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RESEARCH ARTICLE

Plasticity in Na^+/K^+-ATPase thermal kinetics drives variation in the temperature of cold-induced neural shutdown of adult *Drosophila melanogaster*

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ABSTRACT

Most insects can acclimate to changes in their thermal environment and counteract temperature effects on neuromuscular function. At the critical thermal minimum, a spreading depolarization (SD) event silences central neurons, but the temperature at which this event occurs can be altered through acclimation. SD is triggered by an inability to maintain ion homeostasis in the extracellular space in the brain and is characterized by a rapid surge in extracellular K^+ concentration, implicating ion pump and channel function. Here, we focused on the role of the Na^+/K^+-ATPase specifically in lowering the SD temperature in cold-acclimated *Drosophila melanogaster*. After first confirming cold acclimation altered SD onset, we investigated the dependency of the SD event on Na^+/K^+-ATPase activity by injecting the inhibitor ouabain into the head of the flies to induce SD over a range of temperatures. Latency to SD followed the pattern of a thermal performance curve, but cold acclimation resulted in a left-shift of the curve to an extent similar to its effect on the SD temperature. With Na^+/K^+-ATPase activity assays and immunoblots, we found that cold-acclimated flies have ion pumps that are less sensitive to temperature, but do not differ in their overall abundance in the brain. Combined, these findings suggest a key role for plasticity in Na^+/K^+-ATPase thermal sensitivity in maintaining central nervous system function in the cold, and more broadly highlight that a single ion pump can be an important determinant of whether insects can respond to their environment to remain active at low temperatures.

KEY WORDS: Critical thermal minimum, Chill coma, Sodium pump, Enzyme kinetics, Ionoregulation, Glia

INTRODUCTION

Temperature directly influences insect performance; as temperature is lowered, most biological processes are slowed (Lee, 2012). Most insects are chill susceptible, meaning that adverse effects of cold on physiology begin to manifest at temperatures above those that cause any freezing (Bale, 1996; Sinclair, 1999; Overgaard and MacMillan, 2017). When cooled to their critical thermal minimum (CT_{min}), these insects lose the ability to perform coordinated movements and shortly after enter a paralytic state known as chill coma (Mellanby, 1939; Hazell and Bale, 2011; MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). While in chill coma, an insect's potential fitness is effectively zero, and it is therefore not surprising that this temperature threshold closely relates to geographical distribution (Addo-Bediako et al., 2000; Bale, 2002; Sunday et al., 2011). Within the genus *Drosophila*, for example, chill coma temperatures vary markedly among species and closely correlate with poleward limits to species distribution (Kimura, 2004; Kellermann et al., 2012; Andersen et al., 2015b).

The current mechanistic model for chill coma onset posits that a cold-induced spreading depolarization (SD) event shuts down nervous system function, causing the loss of coordinated movements observed at the CT_{min} (Robertson et al., 2017; Andersen et al., 2018). Subsequently, low temperature decreases muscle excitability, which ultimately leads to complete paralysis and chill coma onset (Hosler et al., 2000; MacMillan et al., 2014; Findsen et al., 2016). However, not all insect species experience this loss of muscle excitability despite still entering chill coma (Andersen et al., 2015a), and as such only experience a relatively 'shallow' coma caused by the SD event in the central nervous system (Andersen and Overgaard, 2019). Similar observations have been made for insect comas induced by heat (Money et al., 2005; Jørgensen et al., 2020) and anoxia (Rodgers et al., 2007), implying SD is a common consequence of abiotic stress in insects.

Cold-induced SD and chill coma are plastic traits that can vary greatly within and among species. This is also true for *Drosophila*, where the chill coma and SD temperatures vary by more than 10°C among species (Mellanby, 1954; Kellermann et al., 2012; Andersen et al., 2015b; MacMillan et al., 2015b) and by several degrees within a species depending on thermal history (Kelty and Lee, 1999; Overgaard et al., 2011; Ransberry et al., 2011; Armstrong et al., 2012; MacMillan et al., 2015a; Schou et al., 2017; Andersen et al., 2018). This variation, along with a long history of extensive research, a multitude of high-throughput datasets (e.g. 'omics') and molecular methodologies, makes drosophilids, such as *Drosophila melanogaster*, excellent model systems for studying the physiological mechanisms underlying variation in cold tolerance. Indeed, research conducted over the last decade has relied heavily on this clade to make great progress in describing the processes that underlie improved organismal cold tolerance (see reviews by Overgaard and MacMillan, 2017; Overgaard et al., 2021).

While SD is a well-described phenomenon, the specific mechanism underlying the SD event itself remains unknown despite nearly eight decades of research in mammalian model systems (Leao, 1944) and two decades in insects (Robertson, 2004). A central feature of SD is a rapid disruption of extracellular ion homeostasis in the interstitium of the brain (Hansen and Zeuthen, 1981; Robertson, 2004; Rodgers et al., 2010). This disruption is characterized by a surge in extracellular K^+ concentration associated with a near-complete depolarization of neurons and glia that

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effectively silences the area of the central nervous system experiencing the SD (Pietrobon and Moskowitz, 2014; Robertson et al., 2020). Thus, transporters responsible for maintaining extracellular ion homeostasis in the brain appear to be at least partially involved in the SD mechanism. While SD can be induced by abiotic stress, it can also be induced by a range of pharmacological interventions that either directly or indirectly inhibit the activity of Na^+/K^+-ATPase, a key regulator of ion balance in the central nervous system (Treherne and Schofield, 1981; Rodgers et al., 2010; Spong et al., 2016a; Andrew et al., 2022). Therefore, Na^+/K^+-ATPase plays a central role in the dynamics of SD induction (Andrew et al., 2022), and it is hypothesized that the maintenance of Na^+/K^+-ATPase activity when homeostasis is challenged mitigates or delays the onset of SD (Rodgers et al., 2010; Spong and Robertson, 2013). In support of this hypothesis, a cold acclimation treatment that lowers the SD temperature also lowers the temperature sensitivity of Na^+/K^+-ATPase activity (Cheslock et al., 2021). Thus, the ability to maintain ion homeostasis, both locally and systemically, appears critical for insect cold tolerance (Overgaard and MacMillan, 2017; Overgaard et al., 2021).

In the present study, we investigated the proposed link between improved cold tolerance (specifically lower cold-induced SD temperature) and thermal sensitivity of brain Na^+/K^+-ATPase. To do so, we induced phenotypic plasticity in the cold tolerance of *D. melanogaster* by acclimating them to either 15 or 25°C throughout development. Once variation in cold tolerance was confirmed, we investigated how temperature affected Na^+/K^+-ATPase in *D. melanogaster* brains and how this might relate to the SD temperature. We hypothesized that improved cold tolerance is enabled, in part, by a lowered thermal sensitivity of Na^+/K^+-ATPase in the brain, serving to maintain neural function at critically low temperatures. We further hypothesized that cold tolerance plasticity is at least partly facilitated by a greater abundance of Na^+/K^+-ATPase protein itself in the brain. We investigated this by (1) estimating how effectively Na^+/K^+-ATPase assisted in preventing SD *in vivo* across a range of temperatures, (2) measuring the activity of Na^+/K^+-ATPase in an enzyme-linked assay *in vitro* at temperatures ranging from room temperature to SD-inducing temperatures, and (3) determining the relative abundance of Na^+/K^+-ATPase protein in brains collected from cold- and warm-acclimated flies.

MATERIALS AND METHODS

Animal husbandry

The line of *Drosophila melanogaster* Meigen 1830 used in this study was established from isofemales collected in London and Niagara on the Lake (ON, Canada) in 2007 (Marshall and Sinclair, 2010). Adult flies were reared in 200 ml bottles containing ∼40 ml banana-based medium (recipe: 0.95 l of water, 8 g agar, 27.5 g active yeast, 2 g methylparaben, 137.5 g organic banana, 47.5 g corn syrup, 30 g liquid malt, 3 ml propionic acid) and kept at 18°C on a 12 h:12 h light:dark cycle. Flies for experiments were produced by letting adult flies oviposit for 4–6 h in a new bottle with medium under rearing conditions, which resulted in rearing densities of 150–200 flies per bottle. The new egg-containing bottle was then transferred to a temperature-controlled incubator (MIR-154-PA, Panasonic) where the eggs were allowed to develop into adults at 15 or 25°C. Newly emerged adults were transferred to 40 ml vials containing ∼7 ml of the same medium. After maturing for 6–9 days at their respective acclimation temperatures, males were discarded and only females were used in experiments (to avoid potential sex-specific differences and because the larger size of females makes dissections easier). No anaesthesia was used to separate females from males in the present study as all experiments involved individual flies being handled under a microscope or dissected, meaning that flies were free from potential effects of sorting under CO_2 (Nilson et al., 2006; MacMillan et al., 2017).

Preparation for electrophysiology

To prepare for electrophysiological experiments, flies were gently held by the head with a 100 µl pipette tip attached to an aspirator, immobilized in a thin layer of wax on a glass cover slide and further secured with a bent minuten pin gently positioned between the thorax and abdomen and fixed in the wax. After immobilization, micro-scissors were used to cut a small hole in the head cuticle to access the brain, and a small incision was made in the abdomen to insert an Ag/AgCl wire for grounding (Spong et al., 2016b).

Measurement of cold-induced SD

In this study, the shutdown of neural function by a SD event was measured electrophysiologically using glass microelectrodes. Filamented glass capillaries [1B100F-4, World Precision Instruments (WPI), Sarasota, FL, USA] were pulled on a Flaming-Brown P-1000 micropipette puller (Sutter Instruments, Novato, CA, USA) to a tip resistance of 5–7 MΩ when backfilled with 500 mmol l^{-1} KCl. The filled glass microelectrodes were placed in electrode holders containing an Ag/AgCl wire, which were attached to a micromanipulator (M3301-M3-L, WPI) and connected to a Duo 773 electrometer (WPI). Raw voltage outputs from the electrometer were digitized using a PowerLab 4SP A/D converter (ADInstruments, Colorado Springs, CO, USA) before being read by a computer running LabChart software (version 4.0, ADInstruments).

