

## RESEARCH ARTICLE

# An impressive capacity for cold tolerance plasticity protects against ionoregulatory collapse in the disease vector *Aedes aegypti*

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## ABSTRACT

The mosquito *Aedes aegypti* is largely confined to tropical and subtropical regions, but its range has recently been spreading to colder climates. As insect biogeography is tied to environmental temperature, understanding the limits of *A. aegypti* thermal tolerance and their capacity for phenotypic plasticity is important in predicting the spread of this species. In this study, we report on the chill coma onset (CCO) and recovery time (CCRT), as well as low-temperature survival phenotypes of larvae and adults of *A. aegypti* that developed or were acclimated to 15°C (cold) or 25°C (warm). Cold acclimation did not affect CCO temperatures of larvae but substantially reduced CCO in adults. Temperature and the duration of exposure both affected CCRT, and cold acclimation strongly mitigated these effects and increased rates of survival following prolonged chilling. Female adults were far less likely to take a blood meal when cold acclimated, and exposing females to blood (without feeding) attenuated some of the beneficial effects of cold acclimation on CCRT. Lastly, larvae suffered from haemolymph hyperkalaemia when chilled, but cold acclimation attenuated the imbalance. Our results demonstrate that *A. aegypti* larvae and adults have the capacity to acclimate to low temperatures, and do so at least in part by better maintaining ion balance in the cold. This ability for cold acclimation may facilitate the spread of this species to higher latitudes, particularly in an era of climate change.

**KEY WORDS:** Chill tolerance, Ionoregulation, Mosquito, Thermal plasticity

## INTRODUCTION

The mosquito *Aedes aegypti* is abundant in tropical and subtropical regions where it is an arboviral disease vector for Zika, chikungunya, yellow fever and dengue (Bhatt et al., 2013; Kraemer et al., 2015, 2019). The global distribution of *A. aegypti* is closely related to environmental temperatures (Brady et al., 2013, 2014; Kraemer et al., 2019). This pattern suggests that like other small dipterans such as *Drosophila*, the inability of *Aedes* to survive cold winters in poleward latitudes limits its ability to colonize these areas (Andersen et al., 2015; Kellermann et al., 2012; Overgaard et al., 2014).

In recent years, however, *A. aegypti* and the closely related vector *Aedes albopictus* have spread through northeastern USA. Adults of

both species have even begun to appear in southern Ontario, Canada, early in spring (CBC News report 2017, <https://www.cbc.ca/news/canada/windsor/mosquito-responsible-for-majority-of-zika-infections-found-in-canada-for-first-time-1.4257135>), which suggests successful local overwintering of at least some adults, late-stage pupae, or eggs that were able to find warmer sheltered habitats and develop rapidly in the spring (Lima et al., 2016). The ability to overwinter in northern climates is completely at odds with our understanding of *A. aegypti* as a cold-intolerant species with little to no ability for seasonal quiescence or diapause (Diniz et al., 2017). Current climate models predict continued increases in average global temperatures and a greater frequency of extreme thermal events during winter (Easterling et al., 2000; Williams et al., 2015), but predictive models of *A. aegypti* distribution do not currently consider the possibility of phenotypic plasticity in this species (e.g. Kamal et al., 2018), because no such plasticity has been described.

Because of their global importance as disease vectors and their demonstrated potential for invasion, there is growing interest in understanding the limits of mosquito thermal tolerance. Low temperatures adversely affect life history traits throughout the *A. aegypti* life cycle, including development rate, reproductive success and survival (Carrington et al., 2013; Davis, 1931; Yang et al., 2009). The effects of temperature on *A. aegypti* developmental success appear quite pronounced, as survival from egg to adult drops from 92% at 20°C to only 3% at 15°C (Rueda et al., 1990). Of the different life stages of *A. aegypti*, the eggs appear tolerant to cold, surviving and hatching following cold exposures for up to 24 h at -2°C or 1 h at -17°C (Davis, 1931; Thomas et al., 2012). Accordingly, *A. aegypti* have been documented to successfully overwinter as far north as Washington DC and Indiana, and as far south as Buenos Aires, and there is evidence of cold adaptation occurring in these temperate populations (De Majo et al., 2017; Fischer et al., 2011; Hawley et al., 1989; Lima et al., 2016). Like many other poikilotherms, larval *A. aegypti* experience slowed development, delayed and decreased pupation, and increased mortality with decreasing temperatures (Brady et al., 2014; Carrington et al., 2013; De Majo et al., 2017; Tun-Lin et al., 2000; Yang et al., 2009). Similarly, adult *A. aegypti* experience increased mortality, decreased oviposition rate and overall reduced fecundity at 15°C (Tun-Lin et al., 2000). To date, however, studies of temperature effects on *A. aegypti* have largely focused on consequences to reproductive success, growth and development, and there has been little work focused on the extreme limits of thermal tolerance or the potential for thermal plasticity, particularly in later life stages. This gap in knowledge represents a considerable risk, particularly considering recent reports from *Drosophila* that thermal limits may better predict species distribution and abundance than optimal temperatures or

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rates of growth and reproduction at more favourable temperatures (MacLean et al., 2019; Overgaard et al., 2014).

*Aedes aegypti* is a chill-susceptible insect, meaning it succumbs to the effects of exposure to low temperatures well above the freezing temperature of its bodily fluids. The cold tolerance of chill-susceptible insects can vary widely, both among and within species. Broad differences in basal cold tolerance can exist among populations or species (Gibert et al., 2001; Kellermann et al., 2012; Vorhees et al., 2013; Warren and Chick, 2013), and many insects can also drastically alter their cold tolerance within their lifetime. For example, insects can undergo thermal acclimation in response to chronic low-temperature exposure, or rapidly harden in response to an acute temperature change (e.g. rapid cold hardening) (Colinet and Hoffmann, 2012; Hoffmann et al., 2003; Kellermann et al., 2012; Kelty and Lee, 2001; Sinclair et al., 2006). To date, a capacity for thermal plasticity at low temperature (cold acclimation) has been demonstrated in many chill-susceptible insects, such as fruit flies, cockroaches, locusts and crickets (Andersen et al., 2017a; Coello Alvarado et al., 2015; Colinet and Hoffmann, 2012; Košťál et al., 2006).

