPA impair cold tolerance by slowing rates of ion (particularly K^+) and water flux through the Malpighian tubules upon rewarming, thereby reducing the speed at which flies can re-establish osmotic and ionic balance following cold exposure.

In contrast to several previous studies on *D. melanogaster* (Davies et al., 1995; Davies et al., 1997; Kean et al., 2002; MacPherson et al., 2001), we found that exposing tubules to micromolar (10^{-6} mol l^{-1}) doses of CAPA did not stimulate fluid secretion (Fig. 3). We cannot account for this discrepancy. In the present study, however, despite failing to stimulate fluid secretion, 10^{-6} mol l^{-1} CAPA instead led to kaliuresis (higher [K^+] in the secreted fluid). This finding is significant as it presents a plausible mechanism for increased chill tolerance following injection of high doses of CAPA peptide. In studies conducted on *D. melanogaster* to date, improvements in chill tolerance are associated with increased rates of K^+ clearance by the tubules. Acclimation of flies to 10°C is associated with a compensatory increase in the rates of K^+ secretion by the tubules (Yerushalmi et al., 2018). Similarly, cold-adapted *Drosophila* species maintain rates of tubule K^+ secretion in the cold (3°C) that are higher than in species adapted to warmer climates (MacMillan et al., 2015d). Either of these strategies would help flies to avoid hyperkalaemia in the cold and/or enable them to recover more rapidly from ionic imbalance upon rewarming, provided that rates of K^+ reabsorption are simultaneously kept constant or reduced along the gut epithelia, as is the case for both cold acclimated and cold adapted *Drosophila* (Andersen et al., 2017c; Yerushalmi et al., 2018). Thus, although the direct effects of 10^{-6} mol l^{-1} CAPA on Malpighian tubule activity observed herein are not in line with previous reports in *Drosophila*, they are internally consistent. We therefore argue that micromolar doses of CAPA peptide improve chill tolerance via kaliuretic activity (i.e. by stimulating K^+ secretion), with or without concurrent stimulation of fluid secretion.

If femtomolar doses of CAPA impair chill tolerance in *Drosophila* and do so by inhibiting fluid and ion secretion in the Malpighian tubules, we predicted that they may do so via the NOS–cGMP–PKG pathway. In larval and adult mosquitoes (*A. aegypti*), low doses of cGMP (10^{-9} to 10^{-6} mol l^{-1}) mimic the anti-diuretic effects of low doses of CAPA (10^{-15} mol l^{-1}), with maximal inhibition of secretion observed at 10^{-8} mol l^{-1} cGMP (Ionescu and Donini, 2012; Sajadi et al., 2018). In the case of larval mosquitoes, higher doses of cGMP (10^{-3} mol l^{-1}) induce a very modest (non-significant) increase in fluid secretion (Ionescu and Donini, 2012), while in adult mosquitoes no such stimulation could be induced with higher levels of cGMP (Sajadi et al., 2018). Accordingly, in the present study we tested whether similar low (10^{-8} mol l^{-1}) and high (10^{-3} mol l^{-1}) doses of cGMP could mimic these effects in *Drosophila* (Fig. 4). Although the effects of 10^{-8} mol l^{-1} cGMP mirrored the effects of a low dose (10^{-15} mol l^{-1}) of CAPA in *Drosophila* (reduced rates of fluid, Na^+ and K^+ secretion), 10^{-3} mol l^{-1} cGMP did not stimulate fluid secretion or induce kaliuresis. Indeed, exposure of *D. melanogaster* tubules to 10^{-3} mol l^{-1} cGMP had no significant effects on tubule secretion rates, ion concentrations in the secreted fluids or rates of ion flux by the tubules (Fig. 4). Thus, the impact of cGMP on tubule secretion in adult *Drosophila* appears to mimic those observed in *A. aegypti* (Ionescu and Donini, 2012; Massaro et al., 2004; Sajadi et al., 2018), as well as many other insects, including beetles (Eigenheer et al., 2002; Wiehart et al., 2002) and hemipterans (Paluzzi and Orchard, 2006; Quinlan and O'Donnell, 1998). Our results support the idea that low doses of CAPA peptide slow rates of fluid secretion through cGMP signalling, since this second messenger mimicked the anti-diuretic activity of this neuropeptide. In larval *A. aegypti*, stimulatory effects of high doses of AedesCAPA–PVK-1 or high doses of cGMP can be reversed by addition of specific inhibitors of protein kinase A (Ionescu and Donini, 2012), which suggests that high levels of CAPA peptide may be pharmacological, inadvertently activating the signalling cascade that drives diuresis and thereby overwhelming any effects of cGMP. As we did not observe diuretic effects following application of high titres of CAPA peptide in the present study, we were unable to test whether a similar effect can explain CAPA-induced diuresis in *D. melanogaster*.

