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

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Abstract

1. 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 closely tied to environmental temperature, understanding the limits of *Ae. aegypti* thermal tolerance and their capacity for phenotypic plasticity is important in predicting the spread of this species.

2. In this study we report on the chill coma onset and recovery, as well as low temperature survival phenotypes of larvae and adults of *Aedes aegypti* that developed or were acclimated to 15°C (cold) or 25°C (warm).

3. Developmental cold acclimation did not affect chill coma onset of larvae but substantially reduced chill coma onset temperatures in adults. Chill coma recovery time was affected by both temperature and the duration of exposure, and developmental and adult acclimation both strongly mitigated these effects and increased rates of survival following prolonged chilling.

4. Female adults were far less likely to take a blood meal when cold acclimated and simply exposing females to blood (without feeding) attenuated some of the beneficial effects of cold acclimation on chill coma recovery time.

5. Lastly, larvae suffered from hemolymph hyperkalemia when chilled, but development in the cold attenuated the imbalance, which suggests that acclimation can prevent cold-induced ionoregulatory collapse in this species.

6. Our results demonstrate that *Aedes aegypti* larvae and adults have the capacity to acclimate to cold 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.

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., 2019, 2015). The global distribution of *Ae. aegypti* is closely related to environmental temperatures (Brady et al., 2014, 2013; 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 (J. L. Andersen et al., 2015; Kellermann et al., 2012; Overgaard, Kearney, & Hoffmann, 2014).

In recent years, however, *Ae. aegypti*, and the closely-related vector *Ae. albopictus*, have spread through the northeastern United States. Adults of both species have even begun to appear in southern Ontario, Canada early in spring (mosquito responsible for majority of Zika infections found in Canada for the first time, 2017), which suggests successful local overwintering of at least some adults or late-stage pupae. The ability to overwinter in northern climates is completely at odds with our understanding of *Ae. aegypti* as a cold-intolerant species with little to no ability for seasonal quiescence or diapause (Diniz, Albuquerque, Oliva, Melo-santos, & Ayres, 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, Henry, & Sinclair, 2014), but predictive models of *Ae. 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 *Ae. aegypti* life cycle, including development rate, reproductive success, and survival (Carrington, Armijos, Lambrechts, Barker, & Scott, 2013; Davis, 1931; Yang, Macoris, Galvani, Andrighetti, & Wanderly, 2009). The effects of temperature on *Ae. 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, Patel, Axtell, & Stinner, 1990). Of the different life stages of *Ae. aegypti*, the eggs appear tolerant to cold, surviving and hatching following cold exposures up to 24 h at -2°C or 1 h at -17°C (Davis, 1931; Thomas, Obermayr, Fischer, Kreyling, & Beierkuhnlein, 2012). Accordingly, *Ae. aegypti* have been documented to successfully overwinter as far north as Washington D.C. and Indiana, and as far south as Buenos Aires, and there is evidence of cold adaptation occurring in these temperate populations (De Majo, Montini, & Fischer, 2017; Fischer, Alem, De Majo, Campos, & Schweigmann, 2011; Hawley, Pumpuni, Brady, & Craig, 1989; Lima, Lovin, Hickner, & Severson, 2016). Like many other poikilotherms, larval *Ae. 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, Burkot, & Kay, 2000; Yang et al., 2009). Similarly, adult *Ae. 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 *Ae. 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

83 species distribution and abundance than optimal temperatures or rates of growth and
84 reproduction at more favourable temperatures (MacLean et al., 2019; Overgaard et al., 2014).

85 *Ae. aegypti* is a chill susceptible insect, meaning it succumbs from exposure to low temperatures
86 well above the freezing of its bodily fluids. The cold tolerance of chill susceptible insects can
87 vary widely, both among and within species. Broad differences in basal cold tolerance can exist
88 among populations or species (Gibert, Moreteau, Pétavy, Karan, & David, 2001; Kellermann et
89 al., 2012; Vorhees, Gray, & Bradley, 2013; Warren & Chick, 2013), and many insects can also
90 drastically alter their cold tolerance within their lifetime. For example, insects can undergo
91 thermal acclimation in response to chronic low temperature exposure, or rapidly harden in
92 response to an acute temperature change (e.g. rapid cold-hardening) (Colinet & Hoffmann, 2012;
93 Hoffmann, Scott, Partridge, & Hallas, 2003; Kellermann et al., 2012; Kelty & Lee, 2001;
94 Sinclair et al., 2006). To date, a capacity for thermal plasticity at low temperature (cold
95 acclimation) has been demonstrated in many chill-susceptible insects, such as fruit flies,
96 cockroaches, locusts, and crickets (M. K. Andersen, Folkersen, MacMillan, & Overgaard, 2017;
97 Coello Alvarado, MacMillan, & Sinclair, 2015; Colinet & Hoffmann, 2012; V Koštál,
98 Yanagimoto, & Bastl, 2006).