For experiments, the glass cover slide with a fly attached was placed on a custom-built thermoelectrically cooled Peltier plate. Next, a type K thermocouple was placed immediately next to the fly's head to monitor brain temperature (connected to the A/D converter with a thermocouple-to-analog converter; TAC80B-K, Omega, Stamford, CT, USA) and a glass microelectrode was inserted into the brain, through the hole cut in the head capsule, by gently advancing the electrode with the micromanipulator. Successful placement of the electrode was determined when the advancement of the microelectrode resulted in a small increase in the recorded voltage (5–10 mV); this was interpreted as the electrode having penetrated the glial blood–brain barrier such that it was monitoring the transperineurial potential (Robertson et al., 2020; Cheslock et al., 2021). Subsequently, the temperature of the fly's head was lowered from room temperature (22–23°C) by ∼1°C min^{-1} under manual control until an abrupt drop in the transperineurial potential was observed (∼30–50 mV in ∼5–10 s), indicative of SD having occurred. The temperature of SD onset was measured at the half-amplitude of the drop in transperineurial potential, and the amplitude of the event was calculated as the difference in potential before and after SD onset. Furthermore, the descending slope at the half-amplitude of the drop in transperineurial potential was recorded (using the *Slope* function in LabChart). After SD onset, temperature was further lowered by 1°C after which flies were returned to room temperature, during which the transperineurial potential was also monitored to ensure that they could recover from the SD (i.e. the cold exposure was not terminal). The SD temperature and amplitude were measured in 10 flies from each of the 15 and 25°C acclimation groups.

Latency to SD following ouabain injection

To determine whether SD onset characteristics were dependent on Na^+/K^+-ATPase activity, we injected ouabain into the head of the fly while continuously monitoring the transperineurial potential (as described above) (Rodgers et al., 2007, 2010; Spong et al., 2016b). Ouabain (O3125, Sigma-Aldrich, St Louis, MO, USA) was diluted in saline (147 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 4 mmol l^{-1} CaCl and 10 mmol l^{-1} Hepes at pH 7.2) to a concentration of 0.1 mmol l^{-1} and slowly injected into the head of the fly in a small aliquot (~14–15 nl) using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). Following injection, we measured the latency to the first SD event (there were often multiple events following the first one). This measurement was performed at temperatures ranging from 10 to 35°C in 5°C increments, and fly preparations were allowed to equilibrate at this temperature for 5–10 min before injection. We found that 10°C was too close to the SD temperature for the warm-acclimated group as it often led to a cold- rather than ouabain-induced SD, so 35°C was included for the warm-acclimated group so that the same number of temperatures was tested in each group. Six to eight flies from each acclimation group were measured at each temperature (N=67 flies in total).

Collection of brain samples

Flies were briefly sedated in 70% ethanol and subsequently dissected in standard *Drosophila* saline (in mmol l^{-1}: 122.5 NaCl, 15 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 15 MOPS, 20 glucose, 10 glutamine, at pH 7.0). Brains were quickly transferred to 10 µl of the same saline in a 200 µl microcentrifuge tube kept at 2–3°C in a Boekel Scientific TropiCooler Benchtop Incubator (model 260014, Fisher Scientific, Ottawa, ON, Canada), and once 30 brains had been collected (2–2.5 min per brain, ~70 min total), samples were flash frozen in liquid N_2 and stored at −80°C until use.

Brain Na^+/K^+-ATPase activity

Samples were thawed on ice and mixed with 140 µl SEID buffer (in mmol l^{-1}: 150 sucrose, 10 EDTA, 50 imidazole, with 0.1% w/v deoxycholic acid) to a volume of 150 µl. Next, samples were transferred from the 200 µl tubes to 1.7 ml microcentrifuge tubes by centrifuging them upside-down inside the 1.7 ml tubes at 2000 ***g*** for 1 min, and mixed with a further 100 µl of SEID buffer to a final volume of 250 µl (if any brains could not be transferred, this was noted). Here, samples were first ground with a polypropylene pestle and then sonicated (Model Q55, QSonica Sonicators, Newtown, CT, USA) twice for 5–6 s with a 25–30 s break on ice to avoid overheating. Lastly, crude homogenates were vortexed, split into five aliquots and stored at −80°C until use.

To normalize Na^+/K^+-ATPase activity to protein content, we first ran a Bradford assay on one of the five aliquots. In a clear 96-well plate, 5 µl of crude homogenate and protein standards (0–2 mg ml^{-1} bovine serum albumin, ALB001.25, Bioshop Canada, Burlington, ON, Canada) was loaded into wells in triplicate followed by 250 µl of Bradford reagent (B6916, Sigma-Aldrich). After incubating for 10 min, the absorbance of both samples and standards was measured in a microplate spectrophotometer (BioTek Epoch, Winooski, VT, USA) at 595 nm, and sample protein contents were calculated by reference to the standards.

The activity of Na^+/K^+-ATPase was measured in the remaining four aliquots using a continuous, spectrophotometric assay at 5, 10, 15 and 25°C following the approach of McCormick and Bern (1989), modified by Jonusaite et al. (2011), and following the best approaches for optimizing activity (Moyes et al., 2021): solution A [4 units ml^{-1} lactate dehydrogenase (L2500, Sigma), 5 units ml^{-1} pyruvate kinase (P1506, Sigma), 2.8 mmol l^{-1} phosphoenolpyruvate (cyclohexylammonium salt, P3637, Sigma), 3.5 mmol l^{-1} ATP (AD0020, Bio Basic, Markham, ON, Canada), 0.3 mmol l^{-1} NADH (NBO642, Bio Basic) and 50 mmol l^{-1} imidazole, pH 7.5] was prepared and activated by mixing with a salt solution (in mmol l^{-1}: 189 NaCl, 42 KCl, 10.5 $MgCl_2$ and 50 mmol l^{-1} imidazole) at a ratio of 3:1 (solution A:salt) to final salt concentrations of 47.25 mmol l^{-1} NaCl, 10.5 mmol l^{-1} KCl and 2.63 mmol l^{-1} $MgCl_2$. Next, the quality of solution A was tested by creating a standard curve to describe the disappearance of NADH given the addition of specific amounts of ADP (to simulate hydrolysis of ATP to ADP by the Na^+/K^+-ATPase). This was done by adding known amounts of ADP (AD0016D, Bio Basic) in 10 µl aliquots (in triplicate) to a clear 96-well plate, followed by 200 µl of solution A, after which the depletion of NADH was measured at 340 nm every minute for 30 min in the microplate spectrophotometer. If a negative relationship between final absorbance (ABS) and ADP addition equal to or steeper than −0.013 ABS $nmol^{-1}$ ADP was observed, solution A was deemed satisfactory (see an example in Fig. S1), and an accompanying solution B was created as described above with the addition of 5 mmol l^{-1} ouabain (to specifically inhibit Na^+/K^+-ATPase). To measure sample Na^+/K^+-ATPase activity, four 10 µl aliquots of sample homogenate were loaded into a clear 96-well plate followed by the addition of 200 µl of solution A to two wells and 200 µl of solution B to the remaining two, and again the depletion of NADH was monitored in the microplate spectrophotometer at 340 nm for 30 min. The activity of Na^+/K^+-ATPase was calculated as follows:

$$\text{Na}^+/\text{K}^+\text{-ATPase activity} = \frac{\left(\frac{\text{Slope A}-\text{Slope B}}{\text{ADP standard curve slope}}\right)}{\text{Sample protein content}}, \quad (1)$$

where Slope A and Slope B refer to the slope of disappearance of NADH (i.e. production of ADP) in the sample wells (A) and in sample wells with the Na^+/K^+-ATPase blocked (B) and the ADP standard curve slope is the slope of the relationship between ADP addition and NADH disappearance (−0.013 and −0.015 ABS $nmol^{-1}$ ADP). Slopes of NADH disappearance in samples (A and B) were calculated on a linear part of the reading lasting at least 10 min. The initial part of readings was often not linear when the temperature of the plate was still stabilizing (~1–3 min). Eight biological replicates were measured for both warm- and cold-acclimated flies.

The thermal sensitivity of Na^+/K^+-ATPase was estimated by calculating the Q_{10} values using the Q10() function from the '*respirometry*' package in R (http://cran.r-project.org/package=respirometry), which fits an exponential curve and subsequently calculates Q_{10} using the formula:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10°C}{(T_2-T_1)}}, \quad (2)$$

where R_2 and R_1 denote Na^+/K^+-ATPase activity at temperatures T_2 and T_1, respectively, with T_2 being the higher temperature. This calculation was applied to individual samples (each sample was measured at all four temperatures), which allows us to statistically compare the thermal sensitivity of Na^+/K^+-ATPase in the brains of 15 and 25°C-acclimated flies.