Cold acclimation typically affects a variety of cold-tolerance phenotypes in chill-susceptible insects. For example, cold-acclimated insects commonly have a lower temperature of chill coma onset (CCO; see below), more rapidly recover from chill coma following rewarming (a lower chill coma recovery time, CCRT), and avoid the development of cold-induced injury better than warm-acclimated conspecifics (Coello Alvarado et al., 2015; MacMillan et al., 2015a; Ransberry et al., 2011). While little is known about cold acclimation in *A. aegypti*, eggs of *A. albopictus* have increased cold tolerance following cold acclimation (Hanson and Craig, 1995). The magnitude of cold plasticity can vary among and within populations (Nyamukondiwa et al., 2011; Sørensen et al., 2016). In the case of *A. albopictus*, cold acclimation was only noted in temperate populations and not tropical populations, so this capacity for plasticity is thought to be facilitating the northward expansion of the species' range (Hanson and Craig, 1995; Rochlin et al., 2013; Romi et al., 2006).

While tolerance to extreme cold relies on a physiological capacity to avoid or survive ice formation inside the body, tolerance to chilling requires a physiological capacity to resist the effects of low temperature per se on organ, tissue and cellular biochemistry (MacMillan, 2019; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013). Consequently, measures of cold tolerance relevant to freeze-avoidant and freeze-tolerant insects, such as the supercooling point (the temperature of spontaneous ice formation within the body) or survival following freezing, are irrelevant to characterizing the thermal limits of chill-susceptible insects (Overgaard and MacMillan, 2017). When cooled below a critical threshold temperature, chill-susceptible insects suffer a local loss of ion homeostasis in the nervous system, leading to nerve depolarization (spreading depression) and a state of complete neuromuscular silence termed chill coma (MacMillan and Sinclair, 2011a; Mellanby, 1939; Robertson et al., 2017). The temperature at which this paralytic state occurs is called the chill coma onset (CCO) temperature (Overgaard and MacMillan, 2017). With time spent at low temperatures, chill-susceptible insects lose ion and water balance and suffer from haemolymph hyperkalaemia (high  $[K^+]$ ), which further depolarizes cells and activates voltage-gated calcium channels, driving rampant cellular apoptosis (Bayley et al., 2018; MacMillan et al., 2015b,c). The severity of this loss of homeostasis increases with longer or lower temperature exposures, and the tissue damage that accrues while an insect is in this state is

thought to largely determine its survival and fitness following rewarming (Overgaard and MacMillan, 2017). Species that are more cold tolerant, or individuals that have acclimated to low temperatures, are better able to maintain ion and water balance during cold exposure (Andersen et al., 2017a; Coello Alvarado et al., 2015; Košťál et al., 2006; MacMillan et al., 2015a).

Here, we used a laboratory-bred population of *A. aegypti* to determine chill coma onset and recovery phenotypes of larvae and adults of this species. We allowed larval and adult mosquitoes to undergo either warm (25°C) or cold acclimation (15°C) to test whether this species is capable of acclimating to sub-optimal thermal conditions. Cold acclimation led to significant changes in the cold tolerance of both larvae and adults, so we used larvae to test whether improvements in cold tolerance following cold acclimation are driven by an improved ability to maintain ion balance in the cold.

## MATERIALS AND METHODS

### Animal husbandry

A colony of *Aedes aegypti* mosquitoes (Linnaeus) was established in 2007 at York University, Toronto, from eggs provided by M. Patrick (San Diego, CA, USA) and supplemented with eggs from Liverpool strain provided by C. Lowenberger (Simon Fraser University, Burnaby, BC, Canada). Our mosquitoes were reared as described by Misyura et al. (2017) with slight modifications. Briefly, eggs were hatched in 2 l of dechlorinated tap water (water changed every 4 days) and fed 6 ml of a premade food solution composed of 1.8 g liver powder and 1.8 g of inactive yeast in 500 ml of reverse-osmosis water daily. The population was maintained at room temperature (22±1°C) with a 12 h:12 h light:dark cycle.

To obtain larvae for experiments, eggs were added to 1.5 l of dechlorinated tap water with 2 ml of the liver–yeast diet. Containers of water and eggs were kept in a 25±0.5°C incubator (12 h:12 h light:dark). The next day, hatching was confirmed through visual inspection and 2 ml of food was added. One day later, all larvae in each bin were randomly assigned to one of two larval acclimation treatments, 15 or 25°C, such that the two treatments had approximately equal numbers of larvae. The larvae assigned to each treatment were transferred to a new container filled with 1.5 l dechlorinated tap water and 2 ml food and placed in either the 15 or 25°C incubator. Larvae were fed 2 ml liver–yeast food mix every day until the first pupa was spotted. Water was changed as needed, with food always added after a water change. When the first pupae were observed, 4th instar larvae were collected to be used in experiments.

To acclimate adults for experiments, pupae (reared under standard colony conditions as described above) were isolated daily and given 1–2 days to mature prior to the placement of 40±10 pupae in small open-top containers with ~60 ml of dechlorinated tap water. The open-top containers were then placed within custom-made (18 cm long×15 cm wide×10 cm tall) enclosed containers (with a netted section to allow for air flow), allowing the pupae to emerge over a period of 48 h. A premade sugar–water solution (40 g of sucrose in 250 ml of tap water) was placed in each container to allow adults to feed. Following 48 h for emergence, any remaining pupae were removed, and the containers were separated into two different acclimation treatments: cold acclimation (15°C) and warm acclimation (25°C). This ensured all adults were 1–2 days old upon the initiation of the acclimation treatments. Both acclimation groups were maintained on a 12 h:12 h light:dark cycle. Adult mosquitoes were left at their respective acclimation temperatures for 5 days, and thus all adults were 6–7 days post-emergence when used in experiments.

## CCO

To assess CCO temperature, individual larvae were collected from the larval acclimation treatments using a pipette and transferred to 4 ml glass vials along with 2 ml of their rearing water. The vials were affixed to a custom-made aluminium rack that was submerged in a glass aquarium containing a 1:1 mixture of ethylene glycol and water, which was circulated by a programmable refrigerated bath (Model AP28R-30, VWR International, Mississauga, ON, Canada). The temperature of the bath was independently monitored with a pair of type-K thermocouples connected to a computer running Picolog (version 5.25.3) via a Pico TC-07 interface (Pico Technology, St Neots, UK). The larvae were held at 20°C for 15 min then the temperature was ramped down at 0.1°C min<sup>-1</sup>. We recorded the temperature at which each larva completely stopped responding to vibrational and light stimuli. As larvae would often ignore a stimulus during one scan only to respond strongly on the next, all larvae, including those that had been recorded as being in chill coma, were tested for a response throughout the experiment to ensure the accuracy of the recorded CCO temperature.