99 Cold acclimation typically affects a variety of cold tolerance phenotypes in chill susceptible
100 insects. For example, cold-acclimated insects commonly have a lower temperature of chill coma
101 onset (CCO), more rapidly recover from chill coma following rewarming (a lower chill coma
102 recovery time; CCRT), and avoid the development of cold-induced injury better than warm-
103 acclimated conspecifics (Coello Alvarado et al., 2015; MacMillan, Andersen, Loeschcke, &
104 Overgaard, 2015; Ransberry, MacMillan, & Sinclair, 2011). While little is known about cold

105 acclimation in *Ae. aegypti*, eggs of *Ae. albopictus* have increased cold tolerance following cold
106 acclimation (Hanson & Craig, 1995). The magnitude of cold plasticity can vary among and
107 within populations (Nyamukondiwa, Terblanche, Marshall, & Sinclair, 2011; Sørensen,
108 Kristensen, & Overgaard, 2016). In the case of *Ae. albopictus*, cold acclimation was only noted
109 in temperate populations and not tropical populations, so this capacity for plasticity is thought to
110 be facilitating the northward expansion of the species' range (Hanson & Craig, 1995; Rochlin,
111 Ninivaggi, Hutchinson, & Farajollahi, 2013; Romi, Severlini, & Toma, 2006).

112 While tolerance to extreme cold relies on a physiological capacity to avoid or survive ice
113 formation inside the body, tolerance to chilling requires a physiological capacity to resist the
114 effects of low temperature *per se* on organ, tissue, and cellular biochemistry (MacMillan, 2019;
115 Overgaard & MacMillan, 2017; Teets & Denlinger, 2013). Consequently, measures of cold
116 tolerance relevant to freeze avoidant and freeze tolerant insects, such as the supercooling point
117 (the temperature of spontaneous ice formation within the body) or survival following freezing,
118 are irrelevant to characterizing the thermal limits of chill susceptible insects (Overgaard &
119 MacMillan, 2017). When cooled below a critical threshold temperature, chill susceptible insects
120 suffer a local loss of ion homeostasis in the nervous system, leading to nerve depolarization
121 (spreading depression) and a state of complete neuromuscular silence termed chill coma
122 (MacMillan & Sinclair, 2011a; Mellanby, 1939; Robertson, Spong, & Srithiphaphirom, 2017).
123 The temperature at which this paralytic state occurs is called the chill coma onset temperature
124 (CCO) (Overgaard & MacMillan, 2017). With time spent at low temperatures, chill susceptible
125 insects lose ion and water balance and suffer from hemolymph hyperkalemia (high $[K^+]$), which
126 further depolarizes cells and activates voltage-gated calcium channels, driving rampant cellular
127 apoptosis (Bayley et al., 2018; MacMillan, Andersen, Davies, & Overgaard, 2015; MacMillan,

Baatrup, & Overgaard, 2015). 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 & 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 (M. K. Andersen, Folkersen, et al., 2017; Coello Alvarado et al., 2015; V Koštál et al., 2006; MacMillan, Andersen, Loeschcke, et al., 2015).

Here, we use a laboratory-bred population of *Ae. 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 from eggs provided by M. Patrick (San Diego) and supplemented with eggs from Liverpool strain provided by C. Lowenberger (Simon Frasor, 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 d) 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 is maintained at room temperature (22±1°C) with a 12:12 h light:dark cycle.

To obtain larvae for experiments, eggs were added to 1.5 L of dechlorinated tap water along 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 developmental acclimation treatments, 15°C or 25°C, such that each treatment had approximately equal numbers. 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°C or the 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 x 15 cm wide x 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 for adults to feed. Following 48 h given 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

172 groups were maintained on a 12:12 h light:dark cycle. Adult mosquitoes were left at their
173 respective acclimation temperatures for five days, and thus all adults were 6-7 days post-
174 emergence when used in experiments.