Brain Na^+/K^+-ATPase abundance

The abundance of Na^+/K^+-ATPase was compared between warm- and cold-acclimated flies using western blots. Samples were dissected and stored as described for the Na^+/K^+-ATPase activity assay above but with only 10 brains per sample. Samples (10 µl) were thawed on ice and mixed with 90 µl RIPA buffer [50 mmol l^{-1} Tris-HCl, 150 mmol l^{-1} NaCl, 0.1% SDS, 1% deoxycholic acid, 1% Triton X-100, 1 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF and 1:200 protease inhibitor cocktail (P1860, Sigma-Aldrich), pH 7.5] and transferred to a 1.7 ml microcentrifuge tube and homogenized as described above. Sample homogenates were centrifuged at 10,000 ***g*** for 10 min and supernatant was transferred to 200 µl tubes and kept on ice before sample protein content was estimated using a Bradford assay (as described above). Before running samples through SDS-PAGE, a pilot experiment was run (using the procedure described below) to determine the amount of sample protein to load to optimize signal-to-noise ratios and avoid oversaturation of the final blot (Taylor et al., 2013; Taylor and Posch, 2014; Bass et al., 2017). Ultimately, protein samples were diluted with incomplete RIPA buffer (RIPA without DTT, PMSF and protease inhibitor) and added to a 6× Laemmli loading buffer (375 mmol l^{-1} Tris-HCl, 9% SDS, 50% glycerol and 0.03% Bromophenol Blue mixed 9:1 with β-mercaptoethanol) such that a total of 2 µg protein in 20 µl of buffer was loaded into wells of a Mini-PROTEAN TGX Stain-Free gel (5–14%; Bio-Rad, Mississauga, ON, Canada). Protein ladder (5 µl of Precision Plus Protein™ All Blue Prestained Protein Standards, Bio-Rad) was added to wells to the left and right of protein samples. Next, proteins were separated by running the gel in an electrophoresis chamber (Mini-PROTEAN Tetra Vertical Electrophoresis Cell, Bio-Rad) at 200 V for 30 min. Following electrophoresis, the gel was extracted and imaged on a ChemiDoc MP Imaging System (Bio-Rad) to confirm protein separation, after which the separated protein was transferred to a polyvinylidene fluoride (PVDF) membrane in a Trans-Blot Turbo System (Bio-Rad) at 25 V (1.3 A) for 7 min. Before blocking the PVDF membrane, it was imaged in the ChemiDoc MP Imaging System as the use of TGX Stain-Free gel allowed for imaging of membrane-bound total protein without subsequent staining (setting: Stain-free blot, 7.5 s exposure time). After imaging, the PVDF membrane was blocked in a 5% skim milk solution [w/w in TBS-T buffer: 20 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 0.1% Tween-20 (v/w)] at room temperature (22–23°C) for 1 h. Blocked membranes were briefly rinsed in TBS-T buffer (3×5 min) and probed in a 500:1 solution of blocking buffer and primary antibody targeting the α-subunit of the Na^+/K^+-ATPase (mouse anti-Na^+/K^+-ATPase; a5, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) at 4°C for 20 h overnight on a rotating stage. The next morning, probed membranes were washed in TBS-T (3×5 min) and incubated with the secondary antibody (StarBright Blue 700 Goat Anti-Mouse IgG) in blocking buffer (1:3000) at room temperature for 1 h on a rotating stage and subsequently washed (3×15 min) in TBS-T. Lastly, the blotted membrane was imaged in the ChemiDoc MP Imaging System to assess the fluorescence signal of the secondary antibody (setting: Starbright B700, 3 s exposure time). Densitometric quantification of total protein load from the Stain-free blot (for normalization) and fluorescence signal from the secondary antibody was performed by measuring lane and band intensity, respectively, using ImageLab software (v6.0.1, Bio-Rad) and following the approach outlined by Taylor et al. (2013) and Taylor and Posch (2014) (blot images are available in the Supplementary Materials and Methods). Briefly, lanes and bands were marked in ImageLab to create a lane profile, after which the background signal was omitted (disk size=10.0 for all lanes and blots). By inspecting each lane profile, we ensured that only relevant bands were marked [for example, the long tail in the fluorescent antibody signal (>150 kDa) was excluded], after which the cumulative band and lane intensities were extracted for the fluorescent antibody signal and total protein signal, respectively. For each sample, the fluorescence signal was then normalized to its total protein signal. Six biological replicates were measured for each acclimation group.

Statistical analysis

All statistical analyses were performed in R version 4.2.1 (http://www.R-project.org/). All datasets were tested for normality with a combination of Shapiro–Wilk tests and inspection of boxplots and residuals. Where the normality was violated, non-parametric tests were used to investigate differences between acclimation groups.

SD temperature and amplitude were confirmed to have similar variances using *F*-tests, after which the means were compared using Student's *t*-tests. This was followed by a linear regression to investigate the relationship between the temperature and amplitude of the SD events. Descending slopes of the SD events were found to have different variances between the two acclimation groups, and means were therefore compared using a Welch *t*-test. Subsequently, a linear regression was used to investigate the relationship between temperature and slope of the events.

To describe the relationship between temperature and reliance on Na^+/K^+-ATPase (i.e. latency), we fitted a three-parameter Gaussian model to the dataset on the effect of temperature on the latency to ouabain-induced SD with a non-linear least squares approach using the nls() function and the following formula:

$$\text{Latency} = M\, e^{-\frac{(\text{Temperature} - \mu)^2}{2\sigma^2}}, \quad (3)$$

where M is the maximum latency (in min), μ is the temperature where latency is maximized (in °C) and σ is the 'standard deviation' (in °C) which here represents a measure of 'thermal insensitivity',

Table 1. Non-linear least squares regression to a Gaussian model used to estimate the effect of acclimation on the thermal performance curve of the latency to spreading depolarization dataset

Main parameter	Model output	Acclimation parameter	Effect of acclimation	*t*	*P*	Acclimation group estimates	
						15°C	25°C
M	47.0±11.0 min	a_M	1.3±0.5 min °C^{-1}	2.5	0.017	67.1±3.4 min	80.4±4.3 min
μ	18.2±1.8°C	a_μ	0.4±0.1°C °C^{-1}	5.2	<0.001	24.1±0.6°C	28.0±0.4°C
σ	11.3±2.0°C	a_σ	–0.2±0.1°C °C^{-1}	–2.5	0.016	8.1±0.7°C	6.0±0.4°C

The output parameters from the model include the maximum latency (M), the temperature of the maximum latency (μ, midpoint of the distribution), a measure of the thermal insensitivity (σ, the 'standard deviation'), and accompanying acclimation parameters (a_μ, a_μ and a_σ) to estimate how acclimation affected the main parameters (see Materials and Methods, 'Statistical analysis' for more details). Values in the table represent means±s.e.m. The total sample size of 67 results in 61 degrees of freedom for the analysis.

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where a higher value denotes a smaller effect of changing temperature (i.e. high insensitivity) or a broader bell-shaped curve [in this Gaussian curve fit, σ refers to the temperature change that causes a ∼39.3% decrease from the maximum value, rather than the area under the distribution curve, where ±2σ contains 95% of data points in the Gaussian (Normal) distribution]. To compare the effect of acclimation temperature on latency to SD between cold- and warm-acclimated flies, we expanded the outputs of the fitted Gaussian models (M, μ, σ) to include acclimation terms for each of the three above-mentioned parameters [i.e. (M+a_M·Acclimation temperature), (μ+a_μ·Acclimation temperature) and (σ+a_σ·Acclimation temperature)]:

$$\text{Latency} = [M + a_M\ \text{Acclimation}]\, e^{-\frac{(\text{Temperature} - [\mu + a_\mu \text{Acclimation}])^2}{2\,[\sigma + a_\sigma \text{Acclimation}]^2}}. \quad (4)$$

This approach allowed us to use the nls() function to determine whether and how M, μ and σ were affected by thermal acclimation using non-linear least squares regression. A summary of the output parameters (M, μ, σ, a_M, a_μ, a_σ) and statistics (t- and P-values) can be found in Table 1.

The effect of temperature on the activity of brain Na^+/K^+-ATPase in cold- and warm-acclimated flies was analysed using a linear mixed-effect model, where assay temperature and acclimation temperature were treated as fixed factors, while samples were included as random factors (because the same biological replicates were tested at all temperatures). Here, Q_{10} was calculated for each sample to estimate the thermal sensitivity of the brain Na^+/K^+-ATPase in both groups (1) across the entire temperature spectrum (5–25°C), and (2) in the three temperature intervals (5–10°C, 10–15°C and 15–25°C) using the Q10() function from the '*respirometry*' package in R. Q_{10} across the entire spectrum was compared between groups using a Mann–Whitney–Wilcoxon U-test as the data were not normally distributed, while the Q_{10} values in all intervals were analysed using a linear mixed-effect model with temperature and acclimation as fixed factors and the individual sample as a random factor. Q_{10} values were found to vary greatly depending on the temperature interval investigated and Na^+/K^+-ATPase activity did not appear to display an exponential relationship with temperature for either group. We therefore decided to further analyse this dataset similar to how we investigated the effect of acclimation on the latency to ouabain-induced spreading depolarization. Thus, we fitted the three-parameter Gaussian model (Eqn 3) to Na^+/K^+-ATPase activity for each sample, which allowed us to more accurately investigate and compare maximum activity (M), temperature of maximum activity (μ) and thermal sensitivity (σ) of the two groups. Here, group parameters were compared using a Mann–Whitney–Wilcoxon U-test for M (one group was non-normal) and Welch t-tests for μ and σ (F-tests revealed that group variation estimates differed).

Lastly, we compared the abundance of Na^+/K^+-ATPase in the brains of cold- and warm-acclimated flies using western blots. For each sample, the fluorescence signal from the secondary antibody was normalized to the respective total protein signal, after which the protein-normalized signals were normalized to the mean of the 25°C acclimated group. Data were log-transformed during analysis, and groups were compared using a linear mixed-effect model with acclimation temperature as a fixed factor and blot as a random factor (as samples were run on different blots).

A significance level of 0.05 was applied to all analyses, all values mentioned below denote mean±s.e.m., and error bars presented in the figures represent the s.e.m.

RESULTS

Cold acclimation lowers SD temperature, amplitude and descending slope

As expected, cold acclimation reduced the SD temperature to 5.1±0.4°C compared with 9.0±0.4°C after warm acclimation (t_{18}=−7.1, P<0.001; Fig. 1A). Similarly, cold acclimation led to a

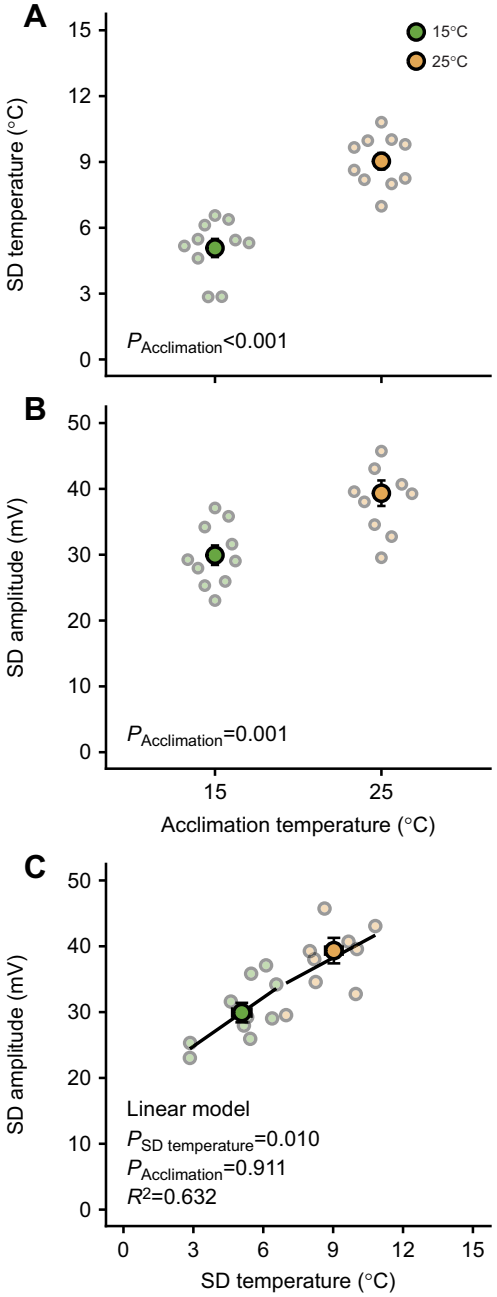

Fig. 1. Cold acclimation lowers spreading depolarization temperature and amplitude. (A) Cold acclimation (15°C, green) of female *Drosophila melanogaster* lowers the spreading depolarization (SD) temperature relative to warm acclimation (25°C, orange). (B) The amplitude of the SD event is similarly lowered by cold acclimation. (C) A statistically significant and positive relationship between SD temperature and amplitude was found among individual flies, but there was no effect of acclimation temperature on the slope of the relationship. N=10 flies per group, which are depicted by small, translucent points (which in A and B have been moved slightly away from the respective acclimation temperatures for clarity). Group means are depicted as large opaque symbols. Error bars represent s.e.m. and error bars that are not visible are obscured by the symbols.