Adult mosquitoes from the 25 and 15°C acclimation groups were briefly anaesthetized under CO<sub>2</sub>, placed in 4 ml glass vials (filled with ambient air) and affixed to a rack that was submerged in a temperature-controlled bath, as described above for the larvae. To record adult CCO, the temperature of the bath was initially set to 25°C for 15 min and then ramped down at a rate of 0.13°C min<sup>-1</sup> while mosquito movement was continuously monitored. The temperature at which movement stopped following perturbation with a plastic probe was recorded as the adult mosquito CCO.

## CCRT

To measure CCRT, larvae were exposed to 2°C for 4, 8, 12 or 16 h. Larvae were cold exposed by transferring each individual to a 1.5 ml open centrifuge tube and incubating the tubes in a refrigerated centrifuge (Thermo Scientific™ Sorvall Legend™ Micro 21R) set to 2°C (temperature was confirmed via independent thermocouples and chosen based on prior trials). After exposure to the cold, each larva was transferred to its own 6.7 cm diameter plastic container, filled with 50 ml room temperature (22°C) dechlorinated water. A timer was set immediately upon placement of the larva into room temperature water. CCRT was assessed as the time taken for the larva to swim a continuous distance of 2 cm (measured using a 1 cm<sup>2</sup> grid lining the bottom of the container). Larvae that could not swim 2 cm within 2 h were considered to have suffered severe injury. Chilling injury was measured as the inability to resume use of the siphon, where larvae that could not use their siphon to ventilate within 2 h were counted as injured.

CCRT was determined in adult mosquitoes following 6 h at 2°C. Individual mosquitoes were sexed and placed in 4 ml enclosed glass containers at room temperature (22±1°C) and observed for 120 min. The duration of time required for a mosquito to stand on all six legs following its removal from the cold was recorded as its CCRT. To assess the effect of blood feeding on CCRT, sugar–water mixture was removed from the cages of warm-acclimated mosquitoes 24 h before blood feeding. Mosquitoes were exposed to warm sheep's blood for 20 min through a thinly stretched Parafilm membrane. The mosquitoes were then given a 0, 40 or 160 min (or alternatively 20, 60 or 180 min from the onset of blood feeding) period prior to the initiation of the cold treatment of 6 h at 2°C. Mosquitoes that did not feed during the 20 min blood exposure period were used as an internal control.

## Low-temperature survival

To measure low-temperature survival of the larvae, individual larvae were isolated into microcentrifuge tubes containing dechlorinated tap water and groups of 24 larvae were exposed to -4, -2, 0, 2, 5 or 10°C for 24 h using a refrigerated centrifuge as described for CCRT. Importantly, the water containing the larval mosquitoes was never observed to freeze under any of these conditions (i.e. the water supercooled). After exposure to the cold, larvae were kept at room temperature for an additional 24 h, and then survival proportion was recorded. Larvae that were able to move when disturbed were counted as alive.

Chilling survival was assessed in adult mosquitoes following 6 h exposure to temperatures between -4 and 2°C. Groups of mosquitoes inside rearing cages were placed inside an incubator pre-cooled to the desired temperature. The exposure temperature varied somewhat between the two acclimation groups to include temperatures that result in survival proportions ranging from 0% to 100%. To this end, cold-acclimated mosquitoes were exposed to -4, -3, -2.5, -2, -1, 0, 1 and 2°C and warm-acclimated mosquitoes were exposed to -2, -1, -0.5, 0, 1 and 2°C. Immediately upon removal from the cold exposure, mosquitoes were individually isolated into 4 ml enclosed glass containers and left at room temperature (22±1°C) for 18 h to recover. Following this, the mosquitoes were assessed such that those that were able to stand were considered alive while those that were unable to stand were considered dead.

## Haemolymph ion concentration

To quantify Na<sup>+</sup> and K<sup>+</sup> concentrations in larval haemolymph, we used the ion-selective microelectrode (ISME) technique. Control larvae were sampled directly from their rearing conditions, while cold-exposed larvae were first exposed to 24 h at 0°C (using a refrigerated centrifuge as described for CCRT), before haemolymph was sampled and immediately measured. Haemolymph was collected by first securing larvae onto lids from 35 mm×10 mm sterile Petri dishes using Murray's<sup>®</sup> pure beeswax. Each larva was immobilized by applying beeswax to the head and terminal segment. A drop of paraffin oil was then applied to the abdomen, and the cuticle of this region was lightly sheared open with a sharp-pointed metal pin. The emerging droplet of haemolymph was collected and placed under mineral oil in a Petri dish coated with a silicone elastomer using a micropipette.

Custom-made ISMEs were constructed and used following previously described methods (Jonusaite et al., 2011). Briefly, borosilicate glass capillaries were pulled to a tip diameter of ~3 µm using a micropipette puller (Flaming Brown P-97, Sutter Instruments, Novato, CA, USA), heated to 300°C and exposed to *N,N*-dimethyltrimethylsilylamine vapour for 1 h. Potassium-sensitive electrodes were backfilled with 100 mmol l<sup>-1</sup> of KCl and front-filled with K<sup>+</sup> ionophore (K<sup>+</sup> ionophore I, cocktail B; Sigma-Aldrich, St Louis, MO, USA). Sodium-sensitive electrodes were backfilled with 100 mmol l<sup>-1</sup> NaCl and front-filled with Na<sup>+</sup> ionophore (Na<sup>+</sup> ionophore II cocktail A; Sigma-Aldrich). The circuit was completed with a reference electrode pulled from filamented glass capillary and back-filled with 500 mmol l<sup>-1</sup> KCl. Signal information was relayed to a PowerLab 4/30 data acquisition device (ADInstruments, Sydney, NSW, Australia) and interpreted by LabChart 6 software (ADInstruments). Voltages obtained from the haemolymph samples were compared with those from calibration solutions of known concentration, and the Nernst slope was applied to determine haemolymph ion concentration ([X])

using the following formula:

$$[X] = C_0 \times 10^{\frac{V-V_0}{S}}, \quad (1)$$

where  $C_0$  is the lower calibration concentration in  $\text{mmol l}^{-1}$ ,  $V$  is the voltage (mV) reading from the haemolymph sample,  $V_0$  is the voltage (mV) reading of the lower calibration concentration, and  $S$  is the slope of the electrode (mV), which is the difference in voltage between the two calibration solutions that differ in concentration by a factor of 10. The following calibration solutions were used:  $\text{Na}^+ - 20 \text{ mmol l}^{-1} \text{ NaCl}/180 \text{ mmol l}^{-1} \text{ LiCl}$ , and  $200 \text{ mmol l}^{-1} \text{ NaCl}$ ; and  $\text{K}^+ - 0.5 \text{ mmol l}^{-1} \text{ KCl}/49.5 \text{ mmol l}^{-1} \text{ LiCl}$ ,  $5 \text{ mmol l}^{-1} \text{ KCl}/45 \text{ mmol l}^{-1} \text{ LiCl}$  and  $50 \text{ mmol l}^{-1} \text{ KCl}$ .