175 *Chill coma onset*

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

189 Adult mosquitoes from the 25°C and 15°C acclimation groups were briefly anesthetized under
190 CO_2 and placed in 4 mL glass vials (filled with ambient air) and affixed to a rack that was
191 submerged in a temperature-controlled bath, as described above for the larvae. To record adult
192 CCO, the temperature of the bath was initially set to 25°C for 15 min and then ramped down at a
193 rate of 0.13°C min^{-1} while mosquito movement was continuously monitored. The temperature at

194 which movement stopped following perturbation with a plastic probe was recorded as the adult
195 mosquito CCO.

196 *Chill coma recovery time (CCRT)*

197 To measure chill coma recovery time (CCRT), larvae were exposed to 2°C for 4, 8, 12, or 16
198 hours. Larvae were cold-exposed by transferring each individual to a 1.5 mL open centrifuge
199 tube and incubating the tubes in a refrigerated centrifuge (Thermo ScientificTM Sorvall
200 LegendTM Micro 21R) set to 2°C (temperature was confirmed via independent thermocouples,
201 and chosen based on prior trials). After exposure to the cold, each larva was transferred to its
202 own 6.7cm diameter plastic container, filled with 50 mL room temperature dechlorinated water.
203 A timer was set immediately upon placement of the larva into room temperature water. CCRT
204 was assessed as the time taken for the larva to swim a continuous distance of 2 cm (measured
205 using a 1 cm^2 grid lining the bottom of the container). Larvae that could not swim 2 cm within 2
206 h were considered to have suffered severe injury.

207 Chill coma recovery time (CCRT) was determined in adult mosquitoes following 6 h at 2°C.
208 Mosquitoes were sexed and placed in 4 mL enclosed glass containers at room temperature
209 (22±1°C) and observed for 120 minutes. The duration of time required for a mosquito to stand on
210 all 6 legs following its removal from the cold was recorded as its CCRT. To assess the effect of
211 blood feeding on CCRT, sugar-water mixture was removed from the cages of warm-acclimated
212 mosquitoes 24 h before blood feeding. Mosquitoes were exposed to warm sheep blood for 20
213 min through a thinly stretched parafilm membrane. The mosquitoes were then either given a 0,
214 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, groups of 24 larvae were exposed to -4°C, -2°C, 0°C, 2°C, 5°C, 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 injury was measured as the inability to resume use of the siphon, where larvae that could not use their siphon to ventilate within 2 hours were counted as injured.

Chilling-survival was assessed in adult mosquitoes following 6 h exposures to temperatures between -4 and 2°C. The exposure temperatures 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°C, -3°C, -2.5°C, -2°C, -1°C, 0°C, 1°C, and 2°C and warm-acclimated mosquitoes were exposed to -2°C, -1°C, -0.5°C, 0°C, 1°C, and 2°C. Immediately upon removal from the cold exposure mosquitoes were isolated in 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.

236 *Hemolymph ion concentration*

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

247 Custom-made ion-selective microelectrodes were constructed and used following previously
248 described methods (Jonusaite, Kelly, & Donini, 2011). Briefly, borosilicate glass capillaries were
249 pulled to a tip diameter of ~3 µm using a micropipette puller (Flaming Brown P-97, Sutter
250 Instruments, Novato, USA), heated to 300°C, and exposed to N,N-dimethyltrimethylsilylamine
251 vapour for 1 h. Potassium-sensitive electrodes were backfilled with 100 mM of KCl and front-
252 filled with K^+ ionophore (K^+ ionophore I, cocktail B, Sigma Aldrich, St. Louis, MO, USA).
253 Sodium sensitive electrodes were backfilled with 100 mM NaCl and front-filled with Na^+
254 ionophore (Na^+ Ionophore II Cocktail A; Sigma Aldrich). The circuit was completed with a
255 reference electrode pulled from filamented glass capillary and back-filled with 500 mM KCl.
256 Signal information was relayed to a PowerLab 4/30 data acquisition device (ADInstruments;
257 Sydney, AUS) and interpreted by LabChart 6 software (ADInstruments). Voltages obtained from

the hemolymph samples were compared to those from calibration solutions of known concentrations, and the Nernst slope was applied to determine hemolymph 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 mM, V is the voltage (mV) reading from the hemolymph 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: Na^+ (20 mM NaCl/180 mM LiCl, and 200 mM NaCl); and K^+ (0.5 mM KCl/49.5 mM LiCl, 5 mM KCl/45 mM LiCl, and 50mM KCl).