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reduced amplitude of the SD event (t_{18}=−3.9, P=0.001) such that the event had an amplitude of 29.9±1.5 and 39.3±1.9 mV in cold- and warm-acclimated flies, respectively (Fig. 1B). Interestingly, there was a linear relationship between SD temperature and amplitude within individuals (t_{17}=2.9, P=0.010), and the slope of this relationship was not affected by acclimation (t_{17}=−0.1, P=0.911) (Fig. 1C).

The last SD parameter we characterized was the slope of the descending down-shift in transperineurial potential (Fig. 2). Here, we found a clear trend that cold acclimation resulted in more gently descending slopes (Fig. 2A), but while this trend was almost twofold it did not reach the level of statistical significance (−6.4±1.1 and −11.3±2.4 mV s^{-1} for cold- and warm-acclimated flies, respectively; $t_{12.8}$=1.9, P=0.085). Furthermore, we noted that SD events occurring at a lower temperature tended to have gentler slopes in general (Fig. 2B), but this too failed to reach statistical significance (t_{18}=−2.0, P=0.062).

Cold acclimation left-shifts the thermal sensitivity of ouabain-induced SD

To investigate how acclimation affected the role of Na^+/K^+-ATPase in preventing SD, we injected the specific Na^+/K^+-ATPase inhibitor ouabain into the heads of flies while monitoring the transperineurial potential. Ouabain injection always induced one or more SD events (see Fig. 3 for example trace), and we used the latency to the first event as a measure of how the event relates to Na^+/K^+-ATPase activity. The overall pattern resembled a thermal performance curve (Fig. 4), which describes the effects of ambient temperature in biological processes (see Angilletta et al., 2002; Schulte et al., 2011), so we decided to use a model-fitting approach to analyse these data (see Materials and Methods, 'Statistical analysis'). Fitting a three-parameter Gaussian model to data from the two groups resulted in high correlation coefficients (R^2) of 0.754 and 0.801 for cold- and warm-acclimated flies, respectively (Eqn 3), and 0.783 for the combined model (Eqn 4). Using the Gaussian model outputs to test how acclimation affected the latency to ouabain-induced SD, we found that (1) the maximum latency at the optimal temperature (M) was 13.3±5.4 min shorter in cold-acclimated flies (a_M; t_{61}=2.5, P=0.017), (2) the optimal temperature (μ) of cold-acclimated flies was left-shifted by 4.3±0.7°C (a_μ; t_{61}=5.2, P<0.001), and (3) the thermal 'insensitivity' (σ) was increased by 1.7±0.8°C (a_σ; t_{61}=−2.5, P=0.016). All model parameters and statistical outputs from this analysis can be found in Table 1.

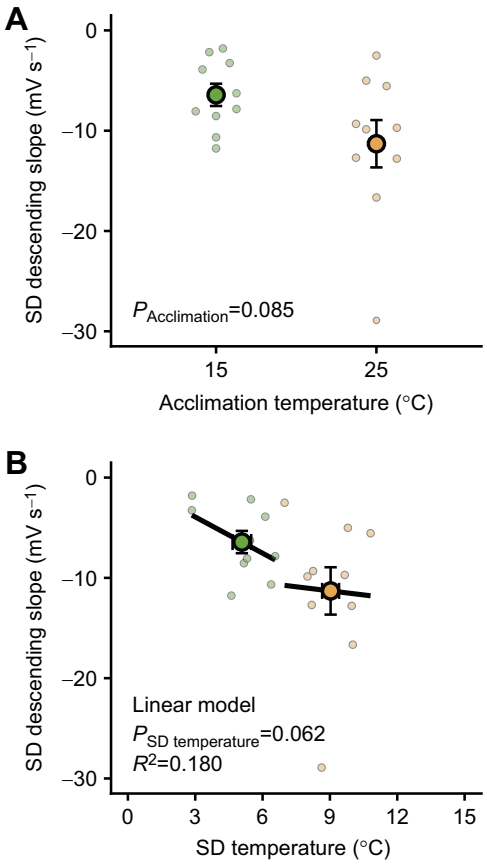

Fig. 2. Cold acclimation reduces the slope of the drop in transperineurial potential during SD. (A) Cold acclimation (15°C, green) of female *D. melanogaster* reduces the magnitude of the SD descending slope relative to warm acclimation (25°C, orange), despite not reaching the level of statistical significance. (B) A negative relationship between SD temperature and descending slope of the transperineurial potential during the SD was evident; however, this too did not reach the level of statistical significance. N=10 flies per group, which are depicted by small, translucent points (spread slightly on the x-axis for clarity in A), and group means are depicted as large opaque symbols. Error bars denote s.e.m.

Thermal kinetics of brain Na^+/K^+-ATPase are altered by cold acclimation

Na^+/K^+-ATPase activity was measured at 5, 10, 15 and 25°C in brain samples from cold- and warm-acclimated flies (Fig. 5). Overall, there was no main effect of acclimation on the activity of Na^+/K^+-ATPase in the brain ($F_{1,42}$=1.5, P=0.248). Activity of the pump was highest at 25°C in both acclimation groups, with warm-acclimated flies having higher activity (38.0±0.9 μmol ADP mg^{-1} h^{-1}) than their cold-acclimated conspecifics (31.4±1.1 μmol ADP mg^{-1} h^{-1}) (Fig. 5). As the temperature was lowered, the activity of the Na^+/K^+-ATPase decreased ($F_{3,42}$=1039, P<0.001), but in an acclimation-specific manner. The magnitude of the temperature effect was significantly mitigated by cold acclimation ($F_{3,42}$=29.7, P<0.001), resulting in cold-acclimated flies having higher brain Na^+/K^+-ATPase activity at 5°C (6.9±0.5 μmol ADP mg^{-1} h^{-1}) than warm-acclimated flies (4.8±0.2 μmol ADP mg^{-1} h^{-1}) (Fig. 5). The smaller effect of cold on brain Na^+/K^+-ATPase activity in cold-acclimated flies resulted in a Q_{10} of 1.83±0.05 across the entire temperature range, which was significantly smaller than the 2.22±0.04 observed in warm-acclimated flies (Mann–Whitney–Wilcoxon U=1, N=16, P<0.001) (Table 2). Calculating Q_{10} in each temperature interval and for every sample revealed a similarly strong effect of acclimation on the temperature sensitivity of the brain Na^+/K^+-ATPase ($F_{1,28}$=39.1, P<0.001); however, this analysis also revealed that Q_{10} for both acclimation groups increased at lower temperatures ($F_{2,28}$=105, P<0.001) and that the degree to which it increased was smaller after cold acclimation ($F_{1,28}$=7.2, P=0.003) (Table 2). Thus, the relationship we observed between temperature and brain Na^+/K^+-ATPase activity appeared non-exponential within the temperature range of this study, and we therefore applied a Gaussian fit to each sample to more accurately describe the relationship and compare acclimation groups (see Fig. S2). Using this approach, we found no evidence of a shift in the Na^+/K^+-ATPase activity performance curve (μ; $t_{7.3}$=1.2, P=0.251), but instead that the cold-acclimated flies had a lower maximum brain Na^+/K^+-ATPase activity (M; Mann–Whitney–Wilcoxon U=12, N=16, P=0.038) and a higher temperature 'insensitivity' (σ; $t_{7.1}$=2.4, P=0.047) (Fig. S2). Thus, both analysis approaches suggest that cold acclimation lowers the thermal sensitivity of Na^+/K^+-ATPase.

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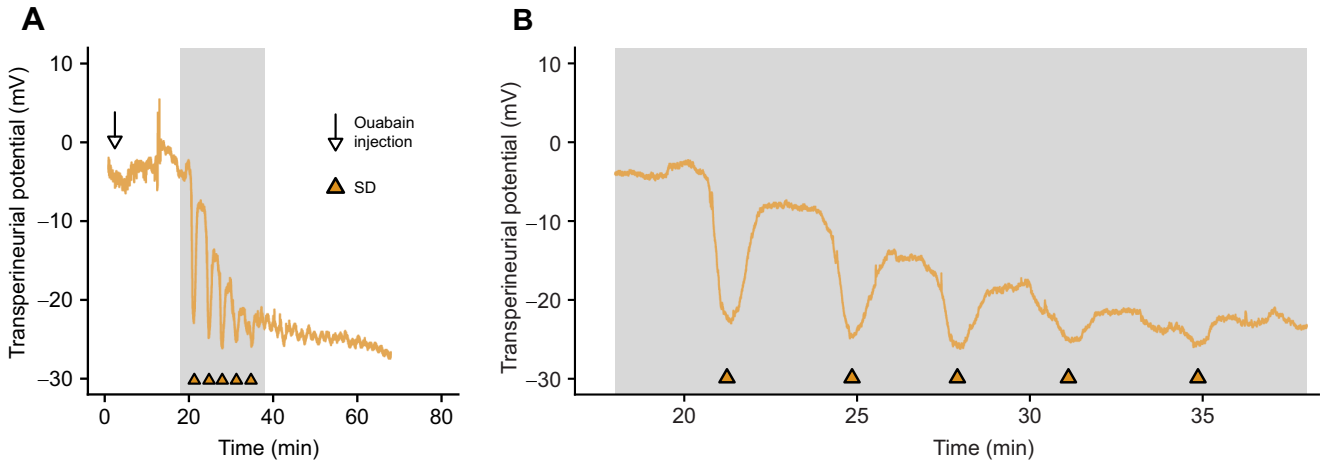

Fig. 3. Example trace of SD events caused by ouabain injection into the head of *D. melanogaster*. (A) Injection of the Na^+/K^+-ATPase-specific blocker ouabain (arrow) into leads to SD in the brain (triangles) after a delay (shown here for a 25°C acclimated fly at 20°C). (B) A closer look at the shaded area in A, showing the SD events, which are characterized by large, rapid negative shifts in the transperineurial potential. In the example depicted, ouabain injection elicited (five) repeated SD events, but the number ranged from one to six. Note that the triangles here point to the transperineurial potential minima of the SD events and not their half-amplitude, which was used in latency estimates.