Given our observations that CAPA peptide can both improve and hinder cold tolerance in *D. melanogaster* depending on the dose applied, we were curious whether flies are capable of reaching micromolar titres of CAPA peptide in the haemolymph. Accordingly, we developed a *D. melanogaster* CAPA peptide-specific ELISA. Despite pooling haemolymph of 60 flies per sample, we were unable to detect CAPA peptides in the haemolymph of *D. melanogaster*, which suggests that total CAPA levels in these samples (collected from flies under control conditions, 23°C) are below our lowest standard (25 fmol). In order for us to detect CAPA in these pooled samples, each fly would have to contribute approximately 0.42 fmol of CAPA, which represents ∼1% of the CAPA peptide quantified in a single thoracicoabdominal ganglion (see below), and our technique of haemolymph extraction typically obtains ∼56 nl of haemolymph from an adult female fly (MacMillan and Hughson, 2014). Thus, in order for us to detect CAPA in the haemolymph, *D. melanogaster* would have to have a mean circulating titre of CAPA peptide ≥7.4 nmol l^{-1}. As we did not detect CAPA in these samples, we suggest that resting titres are below this concentration. Using the same ELISA, however, we were able to detect CAPA peptide in pooled samples of the thoracicoabdominal ganglion (Fig. 5), a region of the CNS that houses the Va neurons, where CAPA is produced and stored in *Drosophila* (Kean et al., 2002; Terhzaz et al., 2015). Based on the abundance of CAPA neuropeptides in the entire nervous system where CAPA is produced, we estimate that if all of this peptide was released at once, flies could reach a maximum of ∼500 nmol l^{-1} CAPA peptide circulating in the haemolymph. This *en masse* release of all CAPA content is unlikely however, since neuropeptides are released as neurohormones from specialized neurohaemal organs (Wegener et al., 2006), including the abdominal perivisceral organs where CAPA peptides have been localized and found to be most abundant in a variety of insects (Predel and Wegener, 2006) including *D. melanogaster* (Predel et al., 2004). Importantly, and consistent with earlier observations in

the blowfly *Calliphora erythrocephala* (Duve et al., 1988; Nässel et al., 1988), the *D. melanogaster* adult abdominal neurohaemal organs are directly incorporated into the fused ventral ganglion and localized to the dorsal neural sheath (Predel et al., 2004). In light of this, these results suggest that if flies are capable of reaching micromolar levels of CAPA in the haemolymph, it would likely require, at a minimum, doubling of CAPA peptide abundance above resting levels in the CNS and synchronous release of all CAPA peptides stored within the nervous system. However, this potential complete release *en masse* is unlikely, since *in vitro* induction of neuropeptide from neurohaemal organs has shown to release only fractional amounts compared with the total immunoreactive material present within the nervous system or neurohaemal organ. For example, in the cockroach *Leucophaea maderae*, leucokinin release from the retrocerebral complex induced by depolarization using high potassium saline accounted for only ∼2% of the total immunoreactive material present within the corpora cardiac–corpora allata complex (Muren et al., 1993). Similarly, in the house cricket *Acheta domesticus*, release of achetakinin following depolarization with high potassium saline from the retrocerebral complex, which is the richest source of this neuropeptide, represented <4% (i.e. ∼70 fmol released from ∼1800 fmol stored in each retrocerebral complex) of the total achetakinin immunoreactive material present within this neurohaemal organ (Chung et al., 1994). Finally, we note that both cold and desiccation stress have been demonstrated to cause upregulation of *Capa* mRNA, which may elevate CAPA levels in the CNS, and CAPA has been suggested to be released in *D. melanogaster* only upon removal from the desiccation or cold stress (Terhzaz et al., 2015). Further efforts are thus required to determine whether or not *Drosophila* and other dipterans are capable of reaching levels of CAPA that can stimulate diuresis, and if so, which abiotic conditions specifically lead to this strategy. Critical to this discussion is the direct detection and measurement of circulating levels of CAPA peptide in nanolitre scale haemolymph samples under a variety of highly dynamic conditions, and such an approach in future studies could involve matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Chen et al., 2009; Fastner et al., 2007).