Data analysis

R (version 3.6.1) was used to complete all data analyses (R Development Core Team, 2019). Larval CCO temperatures were compared between acclimation groups using a one-way ANOVA and CCRT temperatures 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 ANOVAs (with acclimation temperature and sex as factors). The effect of blood feeding on CCRT in adult mosquitoes was analyzed for each acclimation group independently (because cold acclimated mosquitoes did not feed) using GLMs. Feeding status and time since feeding were included as factors for warm acclimated mosquitoes, and only time since feeding for cold-acclimated mosquitoes. Survival following cold stress was analyzed for each life stage using

GLMs with a binomial error distribution and a logit-link function. Acclimation treatment and temperature were included as factors for larval survival, and sex, acclimation treatment, and temperature were included for the adults. Initial models were saturated with all potential interactions and were reduced to find the most parsimonious model based on Akaike's Information Criterion (ΔAIC > 2).

Results

Chill coma onset

Developmental acclimation did not significantly affect chill coma onset (CCO) temperatures in larval mosquitoes ($F_{1,67} = 1.3$; $P = 0.265$); both warm- and cold-acclimated mosquitoes had a CCO of ~6°C (Fig. 1A). The majority of larvae were observed to sink to the bottom of the glass vials upon entering chill coma. Although no effect was seen in the larvae, cold acclimation strongly reduced CCO temperatures 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 ~3°C, that of males differed by ~6.4°C (Fig 1B; interaction between acclimation status and sex: $F_{3,27} = 4.8$, $P = 0.037$). In general, females tended to have lower CCO temperatures than males (main effect of sex: $F_{3,27} = 6.8$, $P < 0.014$).

Chill coma recovery and injury

Developmental acclimation strongly impacted chill coma recovery time (CCRT) following exposure to 2°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 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 times, CCRT became increasingly variable

303 in warm-acclimated mosquitoes with increasing duration of cold stress, but the same was not true
304 in the cold-acclimated conspecifics (Fig. 2A). The proportion of larvae that could resume use of
305 their siphon 2 h following recovery was examined in the same individuals (an index of chilling
306 injury). Warm-acclimated larvae suffered clear chilling injury (roughly 30% after 12 or 16 h at
307 2°C), while only slight chilling injury (~2%) was noted in the cold-acclimated larvae following
308 the same exposures (Fig. 2B; interaction between acclimation and exposure duration: $F_{3,20}$ =
309 10.0, P = 0.005).

310 Cold acclimation also significantly improved rates of chill coma recovery in adult mosquitoes;
311 Cold-acclimated mosquitoes recovered from chill coma after 6 h at 0°C approximately 25 min
312 faster than the warm-acclimated conspecifics (Fig. 2C; main effect of acclimation: $F_{3,112}$ = 38.1,
313 P < 0.001). As with CCO, females appeared more cold tolerant based on CCRT, and recovered
314 ~10 min faster than males (on average) from the same cold stress (main effect of sex: $F_{3,112}$ = 5.1,
315 P = 0.026). Unlike CCO, there was no interactive effect of sex and acclimation temperature on
316 chill coma recovery ($F_{3,112}$ = 0.01, P = 0.796), as cold acclimation improved adult CCRT by the
317 same degree (~32-34 min) regardless of sex (Fig. 2C).