Acclimation does not change the abundance of brain Na^+/K^+-ATPase

To investigate whether cold- and warm-acclimated flies differed in Na^+/K-ATPase content, we measured Na^+/K-ATPase abundance using western blots targeting the α-subunit (example blot in Fig. 6A,B). We found no difference in brain Na^+/K^+-ATPase abundance between cold- and warm-acclimated flies (t_8=0.1, P=0.922) (Fig. 6C).

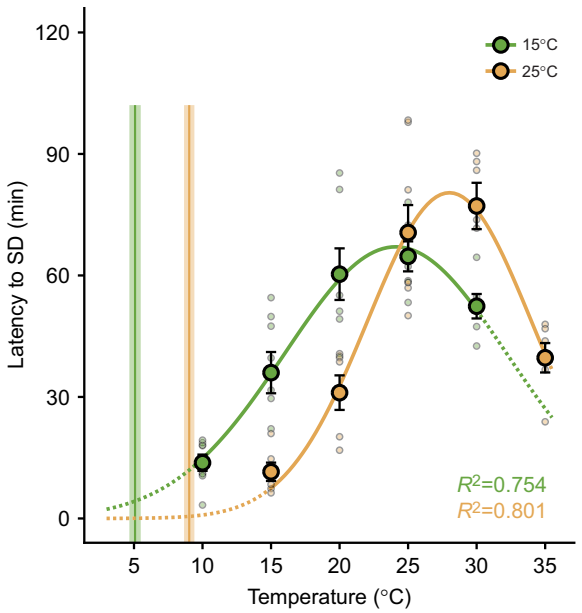

Fig. 4. Cold acclimation causes a leftward thermal shift in the latency to ouabain-induced SD in the brain. Latency to SD after ouabain injection was highly temperature dependent, and this effect of temperature was left-shifted in cold-acclimated (15°C, green) relative to warm-acclimated (25°C, orange) flies (see parameter analysis in Table 1). Note that the latency approached zero (by extrapolation) around the temperature found to cause SD during gradual cooling (data from Fig. 1A; vertical lines and shaded areas depict means and s.e.m.). N=6–8 per temperature and acclimation group. Some data points and error bars (s.e.m.) are obscured by the symbols (all data points can be found in Table S1). Dotted line segments represent model extrapolations beyond the data.

DISCUSSION

Thermal tolerance is broadly considered to be important in determining the distribution of insects (Addo-Bediako et al., 2000; Kimura, 2004; Sunday et al., 2011; Kellermann et al., 2012), and the capacity to respond and adapt to environmental change is therefore likely to shape their future biogeography (Chown and Nicolson, 2004; Ghalambor et al., 2007; Overgaard et al., 2011). In many insects, including *Drosophila*, cold acclimation has repeatedly been shown to induce adaptive improvements in the ability to maintain mobility at low temperatures (Kelty and Lee, 1999; Ransberry et al., 2011; Schou et al., 2017). Here, we reaffirm this cold acclimation capacity and argue that modification to a single ion transporter, the Na^+/K^+-ATPase, is critical to cold acclimation in the brain of *Drosophila* and reduces the CT_{min} by lowering the temperature at which a SD shuts down central nervous system function.

Maintenance of Na^+/K^+-ATPase activity decreases SD susceptibility

A central theme in the current model of insect cold tolerance is that cold-tolerant insects better maintain the function of active ion transporters at low temperature, which allows them to maintain ion balance and avoid tissue damage induced by hyperkalaemia (Overgaard and MacMillan, 2017; Overgaard et al., 2021). In particular, Na^+/K^+-ATPase is central to the maintenance of ion and water balance in the insect central nervous system (Treherne and Schofield, 1981). We therefore chose to investigate the role of Na^+/K^+-ATPase in cold-induced SD by (1) inhibiting Na^+/K^+-ATPase with ouabain to estimate sensitivity to SD *in vivo* and (2) measuring the activity of Na^+/K^+-ATPase *in vitro* at benign and low temperatures after both warm and cold acclimation.

Application of ouabain to the brain *in vivo* resulted in SD after a delay, and this latency appeared to follow the general shape of a thermal performance curve (Angilletta, 2009; Schulte, 2015), which (1) had a lower maximum, (2) was left-shifted, and (3) had a lower thermal sensitivity in the cold-acclimated flies (Fig. 4 and Table 1). This indicates that cold-acclimated *D. melanogaster* are less

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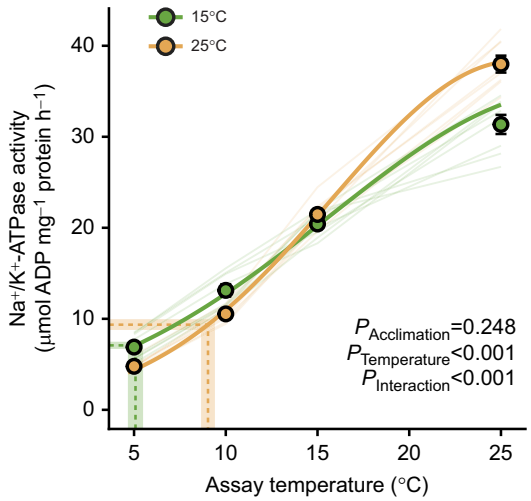

Fig. 5. Cold acclimation lowers the *in vitro* thermal sensitivity of Na^+/K^+-ATPase extracted from the brain of *D. melanogaster*. (A) The overall activity of Na^+/K^+-ATPase from brains of cold-acclimated flies (15°C, green) did not differ from that of warm-acclimated flies (25°C, orange), but their Na^+/K^+-ATPase was less sensitive to temperature such that their activity was higher at 5°C. Thin lines link activity measurements for each biological replicate (*N*=8 per acclimation group), and solid circles with error bars denote group means and s.e.m. at each temperature. *In vitro* pump activity at the SD temperature (based on calculations using Gaussian model parameters; dashed lines and shaded areas denote group means and errors, respectively) was slightly lower in the cold-acclimated group. Error bars that are not visible are obscured by the symbols.

sensitive to SD at low temperature that their warm-acclimated conspecifics, as they have a longer delay to SD after ouabain is applied to the brain in the cold. By extension, this suggests that cold-acclimated insects have a greater capacity to prevent SD in the cold and remain active at low temperature, and that Na^+/K^+-ATPase activity plays a key role in this ability. This is further supported by the shift in the latency to SD curve (4.3±0.7°C) closely matching that of the shift in SD temperature (4.0±0.6°C), and qualitatively similar observations have been made on this relationship between the latency to ouabain-induced SD and insect thermal tolerance previously (Spong and Robertson, 2013; Spong et al., 2014, 2015, 2016b; Gantz et al., 2020).

Interestingly, the binding of ouabain to the Na^+/K^+-ATPase is temperature sensitive, such that the Na^+/K^+-ATPase needs to be active to be inhibited (Clausen and Hansen, 1974; Bayley et al., 2020). This means that the thermal performance curve we observed here at least partly reflects activity of the Na^+/K^+-ATPase itself, and by extension that cold-acclimated *D. melanogaster* are able to maintain Na^+/K^+-ATPase activity at low temperature, supporting our overall hypothesis. This improved cold tolerance may, however, come at the cost of performance at more permissive temperatures or during heat exposure. Specifically, the cold-acclimated flies have a lower maximum latency at their optimum, as well as a lower latency than their warm-acclimated conspecifics when exposed to temperatures above the optimum (see Fig. 4), suggesting a potential trade-off between performance at high and low temperatures (Stillman, 2003; Calosi et al., 2008; Sunday et al., 2019).

Direct measurement of innate Na^+/K^+-ATPase activity in brain homogenates revealed a strong, negative effect of temperature, which was mitigated by cold acclimation (Fig. 5), supporting our observations above. The effect of acclimation on the temperature dependency of Na^+/K^+-ATPase activity was also evident from our

Table 2. Q_{10} estimates for *in vitro* Na^+/K^+-ATPase activity calculated for each acclimation group across different temperature ranges

	Acclimation group Q_{10} estimates		
Temperature range (°C)	15°C	25°C	*P*-value
5–25	1.83±0.05	2.22±0.04	$P_{acclimation}$<0.001
5–10	3.74±0.20	4.94±0.26	$P_{acclimation}$<0.001
10–15	2.56±0.28	4.17±0.14	$P_{temperature}$<0.001
15–25	1.55±0.08	1.76±0.05	$P_{interaction}$=0.003

See Results for more information on statistical analysis.

Q_{10} estimates, which were consistently lower for cold-acclimated flies (Table 2). Taken together, these findings strongly suggest that reductions in the thermal sensitivity of Na^+/K^+-ATPase help delay or prevent SD onset, and by extension help maintain organismal activity in the cold.