### Data analysis

R (version 3.6.1, <http://www.R-project.org/>) was used to complete all data analyses. Larval CCO temperatures were compared between acclimation groups using a one-way ANOVA and CCRT with a generalized linear model (GLM) with exposure time and acclimation temperature as factors. Rates of injury in larval mosquitoes following the CCRT assays were compared using a two-way ANOVA with acclimation temperature and duration of cold exposure included as factors. Adult CCO and CCRT were both compared using two-way ANOVA (with acclimation temperature and sex as factors). The effect of time since exposure to blood on CCRT in adult mosquitoes was analysed for each acclimation group independently (because cold-acclimated mosquitoes did not feed) using GLMs. Feeding status and time since exposure were included as factors for warm-acclimated mosquitoes, and only time since exposure for cold-acclimated mosquitoes. Survival following cold stress was analysed for each life stage using GLMs with a binomial error distribution and a logit-link function. Acclimation treatment, temperature and replicate experimental run were included as factors for larval survival, and sex, acclimation treatment and temperature were included for adults. Initial models were saturated with all potential interactions and were reduced to find the most parsimonious model based on Akaike's information criterion ( $\Delta\text{AIC}>2$ ). Raw data are provided in Dataset 1.

## RESULTS

### CCO

Larval acclimation did not significantly affect CCO temperature ( $F_{1,67}=1.3$ ,  $P=0.265$ ); both warm- and cold-acclimated larvae had a

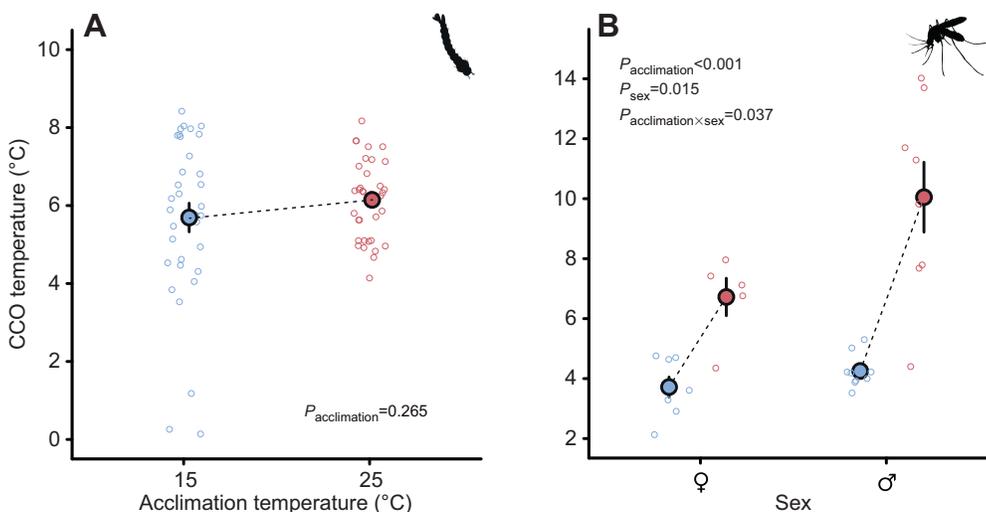
CCO of  $\sim 6^\circ\text{C}$  (Fig. 1A). The majority of larvae were observed to sink to the bottom of the glass vials upon entering chill coma. In contrast, cold acclimation strongly reduced CCO temperature in both male and female adult mosquitoes (Fig. 1B; main effect of acclimation:  $F_{3,27}=48.5$ ,  $P<0.001$ ). The magnitude of this effect differed between the sexes; while the mean female CCO differed by  $\sim 3^\circ\text{C}$ , that of males differed by  $\sim 6.4^\circ\text{C}$  (Fig. 1B; interaction between acclimation status and sex:  $F_{3,27}=4.8$ ,  $P=0.037$ ). In general, females tended to have a lower CCO temperature than males (main effect of sex:  $F_{3,27}=6.8$ ,  $P<0.014$ ).

### Chill coma recovery and injury

Larval acclimation strongly impacted CCRT following exposure to  $2^\circ\text{C}$  in larval mosquitoes. Acclimation temperature and exposure duration interacted to determine CCRT (Fig. 2A;  $F_{3,552}=20.6$ ,  $P<0.001$ ), such that increasing the duration of cold exposure led to longer recovery times in warm-acclimated but not cold-acclimated larval mosquitoes. In addition to increases in mean recovery time, CCRT became increasingly variable in warm-acclimated mosquitoes with increasing duration of cold stress, but the same was not true in the cold-acclimated conspecifics (Fig. 2A). The proportion of larvae that could resume use of their siphon 2 h following recovery was examined in the same individuals (an index of chilling injury). Warm-acclimated larvae suffered clear chilling injury (roughly 30% after 12 or 16 h at  $2^\circ\text{C}$ ), while only slight chilling injury ( $\sim 2\%$ ) was noted in the cold-acclimated larvae following the same exposures (Fig. 2B; interaction between acclimation and exposure duration:  $F_{3,20}=10.0$ ,  $P=0.005$ ).