Chronic exposure to low temperatures suppresses the ability of insects to maintain ion and water homeostasis, causing progressive hyperkalemia and cell death. Our results suggest that CAPA peptides can positively and negatively impact chill tolerance in *D. melanogaster* in a dose-responsive manner. Low (femtomolar) doses of CAPA cause anti-diuresis and limit clearance of K^+ at the Malpighian tubules, limiting the ability of flies to recover ion and water balance upon rewarming and impairing chill tolerance. By contrast, high (micromolar) doses of CAPA cause kaliuresis (and based on previous reports also diuresis), facilitating K^+ clearance from the haemolymph and improving chill tolerance. We argue that the anti-diuretic effects of CAPA operate through cGMP, and question whether levels of CAPA peptide can reach micromolar levels and stimulate diuresis *in vivo*. Although a wide variety of other neuropeptides are known to influence insect ion and water balance through their effects on Malpighian tubule and gut epithelia, none other than CAPA have been tested in the context of chill tolerance.

Acknowledgements
The authors wish to thank Carol Bucking for generously providing space in which to conduct some of these experiments.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.A.M., A.D., J.-P.P.; Methodology: H.A.M.; Formal analysis: H.A.M., B.N.; Investigation: H.A.M., B.N., S.W., G.Y.Y., L.M., J.-P.P.; Resources: A.D., J.-P.P.; Writing - original draft: H.A.M., J.-P.P.; Writing - review & editing: H.A.M., B.N., S.W., G.Y.Y., L.M., A.D., J.-P.P.; Visualization: H.A.M.; Supervision: H.A.M., A.D., J.-P.P.; Project administration: H.A.M., A.D., J.-P.P.; Funding acquisition: H.A.M., A.D., J.-P.P.

Funding
This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to J.-P.P. (RGPIN-2018-05841) and A.D. (RGPIN-2014-06681), a Banting Postdoctoral Fellowship to H.A.M., an NSERC Alexander Graham Bell Canada Graduate Scholarship to G.Y.Y. and York University Faculty of Science Scholarships to G.Y.Y. and L.M.

Data availability
All supporting data have been deposited in the Dryad Digital Repository (MacMillan et al., 2018): http://doi.org/10.5061/dryad.37m5531

References
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.
Andersen, M. K., Jensen, S. O. and Overgaard, J. (2017b). Physiological correlates of chill susceptibility in Lepidoptera. *J. Insect Physiol.* **98**, 317-326.
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. 168518
Baust, J. G. and Rojas, R. R. (1985). Insect cold hardiness: facts and fancy. *J. Insect Physiol.* **31**, 755-759.
Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289-300.
Chen, R., Ma, M., Hui, L., Zhang, J. and Li, L. (2009). Measurement of neuropeptides in crustacean hemolymph via MALDI mass spectrometry. *J. Am. Soc. Mass Spectrom.* **20**, 708-718.
Chung, J., Goldsworthy, G. and Coast, G. (1994). Haemolymph and tissue titres of acetakinins in the house cricket *Acheta domesticus*: effect of starvation and dehydration. *J. Exp. Biol.* **193**, 307-319.
Coast, G. (2007). The endocrine control of salt balance in insects. *Gen. Comp. Endocrinol.* **152**, 332-338.
Coast, G. M., Orchard, I., Phillips, J. E. and Schooley, D. A. (2002). Insect diuretic and antidiuretic hormones. *Adv. Insect Physiol.* **29**, 279-409.
Coast, G. M., TeBrugge, V. A., Nachman, R. J., Lopez, J., Aldrich, J. R., Lange, A. and Orchard, I. (2010). Neurohormones implicated in the control of Malpighian tubule secretion in plant sucking heteropterans: the stink bugs *Acrosternum hilare* and *Nezara viridula*. *Peptides* **31**, 468-473.
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.
Davies, S. A., Huesmann, G. R., Maddrell, S. H., O'Donnell, M. J., Skaer, N. J., Dow, J. A. T. and Tublitz, N. J. (1995). CAP2b, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol.* **269**, R1321-R1326.
Davies, S. A., Stewart, E. J., Huesmann, G. R., Skaer, N. J., Maddrell, S. H., Tublitz, N. J. and Dow, J. A. (1997). Neuropeptide stimulation of the nitric oxide signaling pathway in *Drosophila melanogaster* Malpighian tubules. *Am. J. Physiol.* **273**, R823-R827.
Davies, S.-A., Cabrero, P., Povsic, M., Johnston, N. R., Terhzaz, S. and Dow, J. A. T. (2013). Signaling by *Drosophila* capa neuropeptides. *Gen. Comp. Endocrinol.* **188**, 60-66.
De Haes, W., Van Sinay, E., Detienne, G., Temmerman, L., Schoofs, L. and Boonen, K. (2015). Functional neuropeptidomics in invertebrates. *Biochim. Biophys. Acta* **1854**, 812-826.
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.
Des Marteaux, L. E., Khazraeenia, S., Yerushalmi, G. Y., Donini, A., Li, N. G. and Sinclair, B. J. (2018). The effect of cold acclimation on active ion transport in cricket ionoregulatory tissues. *Comp. Biochem. Physiol. A* **216**, 28-33.
Djamgoz, M. B. A. (1987). Insect muscle: intracellular ion concentrations and mechanisms of resting potential generation. *J. Insect Physiol.* **33**, 287-314.
Donini, A., O'Donnell, M. J. and Orchard, I. (2008). Differential actions of diuretic factors on the Malpighian tubules of *Rhodnius prolixus*. *J. Exp. Biol.* **211**, 42-48.
Dow, J. A. T., Davies, S. A. and Sözen, M. A. (1998). Fluid secretion by the *Drosophila* Malpighian tubule. *Integr. Comp. Biol.* **38**, 450-460.