Interestingly, we found that Q_{10} itself was temperature sensitive, indicating that thermal sensitivity estimates based on Q_{10} alone might be misleading, which also makes comparisons with previous invertebrate literature difficult (Leong and Manahan, 1997; Pan et al., 2016). We therefore decided to fit a three-parameter Gaussian function to extract an objective measure of thermal sensitivity that better fit the relationship between temperature and Na^+/K^+-ATPase activity. We found the same trend of a lower thermal sensitivity in cold-acclimated flies (higher σ-value, larger drop in temperature needed to decrease activity; Fig. S2). Thus, regardless of the approach, our data indicate that acclimation alters the kinetics of the Na^+/K^+-ATPase in a manner than allows cold-acclimated flies to maintain Na^+/K^+-ATPase activity in the cold. Combined, these findings, despite being correlative in nature, strongly suggest that the lowered SD temperature in cold-acclimated *D. melanogaster* is achieved via lowered thermal sensitivity of active transporters involved in maintaining brain ion homeostasis (MacMillan and Sinclair, 2011; Cheslock et al., 2021).

Of course, Na^+/K^+-ATPase can only maintain activity if other cellular processes that modulate its activity and/or transport capacity have been similarly adjusted (e.g. ATP production and K^+ leak). Indeed, cold acclimation has been shown to promote whole-animal mitochondrial ATP production in *D. melanogaster* exposed to stressful cold (Colinet et al., 2017), and mitochondrial ATP-sensitive K^+ channels have been implicated in the modulation of SD susceptibility in locusts (Van Dusen et al., 2020). We also note that cold-acclimated flies appear to have lower Na^+/K^+-ATPase activity than warm-acclimated flies at their respective SD temperatures (see Fig. 5), which indicates changes to processes independent of Na^+/K^+-ATPase. Thus, to fully understand the mechanism(s) by which the brain Na^+/K^+-ATPase of cold-acclimated flies better maintains activity in the cold, and its exact involvement in SD, the upstream effectors and limiters must also be investigated.

It is also interesting to note that while the *in vivo* and *in vitro* measurements of Na^+/K^+-ATPase activity both support maintained activity at low temperature (Figs 4 and 5, respectively), there was no left-shift in the thermal performance curve for the *in vitro* Na^+/K^+-ATPase activity. We speculate that this difference might relate to effects of the membrane environment and cellular localization (e.g. effects of homogenization for *in vitro* measurements; see Moyes et al., 2021), or changes to other ionoregulatory processes (e.g. the K^+ currents described above, or other ATPases involved in SD dynamics; Robertson and Van Dusen, 2021). Lastly, it is worth emphasizing that cold-induced SD might differ mechanistically from ouabain-induced SD.

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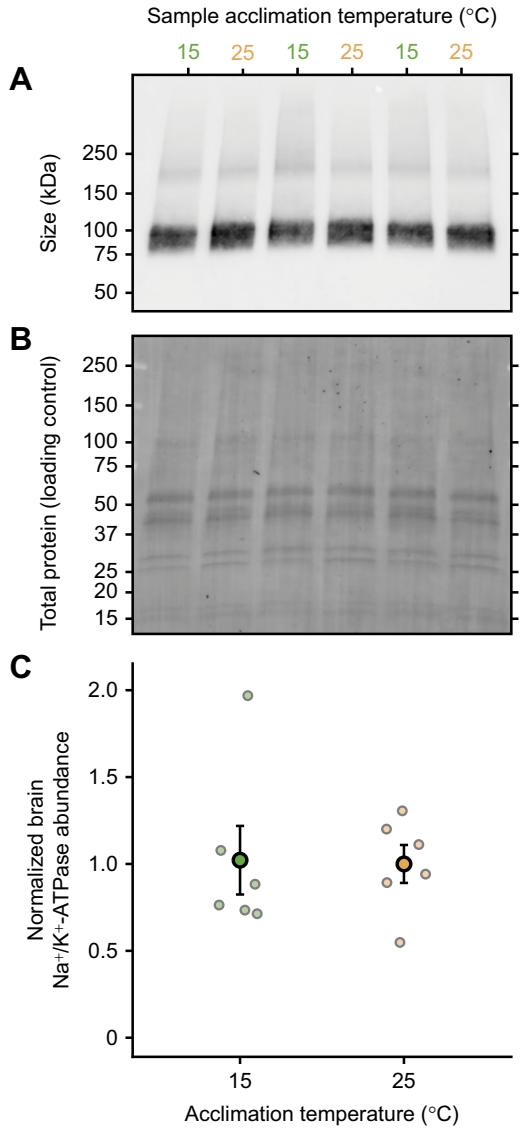

Fig. 6. Acclimation does not alter the abundance of the Na^+/K^+-ATPase α-subunit in the brain of *D. melanogaster*. (A) An example blot showing a prominent band at the expected size of ∼100 kDa. (B) Matching stain-free total protein blot used for normalizing the fluorescence signals from the bands in A to total protein load. (C) Comparing the Na^+/K^+-ATPase α-subunit abundance (normalized to the 25°C acclimated group) revealed no difference between the groups. *N*=6 per group as indicated by the small, translucent points. Group means are depicted as large opaque symbols. Error bars represent s.e.m.

Numerous studies from the 1960s and 1970s investigated how enzymes were adapted to their thermal environment (see Hazel and Prosse, 1974; Hochachka and Somero, 1973). The molecular mechanisms known to modulate innate activity of the Na^+/K^+-ATPase include post-translational modifications, differential transcription of Na^+/K^+-ATPase isoforms, changes in translation and an altered membrane environment (Therien and Blostein, 2000). The insect Na^+/K^+-ATPase has several phosphorylation sites (Emery et al., 1998), and depending on the state of these sites, the activity of the Na^+/K^+-ATPase can be greatly affected (Bertorello et al., 1991). Phosphorylation state of the pump has been linked to seasonal improvements in cold tolerance, albeit in a freeze-tolerant species (McMullen and Storey, 2008). Differential expression of Na^+/K^+-ATPase subunits or modifications to their localization could play critical roles in SD onset under stressful conditions (Blanco and Mercer, 1998; Clausen et al., 2016, 2017; Isaksen and Lykke-Hartmann, 2016; Reiffurth et al., 2020; Larkin et al., 2021). Indeed, Bayley et al. (2020) report that muscle of cold-acclimated locusts has a lower sensitivity to ouabain, which could indicate a change in isoform expression. Thus, similar modifications to the *D. melanogaster* Na^+/K^+-ATPase may underlie the variation in transport capacity and by extension susceptibility to SD (Andrew et al., 2022). Expression of specific Na^+/K^+-ATPase subunits has been investigated in relation to cold acclimation in *D. melanogaster* and a small increase in *nrv2* (a Na^+/K^+-ATPase β-subunit) expression was noted, albeit in whole-animal samples (MacMillan et al., 2015b). Lastly, thermal acclimation or adaptation often results in altered physical properties of the cell membrane that act to defend fluidity during cold exposure (see Hazel, 1995; Koštál, 2010). This is also true for *Drosophila* (Overgaard et al., 2005), and such changes to the membrane environment likely impact activity of the Na^+/K^+-ATPase (Else and Wu, 1999; Habeck et al., 2015).

Unaltered brain Na^+/K^+-ATPase abundance suggests changes to localization

Abundance of the Na^+/K^+-ATPase α-subunit did not differ between cold- and warm-acclimated flies, confirming that the differences in activity measured in the Na^+/K^+-ATPase assay were not related to Na^+/K^+-ATPase content (Fig. 6). It therefore seems most likely that the difference in activity was driven by one of the many factors described above. This is the first time abundance of the Na^+/K^+-ATPase in the brain has been related to a specific measure of insect cold tolerance, but a study on whole-body Na^+/K^+-ATPase α-subunit abundance also reported no difference between cold- and warm-acclimated flies (MacMillan et al., 2015b), and a study on the physiology underlying improved heat tolerance in locusts also found no difference (Hou et al., 2014). Conversely, Hou et al. (2014) found that localization of the Na^+/K^+-ATPase changed drastically following a heat-hardening treatment as more Na^+/K^+-ATPase was incorporated into the membranes of neuronal somata, perineurial cells and nerve axons. Similar observations were made in relation to locust cold hardening more recently (Robertson and Moyes, 2022). In the present study, we did not investigate localization, but we hypothesize that the reduced SD amplitude following cold acclimation could relate to translocation of thermally insensitive Na^+/K^+-ATPases to the adglial membrane of perineurial and subperineurial glia, as such a response would further serve to delay cold-induced SD (see next section).

Thermal acclimation alters the nature of the SD event

Properties of the SD event, and how those properties change with acclimation, can provide important insight into the molecular mechanisms driving changes in the event. Developmental acclimation of flies to 15°C not only lowered the temperature threshold for cold-induced SD onset but also decreased the amplitude and descending slope of the drop in transperineurial potential (see Figs 1 and 2). The transperineurial potential is the electrical potential generated across the blood–brain barrier and is the difference in potential generated across the basolateral (facing the haemolymph) and the adglial (facing the neurons) membranes of the glia (transperineurial=basolateral−adglial) (Schofield and Treherne, 1984). The general consensus is that the large shift in transperineurial potential during SD reflects a near-complete depolarization of the adglial membrane (Robertson et al., 2020). The lower SD amplitude observed for cold-acclimated flies could therefore reflect (1) a less polarized adglial membrane before SD,

(2) a more polarized membrane during SD, or (3) a combination of the two. Similar to us, Cheslock et al. (2021) found a reduced SD amplitude after cold acclimation, but they also found a more positive transperineurial potential at benign temperature, indicating a more polarized adglial membrane prior to SD. However, because both the basolateral and adglial membrane potentials affect the transperineurial potential, it is difficult to determine the exact origin of the change. We speculate that ionoregulatory changes play a key role in determining the abovementioned differences in transperineurial potential and SD amplitude. First, Na^+/K^+-ATPase activity generates a considerable electrogenic potential in insects (Wareham et al., 1974; Bayley et al., 2021) and glial Na^+/K^+-ATPase localization is therefore likely to affect transperineurial potential directly. Specifically, deployment of thermally insensitive Na^+/K^+-ATPases to the adglial membrane would tend to increase adglial polarization and make the transperineurial potential even more positive (as reported by Cheslock et al., 2021), and delay the onset of the SD (see above). Second, the smaller disruption of ion balance experienced by cold-acclimated flies during SD could leave the adglial membrane less depolarized (Andersen et al., 2018). Last, changes to interstitial K^+ clearance, glial K^+ shuttling and basolateral K^+ efflux could be involved (i.e. glial spatial buffering: Orkand et al., 1966; Smith and Shipley, 1990; Kocmarek and O'Donnell, 2011; Chen and Swale, 2018). Indeed, the hypothesized deployment of Na^+/K^+-ATPases to the adglial membrane would facilitate glial K^+ uptake. This could also partly explain the gentler descending slope in the transperineurial potential during SD onset (Fig. 2), as an improved capacity to buffer the initial increase in interstitial K^+ would tend to slow the progress of the event. Again, this is mainly speculation, and more research is needed to investigate the potential links between SD amplitude, adglial and basolateral membrane potentials, Na^+/K^+-ATPase activity, and glial spatial buffering. Furthermore, the two acclimation groups experience SD at different temperatures and we therefore cannot rule out direct effects of temperature on the SD amplitude and descending slope (see Figs 1C and 2B).