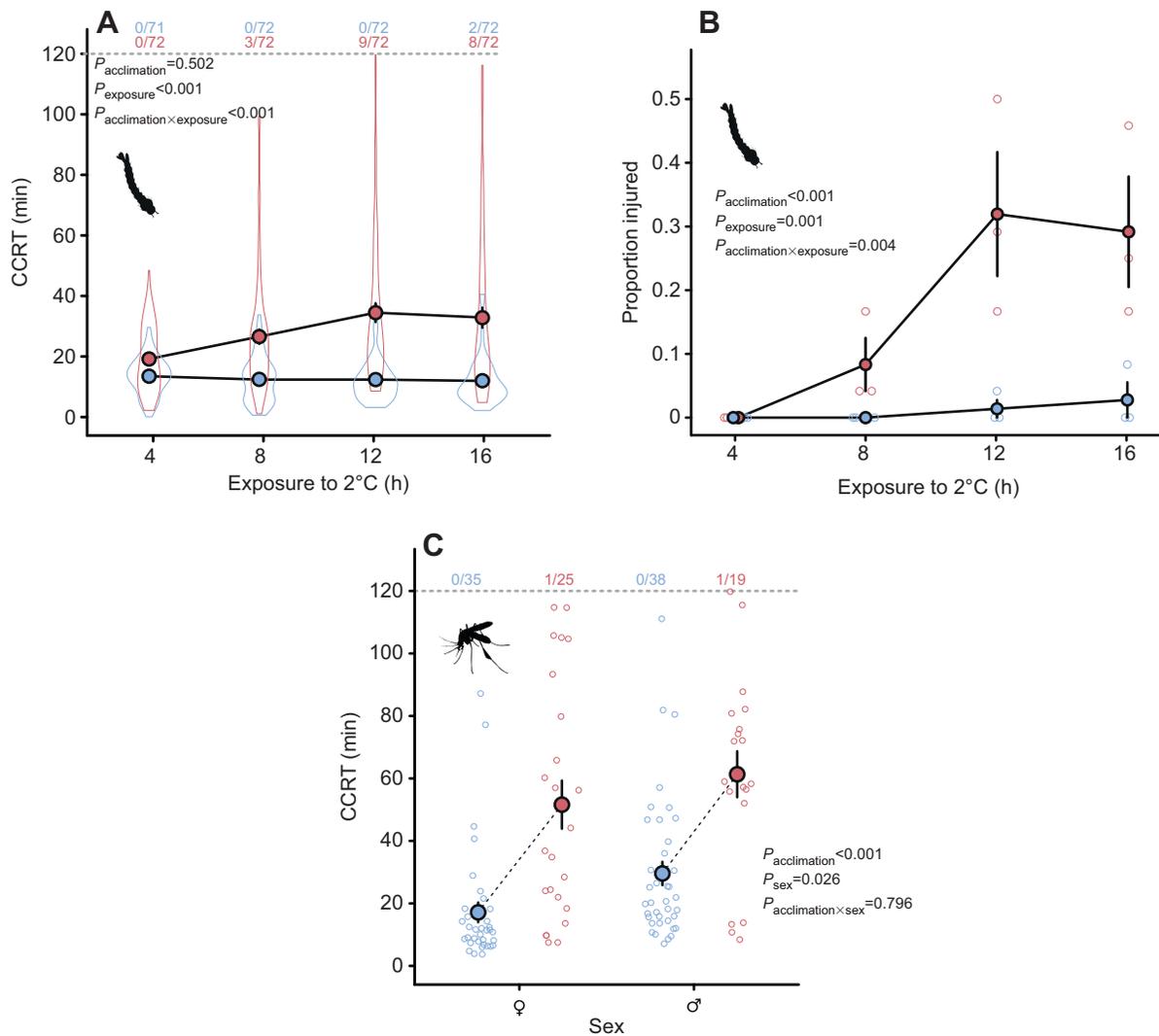
Cold acclimation also significantly improved rates of chill coma recovery in adult mosquitoes; cold-acclimated mosquitoes recovered from chill coma after 6 h at  $0^\circ\text{C}$  approximately 25 min faster than the warm-acclimated conspecifics (Fig. 2C; main effect of acclimation:  $F_{3,112}=38.1$ ,  $P<0.001$ ). As with CCO, females appeared more cold tolerant based on CCRT, and recovered  $\sim 10$  min faster than males (on average) from the same cold stress (main effect of sex:  $F_{3,112}=5.1$ ,  $P=0.026$ ). Unlike CCO, there was no interactive effect of sex and acclimation temperature on chill coma recovery ( $F_{3,112}=0.01$ ,  $P=0.796$ ), as cold acclimation improved adult CCRT by the same degree ( $\sim 32$ – $34$  min) regardless of sex (Fig. 2C).

Warm-acclimated mosquitoes were far more likely to take a blood meal when it was offered (Fig. 3A;  $t=26.3$ ,  $P<0.001$ ); only two cold-acclimated females (out of 65 that were offered it) voluntarily fed on blood within 20 min (Fig. 3A). For cold-acclimated mosquitoes that



**Fig. 1. Chill coma onset in cold- and warm-acclimated *Aedes aegypti*.**

Chill coma onset (CCO) temperature of larval (A) and adult (B) *Aedes aegypti* acclimated to warm ( $25^\circ\text{C}$ ; red) and cool ( $15^\circ\text{C}$ ; blue) conditions. Open circles represent individual mosquitoes and filled circles represent the mean ( $\pm$ s.e.m.) for each acclimation group and life stage. Error bars that are not visible are obscured by the symbols.  $n=34$ – $35$  larvae per acclimation group and  $n=5$ – $9$  adults per sex and acclimation group.



**Fig. 2. Cold acclimation accelerates chill coma recovery and prevents chilling injury in *A. aegypti*.** (A) Chill coma recovery time (CCRT) of larval mosquitoes. Filled circles are means ( $\pm$ s.e.m.); violin plots represent sample distribution (owing to large sample size). (B) Incidence of chilling injury in larval *A. aegypti*. Larvae were exposed to 2°C for one of four different durations (x-axis). Open circles represent the proportion of mosquitoes injured (unable to resume siphon use within 2 h) in three independent trials; filled circles are means ( $\pm$ s.e.m.). (C) CCRT of adult mosquitoes following 6 h at 2°C. Open circles represent individual adult mosquitoes; filled circles are means ( $\pm$ s.e.m.). *Aedes aegypti* were acclimated to warm (25°C; red) and cool (15°C; blue) conditions. Ratios above the dashed lines in A and C represent the number of individuals that did not recover from chill coma within the observation period (120 min). Error bars that are not visible are obscured by the symbols.  $n=71$ –72 larval mosquitoes per acclimation group and treatment in A and B;  $n=19$ –35 adult mosquitoes per sex and acclimation group in C.