Duve, H., Thorpe, A. and Nässel, D. R. (1988). Light- and electron-microscopic immunocytochemistry of peptidergic neurons innervating thoracico-abdominal neurohaemal areas in the blowfly. *Cell Tissue Res.* **253**, 583-595.
Eigenheer, R. A., Nicolson, S. W., Schegg, K. M., Hull, J. J. and Schooley, D. A. (2002). Identification of a potent antidiuretic factor acting on beetle Malpighian tubules. *Proc. Natl. Acad. Sci. USA* **99**, 84-89.
Fastner, S., Predel, R., Kahnt, J., Schachtner, J. and Wegener, C. (2007). A simple purification protocol for the detection of peptide hormones in the hemolymph of individual insects by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **21**, 23-28.
Findsen, A., Andersen, J. L., Calderon, S. and Overgaard, J. (2013). Rapid cold hardening improves recovery of ion homeostasis and chill coma recovery time in the migratory locust, *Locusta migratoria*. *J. Exp. Biol.* **216**, 1630-1637.
Folk, D. G., Han, C. and Bradley, T. J. (2001). Water acquisition and partitioning in *Drosophila melanogaster*: effects of selection for desiccation-resistance. *J. Exp. Biol.* **204**, 3323-3331.
Gerber, L. and Overgaard, J. (2018). Cold tolerance is linked to osmoregulatory function of the hindgut in *Locusta migratoria*. *J. Exp. Biol.* **221**, jeb173930.
Hanrahan, J. and Phillips, J. E. (1983). Mechanism and control of salt absorption in locust rectum. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **244**, R131-R142.
Huesmann, G. R., Cheung, C. C., Kheng, P., Lee, T. D., Swiderek, K. M. and Tublitz, N. J. (1995). Amino acid sequence of CAP_{2b}, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*. *FEBS Lett.* **371**, 311-314.
Ionescu, A. and Donini, A. (2012). *Aedes*CAPA-PVK-1 displays diuretic and dose dependent antidiuretic potential in the larval mosquito *Aedes aegypti* (Liverpool). *J. Insect Physiol.* **58**, 1299-1306.
Kean, L., Cazenave, W., Costes, L., Broderick, K. E., Graham, S., Pollock, V. P., Davies, S. A., Veenstra, J. A. and Dow, J. A. T. (2002). Two nitridergic peptides are encoded by the gene *capability* in *Drosophila melanogaster*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R1297-R1307.
Kellermann, V., Loeschcke, V., Hoffmann, A. A., Kristensen, T. N., Fløjgaard, C., David, J. R., Svenning, J.-C. and Overgaard, J. (2012). Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution* **66**, 3377-3389.
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* **143**, 171-179.
Lakovaara, S. (1969). Malt as a culture medium for *Drosophila* species. *Drosoph. Inf. Serv.* **44**, 128.
Lange, A. B. and Patel, K. (2005). The presence and distribution of crustacean cardioactive peptide in the central and peripheral nervous system of the stick insect, *Baculum extradentatum*. *Regul. Pept.* **129**, 191-201.
MacMillan, H. A. and Hughson, B. N. (2014). A high-throughput method of hemolymph extraction from adult *Drosophila* without anesthesia. *J. Insect Physiol.* **63**, 27-31.
MacMillan, H. A. and Sinclair, B. J. (2011a). Mechanisms underlying insect chill-coma. *J. Insect Physiol.* **57**, 12-20.
MacMillan, H. A. and Sinclair, B. J. (2011b). 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.
MacMillan, H. A., Williams, C. M., Staples, J. F. and Sinclair, B. J. (2012). Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. *Proc. Natl. Acad. Sci. USA* **109**, 20750-20755.
MacMillan, H. A., Findsen, A., Pedersen, T. H. and Overgaard, J. (2014). Cold-induced depolarization of insect muscle: differing roles of extracellular K^+ during acute and chronic chilling. *J. Exp. Biol.* **217**, 2930-2938.