Conclusion

In summary, we demonstrate that phenotypic plasticity in the temperature leading to a loss of neural function of *D. melanogaster*, here estimated as the SD temperature, relates to the ability to maintain active transport capacity of the Na^+/K^+-ATPase in the brain at low temperature *in vivo* and *in vitro* without altering the overall abundance of the Na^+/K^+-ATPase. Considering the central role of the Na^+/K^+-ATPase in regulating extracellular ion homeostasis in the insect central nervous system, it is perhaps not surprising that its activity is essential for preventing SD when exposed to stressful cold. Nonetheless, our findings support a key role of Na^+/K^+-ATPase in modulating SD susceptibility, and adaptations promoting maintained active ion transport capacity therefore appear to be a key pathway by which cold tolerance can be improved at multiple levels of biological organization (see Overgaard et al., 2021). The molecular and/or cellular mechanism by which the Na^+/K^+-ATPase of cold-acclimated flies is able to maintain activity at low temperature remains unknown, but we suggest that future research should focus on investigating the roles of phosphorylation, transcript expression, sequence variation, cellular localization and the membrane environment. Furthermore, investigations into the cellular processes counteracted by the Na^+/K^+-ATPase (i.e. K^+ leak) as well as its upstream modulators of transport capacity (e.g. ATP production) would provide valuable insight into the complex mechanisms modulating its activity. Lastly, we speculate that the observed changes to SD parameters (amplitude and slope) relate to improved ionoregulatory capacity at low temperature and a hypothesized deployment of Na^+/K^+-ATPases to adglial membranes in the blood–brain barrier.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Data availability

All relevant data can be found within the article and its supplementary information. Further western blot analysis files are available from the authors upon request.

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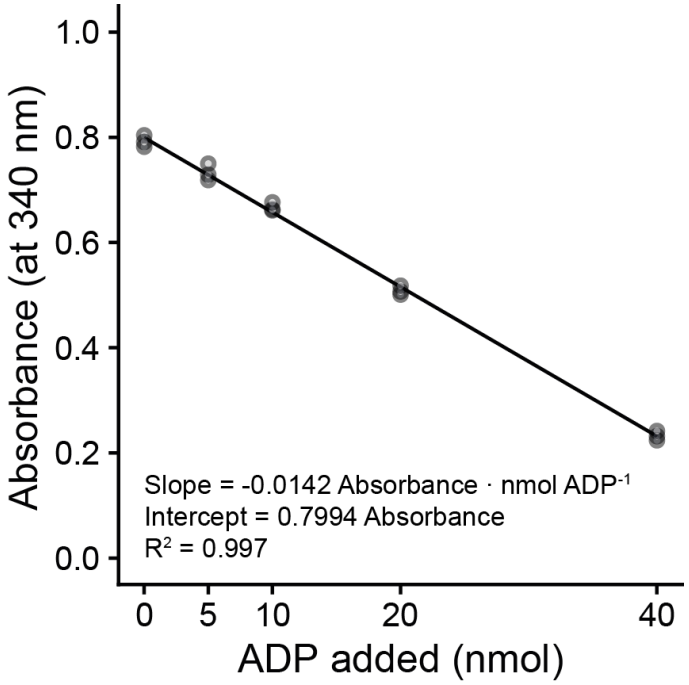


Fig. S1. Example standard curve used to estimate activity of the Na^+/K^+-ATPase. Activity of the Na^+/K^+-ATPase was estimated by measuring depletion of NADH. To translate disappearance of NADH to the production of ADP production by ATPases, we created a standard curve correlating absorbance (i.e. NADH concentration) to predetermined amounts of ADP produced (or added in the case of the standard curve).

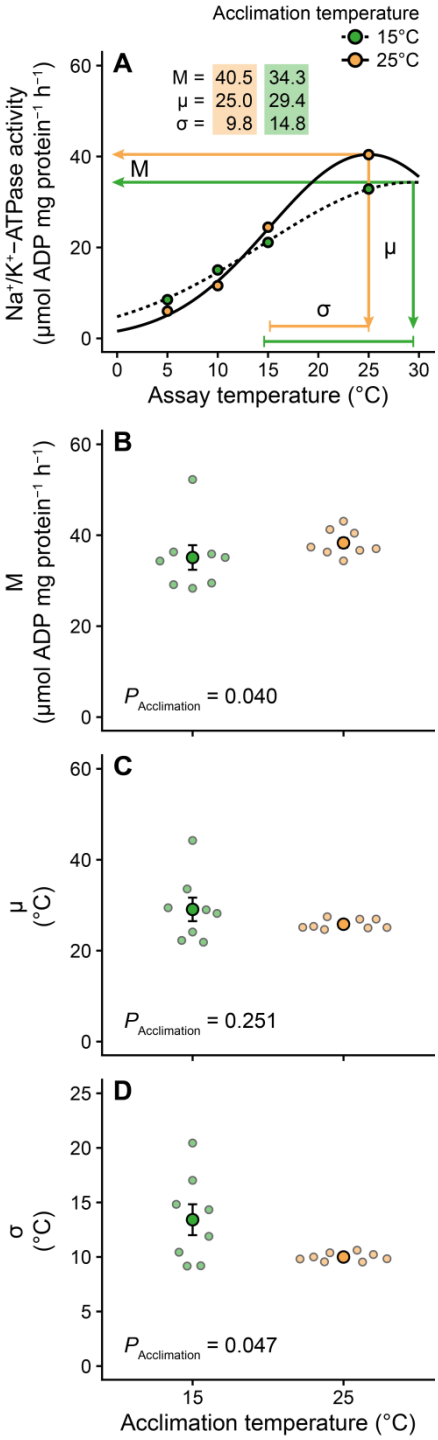


Fig. S2. Three-parameter Gaussian model estimates for the temperature-dependent activity of the Na^+/K^+-ATPase. Because the relation between temperature and Na^+/K^+-ATPase activity was not exponential we decided to also fit a three-parameter Gaussian model to more accurately describe the effects of temperature. (A) A visual representation of each parameter in the model superimposed on data from two samples. This analysis was performed for each sample such that means could be compared between the two acclimation groups. The analysis revealed that (B) cold acclimation led to a lower maximum activity of the Na^+/K^+-ATPase at the optimal temperature, (C) the temperature for optimal Na^+/K^+-ATPase was unaffected by acclimation temperature, and (D) the activity of the Na^+/K^+-ATPase was more resilient to lowered temperature after cold acclimation (larger σ denotes that a bigger change in temperature is needed to reduce activity). Note that the Gaussian model often (10/16 samples) relied on extrapolation beyond the data (more often in the cold-acclimated group, which might explain the higher degree of variation), as is evident from example data from a cold-acclimated fly in panel A. Error bars in panels B-D depict standard errors, and individual data points are spread out on the x-axis for data transparency.

Table S1.

Click here to download Table S1

Supplementary Materials and Methods

Data on image analysis of western blots

This file contains images of the data files used for densitometric quantification of Na^+/K^+-ATPase abundance. Raw data files can also be found in Dataset 1. For each western blot, lane information (sample ID, Acclimation group, amount of protein loaded into lane well, fluorescent signal, and total protein load signal) is listed along with the images from which these numbers were extracted (raw and with lanes marked for analysis in the Image Lab software).

Blot 1

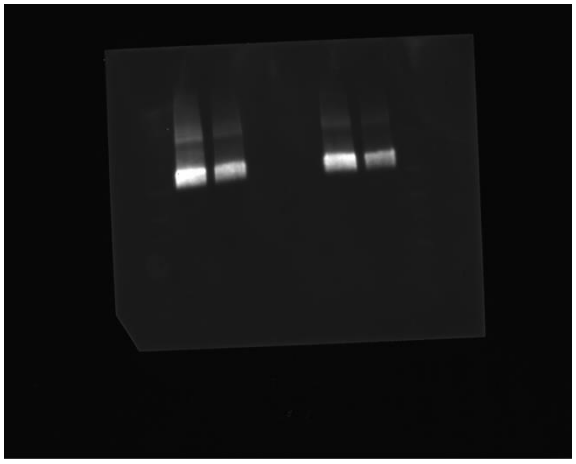
Lane information

Lane	1	2	3	4	5	6	7	8
Sample ID	Ladder	1	1	-	-	2	2	Ladder
Acclimation group	-	15°C	15°C	-	-	25°C	25°C	-
Protein loaded (µg)	-	4	2	-	-	2	1	-
Fluorescent signal (au)	-	496220816	308081770	-	-	416042676	200278658	-
Protein stain signal (au)	-	163596576	55601120	-	-	114112128	54065008	-

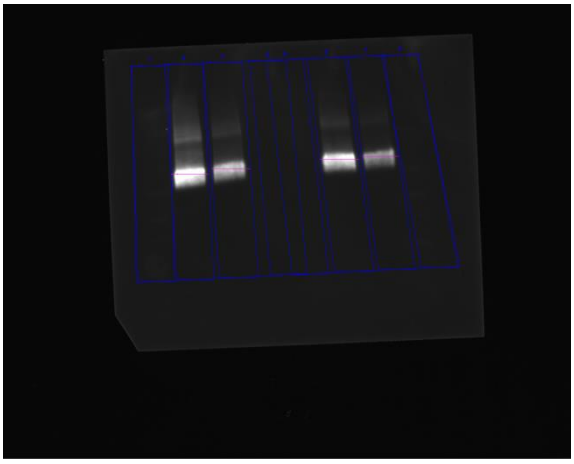
Note! From this particular blot, only data from lanes 3 and 6 were used for the manuscript. Lanes 2 and 7 contain samples with 4 and 1 µg of protein loaded, respectively, rather than the 2 µg loaded for 3 and 6, and were used to test if we needed to load more or less protein. This did not influence the signal from the other lanes as the exposure time was chosen to optimize the signal from lanes 3 and 6.