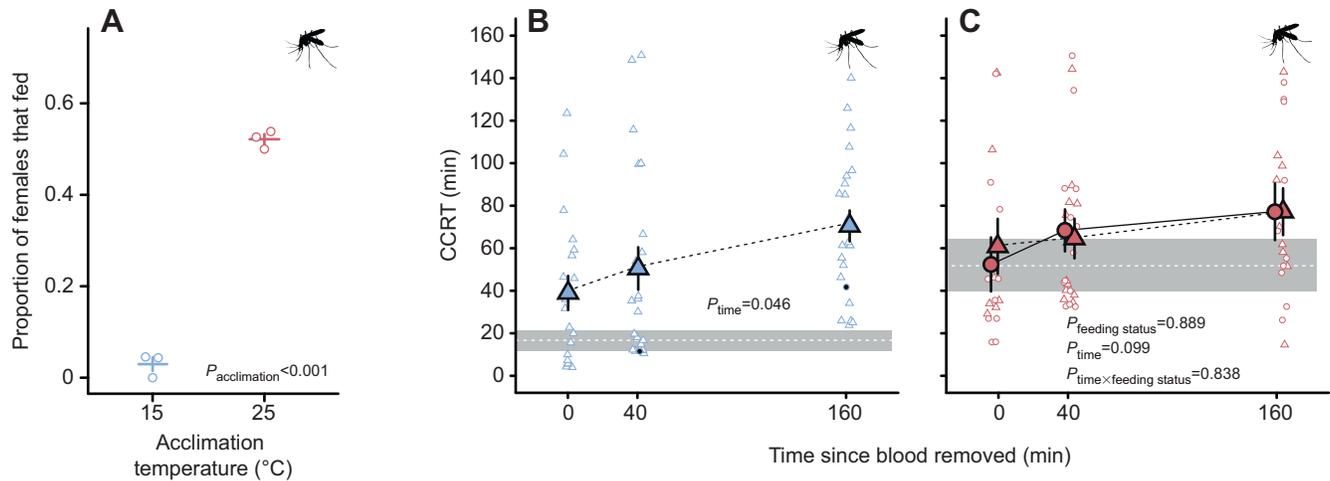
did not feed, exposure to a blood meal still impacted CCRT, as increasing time since exposure to the blood led to longer recovery times (Fig. 3B;  $F_{1,61}=4.1$ ,  $P=0.046$ ). For warm-acclimated mosquitoes, the act of blood feeding had no effect on CCRT (Fig. 3C; main effect of feeding status:  $F_{1,61}=0.2$ ,  $P=0.889$ ), and although there was a slight tendency for CCRT to increase with time since the blood was offered, this effect was not statistically significant (main effect of time:  $F_{1,61}=2.8$ ,  $P=0.100$ ), and there was no significant interactive effect of feeding status and time on CCRT (Fig. 3C;  $F_{1,61}=0.1$ ,  $P=0.838$ ).

#### Low-temperature survival

The most parsimonious model for larval survival retained the interaction between exposure temperature and acclimation temperature, which significantly interacted to determine survival ( $z=2.6$ ,  $P=0.010$ ). Temperature strongly influenced larval survival

in both acclimation groups (main effect of temperature:  $z=9.5$ ,  $P<0.001$ ), with larvae exposed to lower temperatures suffering higher mortality (Fig. 4A). Cold-acclimated larvae survived 24 h exposure to lower water temperatures ( $LT_{50}=-1.64\pm 0.23^\circ\text{C}$ ) than their warm-acclimated conspecifics ( $LT_{50}=0.81\pm 0.18^\circ\text{C}$ ; main effect of acclimation temperature:  $z=7.5$ ,  $P<0.001$ ; Fig. 4A).

The most parsimonious model of adult survival at low temperatures eliminated all interactions between acclimation temperature, exposure temperature and sex, but retained all of these variables as independent effects. Adult mosquitoes exposed to lower temperatures suffered greater mortality (main effect of exposure temperature:  $z=12.0$ ,  $P<0.001$ ), and as was the case for both CCO and CCRT, adult female mosquitoes were consistently more cold tolerant than males (Fig. 4B; main effect of sex:  $z=2.4$ ,  $P=0.014$ ). For both sexes, cold-acclimated adults survived at lower temperatures than warm-acclimated adults (Fig. 4B; main effect of acclimation



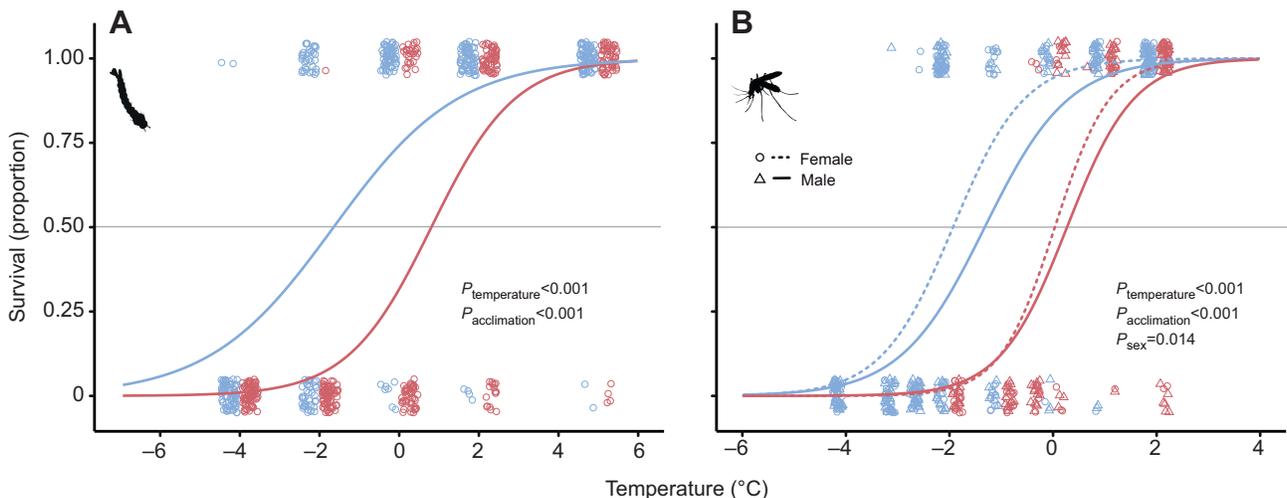
**Fig. 3. Blood feeding and CCRT in cold- and warm-acclimated adult *A. aegypti* females.** (A) Proportion of females that fed following acclimation to cold (15°C; blue) and warm (25°C; red) temperatures. (B,C) CCRT following blood feeding of cold- (B; 15°C) and warm-acclimated (C; 25°C) adults. Mosquitoes of both acclimation groups were offered warm sheep's blood for 20 min before the blood was removed. Individual mosquitoes were then given 0 min or an additional 40 or 160 min at room temperature before they were exposed to 0°C for 6 h. Triangles represent mosquitoes that chose not to feed on the blood while circles represent those that did feed. Open symbols represent CCRT values of individual adult mosquitoes. The two small filled circles in B represent the two cold-acclimated mosquitoes that took a blood meal (see Results). In all cases, filled blue and red symbols represent the mean ( $\pm$ s.e.m.). Error bars that are not visible are obscured by the symbols.  $n=19$ – $26$  female adults per acclimation group and time point.

temperature:  $z=7.1$ ,  $P<0.001$ ). Cold acclimation shifted the female  $LT_{50}$  (following 6 h cold exposure) from  $0.0\pm 0.19$  to  $-1.9\pm 0.15$ °C and the male  $LT_{50}$  from  $0.3\pm 0.18$ °C to  $-1.3\pm 0.19$ °C (Fig. 4B).