MacMillan, H. A., Ferguson, L. V., Nicolai, A., Donini, A., Staples, J. F. and Sinclair, B. J. (2015a). Parallel ionoregulatory adjustments underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance. *J. Exp. Biol.* **218**, 423-432.
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.
MacMillan, H. A., Baatrup, E. and Overgaard, J. (2015c). Concurrent effects of cold and hyperkalaemia cause insect chilling injury. *Proc. R. Soc. B Biol. Sci.* **282**, 20151483.
MacMillan, H. A., Andersen, J. L., Davies, S. A. and Overgaard, J. (2015d). The capacity to maintain ion and water homeostasis underlies interspecific variation in *Drosophila* cold tolerance. *Sci. Rep.* **5**, 18607.
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.
MacMillan, H. A., Nazal, B., Wali, S., Yerushalmi, G. Y., Misyura, L., Donini, A. and Paluzzi, J. (2018). Data from: Anti-diuretic activity of a CAPA neuropeptide can compromise *Drosophila* chill tolerance. *Dryad Digital Repository.* https://doi.org/10.5061/dryad.37m5531.
MacPherson, M. R., Pollock, V. P., Broderick, K. E., Kean, L., O'Connell, F. C., Dow, J. A. T. and Davies, S. A. C. N.-C. (2001). Model organisms: new insights into ion channel and transporter function.: L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster*. *Am. J. Physiol. Cell Physiol.* **280**, C394-C407.
Marshall, K. E. and Sinclair, B. J. (2010). Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. *Proc. R. Soc. B* **277**, 963-969.
Massaro, R. C., Lee, L. W., Patel, A. B., Wu, D. S., Yu, M. J., Scott, B. N., Schooley, D. A., Schegg, K. M. and Beyenbach, K. W. (2004). The mechanism of action of the antidiuretic peptide Tenmo ADFa in Malpighian tubules of *Aedes aegypti*. *J. Exp. Biol.* **207**, 2877-2888.
Muren, J. E., Lundquist, C. T. and Nässel, D. R. (1993). Quantitative determination of myotropic neuropeptide in the nervous system of the cockroach *Leucophaea maderae*: distribution and release of leucokinins. *J. Exp. Biol.* **179**, 289-300.
Nässel, D. R. and Winther, Å. M. E. (2010). *Droso*phila neuropeptides in regulation of physiology and behavior. *Prog. Neurobiol.* **92**, 42-104.
Nässel, D. R., Ohlsson, L. G. and Cantera, R. (1988). Metamorphosis of identified neurons innervating thoracic neurohemal organs in the blowfly: transformation of cholecystokinin-like immunoreactive neurons. *J. Comp. Neurol.* **267**, 343-356.
Nilson, T. L., Sinclair, B. J. and Roberts, S. P. (2006). The effects of carbon dioxide anesthesia and anoxia on rapid cold-hardening and chill coma recovery in *Drosophila melanogaster*. *J. Insect Physiol.* **52**, 1027-1033.
O'Donnell, M. (2008). Insect excretory mechanisms. *Adv. Insect Physiol.* **35**, 1-122.
Overgaard, J. and MacMillan, H. A. (2017). The integrative physiology of insect chill tolerance. *Annu. Rev. Physiol.* **79**, 187-208.
Paluzzi, J.-P. V. (2012). Anti-diuretic factors in insects: the role of CAPA peptides. *Gen. Comp. Endocrinol.* **176**, 300-308.
Paluzzi, J.-P. and Orchard, I. (2006). Distribution, activity and evidence for the release of an anti-diuretic peptide in the kissing bug *Rhodnius prolixus*. *J. Exp. Biol.* **209**, 907-915.
Paluzzi, J.-P., Russell, W. K., Nachman, R. J. and Orchard, I. (2008). Isolation, cloning, and expression mapping of a gene encoding an antidiuretic hormone and other CAPA-related peptides in the disease vector, *Rhodnius prolixus*. *Endocrinology* **149**, 4638-4646.
Phillips, J. E., Thomson, B., Hanrahan, J. and Chamberlin, M. (1987). Mechanism and control of reabsorption in insect hindgut. *Adv. Insect Phys.* **19**, 329-422.