- **Fluorescent signal from secondary antibody bound to the a-subunit of the Na^+/K^+-ATPase**

Raw image

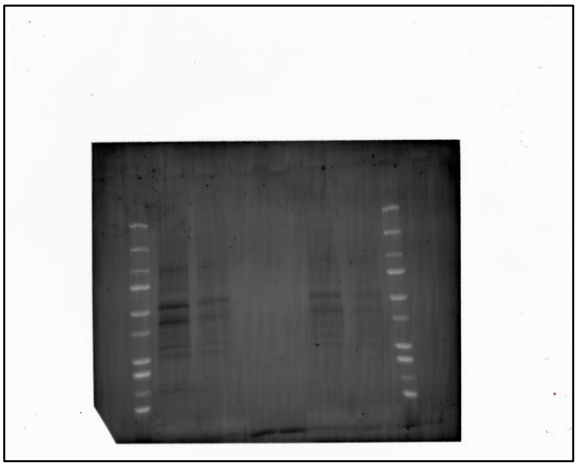


Lanes outlined

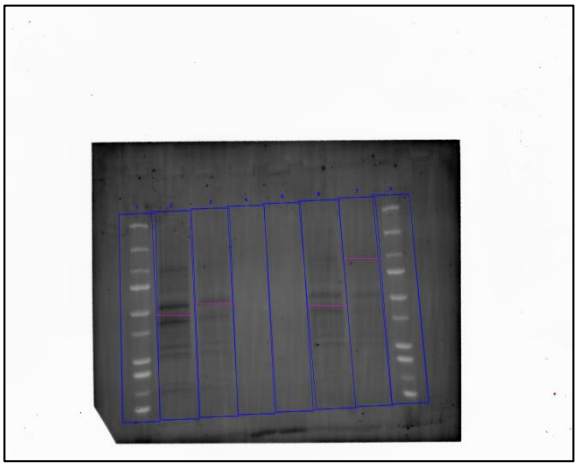


- **Signal from the stain free protein blot (for total protein estimation)**

Raw image



Lanes outlines



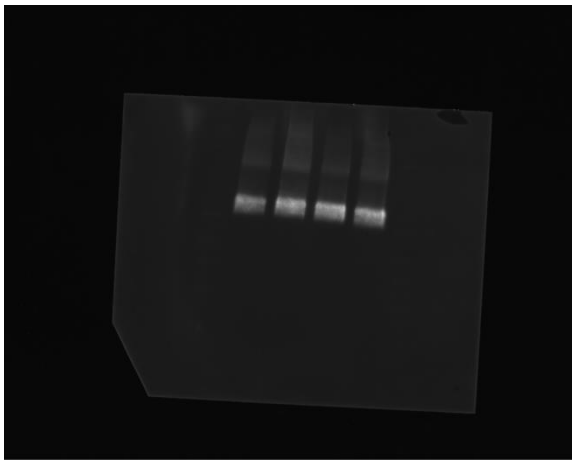
Blot 2

Lane information

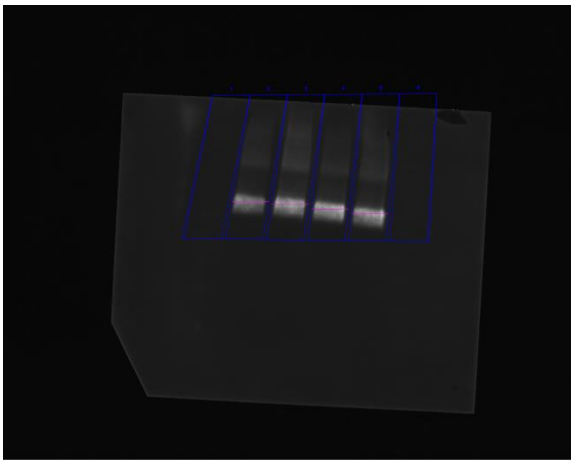
Lane		1	2	3	4	5	6	
Sample ID		Ladder	3	4	5	6	Ladder	
Acclimation group		-	25	15	25	15	-	
Protein loaded (µg)		-	2	2	2	2	-	
Fluorescent signal (au)		-	155363050	217314950	230634161	214989382	-	
Protein stain signal (au)		-	100805637	106308453	91258699	86500551	-	

- **Fluorescent signal from secondary antibody bound to the a-subunit of the Na^+/K^+-ATPase**

Raw image

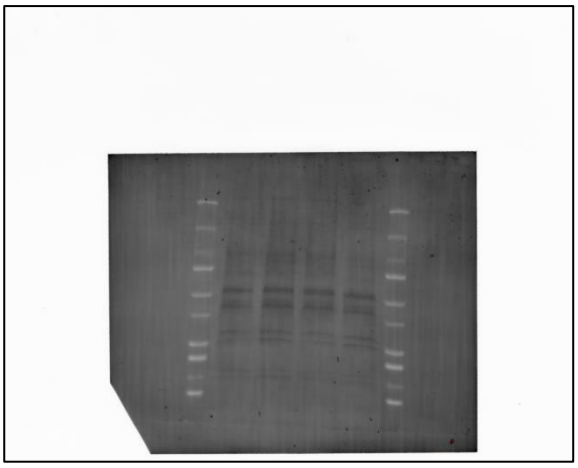


Lanes outlined

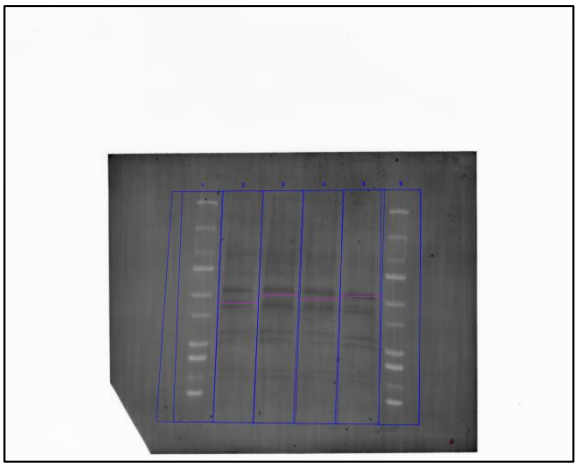


- **Signal from the stain free protein blot (for total protein estimation)**

Raw image



Lanes outlined



Blot 3

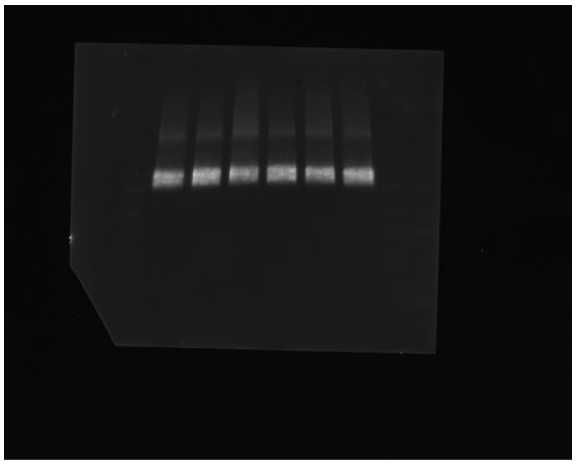
Lane information

Lane	1	2	3	4	5	6	7	8	9	10
Sample ID	-	Ladder	7	8	9	10	11	12	Ladder	Ladder
Acclimation group	-	-	15	25	15	25	15	25	-	-
Protein loaded (µg)	-	-	2	2	2	2	2	2	-	-
Fluorescent signal (au)	-	-	214965012	246832415	194767608	232042000	201616805	234666500	-	-
Protein stain signal (au)	-	-	71752240	92979338	90272302	74315460	100375541	69510903	-	-

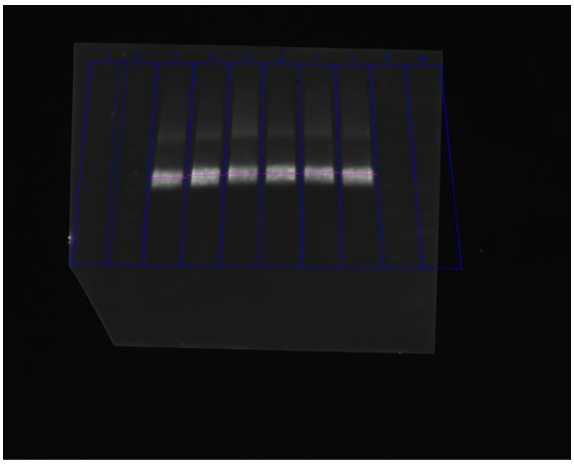
Note! These images are used for Fig. 6 in the main manuscript in slightly cropped versions. Furthermore, the colours have been reversed and light/dark balance changed for the full fluorescent blot for the sake of presentation. No further modifications were applied.

- **Fluorescent signal from secondary antibody bound to the a-subunit of the Na^+/K^+-ATPase**

Raw image

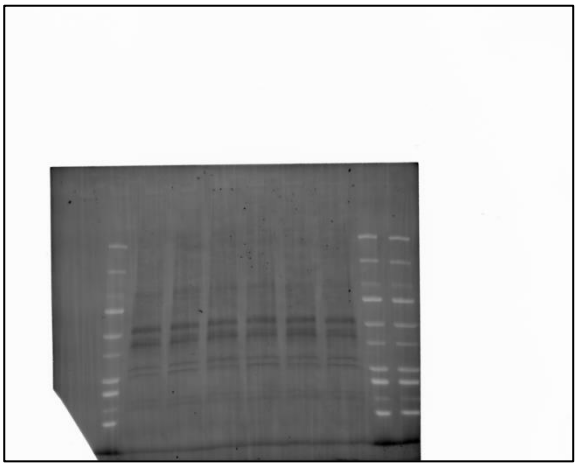


Lanes outlined



- **Signal from the stain free protein blot (for total protein estimation)**

Raw image



Lanes outlined