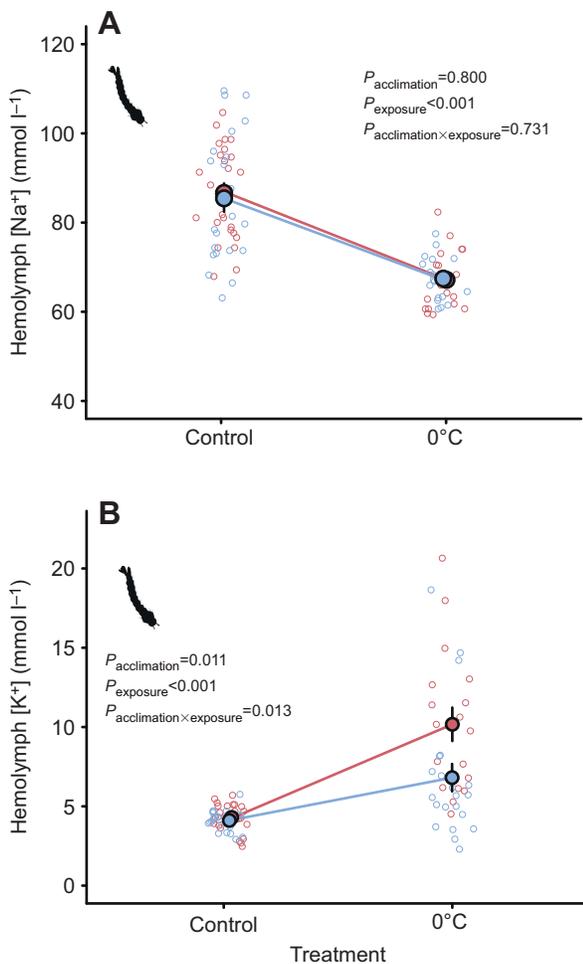
#### Haemolymph ion balance

Exposure to 0°C for 24 h caused both warm- and cold-acclimated larvae to lose haemolymph  $[Na^+]$  balance. The two acclimation groups had similar haemolymph  $[Na^+]$  prior to cold stress, and cold stress caused haemolymph  $[Na^+]$  to significantly decrease in both groups (Fig. 5A; main effect of cold exposure:  $F_{3,83}=74.1$ ,  $P<0.001$ ). There was no main effect of acclimation treatment on  $[Na^+]$  ( $F_{3,83}=0.1$ ,  $P=0.800$ ), nor any interaction between acclimation treatment and cold exposure ( $F_{3,83}=0.1$ ,  $P=0.731$ ). Cold exposure

also caused both warm- and cold-acclimated larvae to lose haemolymph  $K^+$  balance. Cold stress elevated haemolymph  $[K^+]$  in both groups (Fig. 5B; main effect of cold exposure:  $F_{3,84}=43.4$ ,  $P<0.001$ ). Notably, this effect of chilling on haemolymph  $[K^+]$  was more pronounced in warm-acclimated than in cold-acclimated larvae (Fig. 5B; interaction between acclimation group and cold exposure:  $F_{3,84}=6.4$ ,  $P=0.013$ ); 24 h at 0°C elevated mean haemolymph  $[K^+]$  in warm-acclimated mosquitoes by  $\sim 130\%$  but only by 65% in cold-acclimated larvae (Fig. 5B). Because of this difference following cold stress (and because cold-acclimated larvae tended to have very slightly lower mean haemolymph  $[K^+]$  prior to cold stress:  $4.1$  mmol  $l^{-1}$  versus  $4.3$  mmol  $l^{-1}$ ), there was also a significant main effect of acclimation group on haemolymph  $[K^+]$  ( $F_{3,84}=6.8$ ,  $P=0.011$ ).



**Fig. 4. Rate of survival following cold exposure in cold- and warm-acclimated larval and adult *A. aegypti*.** Data are shown for larvae (A) and adults (B) following acclimation to cold (15°C; blue) and warm (25°C; red) temperatures. Open symbols represent individual mosquitoes and are slightly shifted (both vertically and horizontally) for visual clarity. Lines represent models of best fit. Horizontal lines intersect survival curves at the  $LT_{50}$ . Larvae were exposed to treatment temperatures for 24 h and adults for 6 h.  $n=424$  and  $432$  warm- and cold-acclimated larvae, respectively (A);  $n=85$ – $195$  adult mosquitoes per acclimation group and sex (B).



**Fig. 5. Concentration of Na<sup>+</sup> and K<sup>+</sup> following cold stress in larval *A. aegypti*.** (A) [Na<sup>+</sup>] and (B) [K<sup>+</sup>] before and after 24 h at 0°C. *Aedes aegypti* were acclimated to warm (25°C; red) and cool (15°C; blue) conditions. Open circles represent individual samples and filled circles represent the mean ( $\pm$ s.e.m.). Error bars that are not visible are obscured by the symbols.  $n=18$ – $25$  larvae per acclimation and treatment group combination.

## DISCUSSION

Larvae and adults of *A. aegypti* are clearly capable of cold acclimation when presented with a change in larval or adult acclimation temperature. In the present study, we compared the effects of development or adult acclimation at only two temperatures (15 and 25°C), but demonstrate that this difference of 10°C was sufficient to substantially alter chill tolerance in this important vector of disease. Cold-acclimated larvae and adults more rapidly recovered from chill coma following cold stress, and had significantly higher survival following chronic cold. After 12–16 h at 2°C, very few larvae acclimated to 15°C showed any signs of chilling injury while ~30% of larvae acclimated to 25°C were clearly suffering from neuromuscular injury that prevented them moving in a coordinated manner (Fig. 2B).

Chilling injury has been repeatedly associated with a systemic loss of ion balance in several terrestrial insects, including members of Hemiptera, Diptera, Blattodea, Lepidoptera and Orthoptera (Andersen et al., 2017b; Košťál et al., 2004, 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2014, 2015c). Notably, however, all tests of the ionoregulatory collapse model have been previously done on terrestrial insects. Here, we demonstrate a similar inability

to maintain low haemolymph [K<sup>+</sup>] in the cold in an aquatic larval insect (Fig. 5). In terrestrial insects, hyperkalaemia is mitigated (at least in part) through modifications to renal ion and water transport that help to clear excess K<sup>+</sup> ions from the haemolymph and maintain haemolymph volume (Andersen et al., 2017a; MacMillan et al., 2015a; Yerushalmi et al., 2018). Although at present it is unclear whether the same mechanisms underlie improvements in chill tolerance in mosquito larvae, the prevention of hyperkalaemia probably attenuates cold-induced cell membrane depolarization, which would limit cell death and thereby facilitate survival (Andersen et al., 2017a; Bayley et al., 2018; Boutilier, 2001; MacMillan et al., 2015b).