Pollock, V. P., Radford, J. C., Pyne, S., Hasan, G., Dow, J. A. T. and Davies, S.-A. (2003). *norpA* and *itpr* mutants reveal roles for phospholipase C and inositol (1,4,5)- trisphosphate receptor in *Drosophila melanogaster* renal function. *J. Exp. Biol.* **206**, 901-911.
Pollock, V. P., McGettigan, J., Cabrero, P., Maudlin, I. M., Dow, J. A. T. and Davies, S.-A. (2004). Conservation of capa peptide-induced nitric oxide signalling in Diptera. *J. Exp. Biol.* **207**, 4135-4145.
Predel, R. and Wegener, C. (2006). Biology of the CAPA peptides in insects. *Cell. Mol. Life Sci.* **63**, 2477-2490.
Predel, R., Wegener, C., Russell, W. K., Tichy, S. E., Russell, D. H. and Nachman, R. J. (2004). Peptidomics of CNS-associated neurohemal systems of adult *Drosophila melanogaster*: a mass spectrometric survey of peptides from individual flies. *J. Comp. Neurol.* **474**, 379-392.
Predel, R., Neupert, S., Garczynski, S. F., Crim, J. W., Brown, M. R., Russell, W. K., Kahnt, J., Russell, D. H. and Nachman, R. J. (2010). Neuropeptidomics of the mosquito *Aedes aegypti*. *J. Proteome Res.* **9**, 2006-2015.
Quinlan, M. C. and O'Donnell, M. J. (1998). Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stål: antagonistic actions of cAMP and cGMP and the role of organic acid transport. *J. Insect Physiol.* **44**, 561-568.
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.
Rheuben, M. B. (1972). The resting potential of moth muscle fibre. *J. Physiol.* **225**, 529-554.
Rodan, A. R., Baum, M. and Huang, C.-L. (2012). The *Drosophila* NKCC Ncc69 is required for normal renal tubule function. *Am. J. Physiol. Cell Physiol.* **303**, C883-C894.
Rosay, P., Davies, S. A., Yu, Y., Soezen, M. A., Kaiser, K. and Dow, J. A. T. (1997). Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci.* **110**, 1683-1692.
Sajadi, F., Curcuruto, C., Al Dhaheri, A. and Paluzzi, J.-P. V. (2018). Anti-diuretic action of a CAPA neuropeptide against a subset of diuretic hormones in the disease vector, *Aedes aegypti*. *J. Exp. Biol.* **221**, jeb177089.
Sedra, L. and Lange, A. B. (2014). The female reproductive system of the kissing bug, *Rhodnius prolixus*: arrangements of muscles, distribution and myoactivity of two endogenous FMRFamide-like peptides. *Peptides* **53**, 140-147.
Teets, N. M. and Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiol. Entomol.* **38**, 105-116.
Terhzaz, S., Cabrero, P., Robben, J. H., Radford, J. C., Hudson, B. D., Milligan, G., Dow, J. A. T. and Davies, S.-A. (2012). Mechanism and Function of *Drosophila* capa GPCR: a desiccation stress-responsive receptor with functional homology to human neuromedinU receptor. *PLoS ONE* **7**, e29897.
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.

Wegener, C., Reinl, T., Jänsch, L. and Predel, R. (2006). Direct mass spectrometric peptide profiling and fragmentation of larval peptide hormone release sites in *Drosophila melanogaster* reveals tagma-specific peptide expression and differential processing. *J. Neurochem.* **96**, 1362-1374.

Wiehart, U. I., Nicolson, S. W., Eigenheer, R. A. and Schooley, D. A. (2002). Antagonistic control of fluid secretion by the Malpighian tubules of *Tenebrio molitor*: effects of diuretic and antidiuretic peptides and their second messengers. *J. Exp. Biol.* **205**, 493-501.

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**, jeb.174904.

Yi, S.-X., Moore, C. W. and Lee, R. E. (2007). Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. *Apoptosis* **12**, 1183-1193.