We note that the majority of *A. aegypti* larvae tended to sink upon entering chill coma. Mosquito larvae obtain gaseous oxygen from the water surface through a siphon on the posterior end of their abdomen, so sinking during cold stress may limit access to oxygen during cold stress and cause systemic hypoxia. Like cold stress, anoxia has been demonstrated to cause disruption of ion homeostasis, leading to hyperkalaemia in *Drosophila* (Campbell et al., 2018), meaning an inability to access sufficient oxygen during chill coma may further contribute to ionic imbalance and injury in the cold in this aquatic insect. Alternatively, as the metabolic rate of ectotherms is strongly suppressed during cold exposure, larvae may obtain sufficient oxygen from the surrounding water during cold stress to fuel metabolism and avoid the downstream consequences of hypoxia.

We were surprised to find that cold-acclimated adult mosquitoes displayed a very strong aversion to blood feeding when the opportunity was presented (Fig. 3). This could be because the cold-acclimated mosquitoes are at a younger physiological age and are not mature enough to feed on blood, or because cold acclimation triggers a specific aversion to blood feeding. As a tropical and subtropical species, *A. aegypti* is not known to be capable of any form of quiescence or diapause (Diniz et al., 2017), but a reduction in feeding behaviour is one of several hallmarks of insects in a period of dormancy, including mosquitoes. We will not further speculate on whether or not some manner of dormancy is taking place in cold-acclimated adult *A. aegypti* but argue that this subject is worthy of further investigation, particularly given the importance of this species to human health. Despite the adult mosquitoes not feeding, CCRT of females that were simply in the presence of blood increased (became worse) over the 3 h following blood presentation. Most likely, this reduction in cold tolerance was driven by the warmth of the blood, which may induce rapid changes in thermal tolerance in exposed mosquitoes. Drinking a blood meal induces an adaptive heat shock response in *A. aegypti* that protects against the effects of a rise in body temperature on fecundity (Benoit et al., 2011). We thus hypothesize that either the temperature of the warm blood or some other signal of its presence induces a similar response that alters mosquito thermal tolerance. In contrast to cold-acclimated mosquitoes, approximately half of the warm-acclimated females fed on blood within 20 min of its presentation (Fig. 3). Although we hypothesized that the salt load associated with a blood meal would alter ionoregulatory homeostasis and thereby alter chill tolerance, there was no effect of blood feeding on CCRT in warm-acclimated mosquitoes. There was a tendency for the CCRT of warm-acclimated mosquitoes to increase over time following presentation of the blood (as was seen in cold-acclimated adults), but this trend was not statistically significant, possibly because the acclimation temperature (25°C) was closer to the temperature of the blood.

The adult CCO temperature of *A. aegypti* appears highly plastic (Fig. 1B), as cold acclimation reduced the CCO of female and male

mosquitoes by approximately 6.4 and 3°C, respectively. In stark contrast to adults, however, larvae acclimated to 15°C had the same CCO as those acclimated to 25°C (~6°C; Fig. 1A), despite being more tolerant of chilling by every other measure. The CCO, CCRT and chilling injury are all thought to be related to the capacity to maintain ion and water balance, but are mediated by different specific physiological mechanisms of failure occurring in different organs and across different time scales (MacMillan, 2019; Overgaard and MacMillan, 2017; Robertson et al., 2017). Our results in the present study thus suggest that acclimation alters mechanisms underlying CCRT and the development of chilling injury without impacting the temperature that causes paralysis. Further, this result suggests that for larvae, measuring the critical thermal minimum (CT<sub>min</sub>) or CCO alone may strongly underestimate variation in cold tolerance in this species. We thus strongly recommend that other measures of cold tolerance (e.g. survival following cold stress) be included in future comparisons of thermal tolerance among or within populations, particularly in the study of larval thermal tolerance.

Winter temperatures appear to be a critically important predictor of the suitability of environments for the persistence of *A. aegypti* and *A. albopictus* (Johnson et al., 2017) and both species are spreading into habitats that have been previously considered too cold for their permanent establishment. Recent studies in *Drosophila* and other insects have suggested that range limits are closely associated with the frequency and severity of temperatures crossing critical physiological thresholds that mark the boundaries of activity (e.g. CT<sub>min</sub>, CCO) or survival (Andersen et al., 2015; Bozinovic et al., 2011; Calosi et al., 2010; Overgaard et al., 2014). Although typically considered a tropical species with little capacity for survival at low temperatures, *A. aegypti* appears to have a substantial thermal acclimation capacity, and this ability is at least partly associated with an improved ability to prevent cold-induced hyperkalaemia. Given that acclimation can alter thermal limits, thermal plasticity is likely to be an important factor governing the ability of invasive species like *A. aegypti* to survive in new environments and respond to the effects of climate change on the mean and variance of environmental temperatures. The experimental population used in the present study was derived from strains held in a laboratory environment for decades, and acclimation capacity can vary widely in insects in the wild. Eggs of *A. aegypti* at the southern end of their range in the USA appear to have evolved greater cold tolerance than populations previously studied (De Majo et al., 2017), so a careful analysis of population-level variation in thermal tolerance plasticity in *A. aegypti* is overdue, and would serve to inform future models of the distribution of this dangerous disease vector.

#### Acknowledgements

The authors wish to thank Carol Bucking for access to the incubators used for these experiments.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.D., H.A.M.; Methodology: A.J., G.Y.Y., H.E.D., A.D., H.A.M.; Formal analysis: G.Y.Y., H.E.D., H.A.M.; Investigation: A.J., G.Y.Y., H.E.D.; Resources: A.D.; Data curation: H.A.M.; Writing - original draft: A.J., G.Y.Y., H.A.M.; Writing - review & editing: A.J., G.Y.Y., H.E.D., A.D.; Visualization: H.A.M.; Supervision: A.D., H.A.M.; Funding acquisition: A.D., H.A.M.

#### Funding

This research was supported by Natural Sciences and Engineering Research Council of Canada Discovery Grants to both H.A.M. (grant RGPIN-2018-05322) and

A.D. (grant RGPIN-2018-05841), as well as a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship to G.Y.Y. and a Natural Sciences and Engineering Research Council of Canada Graduate Scholarship to H.E.D.

#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.214056.supplemental>

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