318 Warm-acclimated mosquitos were far more likely to take a blood meal when it was offered (Fig.
319 3A; t = 26.3, P < 0.001); only two cold-acclimated females (out of 65 that were offered it)
320 voluntarily fed on blood within 20 min (Fig. 3A). For cold-acclimated mosquitos that did not
321 feed, exposure to a blood meal still impacted CCRT, as increasing time since exposure to the
322 blood led to longer recovery times (Fig. 3B; $F_{1,61}$ = 4.1, P = 0.046). For warm-acclimated
323 mosquitos, the act of blood feeding had no effect on CCRT (Fig. 3C; main effect of feeding
324 status: $F_{1,61}$ = 0.2, P = 0.889), and although there was a slight tendency for CCRT to increase
325 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: $z = 9.4$, $P < 0.001$), with larvae exposed to lower temperatures suffering higher mortality (Fig. 4A). Cold-acclimated larvae survived 24 h exposures to lower water temperatures ($LT_{50} = -1.64 \pm 0.23$°C) than their warm-acclimated conspecifics ($LT_{50} = 0.81 \pm 0.18$°C; main effect of acclimation temperature: $z = 7.49$, $P < 0.001$; 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 chill coma onset and chill coma recovery, 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 to lower temperatures than warm-acclimated adults (Fig. 4B; main effect of acclimation temperature: $z=7.1$, $P < 0.001$). Cold acclimation shifted the female LT_{50} (following 6 h cold exposures) from 0.0 ± 0.19°C to -1.9 ± 0.15°C and the male LT_{50} from 0.3 ± 0.18°C to -1.3 ± 0.19°C (Fig. 4B).

Hemolymph ion balance

Exposure to 0°C for 24 h caused both warm- and cold-acclimated larvae to lose hemolymph $[Na^+]$ balance. Both acclimation groups had similar hemolymph $[Na^+]$ prior to cold stress, and cold stress caused hemolymph $[Na^+]$ 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 hemolymph K^+ balance. Cold stress elevated hemolymph $[K^+]$ in both groups (Fig. 5B; main effect of cold exposure: $F_{3,84} = 43.4$, $P < 0.001$). Notably, this effect of chilling on hemolymph $[K^+]$ was more pronounced in warm-acclimated than 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 cold exposure elevated mean hemolymph $[K^+]$ in warm-acclimated mosquitos by ~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 hemolymph $[K^+]$ prior to cold stress: 4.1 mM vs. 4.3 mM) there was also a significant main effect of acclimation group on hemolymph $[K^+]$ ($F_{3,84} = 6.8$, $P = 0.011$).

Discussion

Larvae and adults of *Aedes aegypti* are clearly capable of cold acclimation when presented with a change in developmental or adult acclimation temperature. In the present study we compared the effects of development or adult acclimation at only two temperatures (15°C and 25°C), but demonstrate that this difference of 10°C was sufficient to substantially alter chilling tolerance in this important vector of disease. Cold-acclimated larvae and adults more rapidly recovered from

372 chill coma following cold stress, and had significantly higher survival following chronic cold.
373 After 12-16 h at 2°C, very few larvae acclimated to 15°C showed any signs of chilling injury
374 while ~30% of larvae acclimated to 25°C were clearly suffering from neuromuscular injury that
375 prevented them moving in a coordinated manner (Fig. 2B).

376 Chilling injury has been repeatedly associated with a systemic loss of ion balance in several
377 terrestrial insects, including members of Hemiptera, Diptera, Blattodea, Lepidoptera, and
378 Orthoptera (M. K. Andersen, Jensen, & Overgaard, 2017; V Koštál et al., 2006; Vladimír Koštál,
379 Vambera, & Bastl, 2004; MacMillan & Sinclair, 2011b; MacMillan, Andersen, Davies, et al.,
380 2015; MacMillan, Findsen, Pedersen, & Overgaard, 2014). Notably, however, all tests of the
381 ionoregulatory collapse model have been previously done on terrestrial insects. Here, we
382 demonstrate a similar inability to maintain low hemolymph [K^+] in the cold in an aquatic larval
383 insect (Fig. 5). In several terrestrial insects, cold acclimation improves chilling tolerance and
384 prevents hyperkalemia. In such cases, hyperkalemia is mitigated (at least in part) through
385 modifications to renal ion and water transport that help to clear excess K^+ ions from the
386 hemolymph and maintain hemolymph volume (M. K. Andersen, Folkersen, et al., 2017;
387 MacMillan, Andersen, Loeschcke, et al., 2015; Yerushalmi, Misyura, MacMillan, & Donini,
388 2018). Although at present it is unclear whether the same mechanisms underlie improvements in
389 chilling tolerance in mosquito larvae, the prevention of hyperkalemia likely attenuates cold-
390 induced cell membrane depolarization, which would limit cell death and thereby facilitate
391 survival (M. K. Andersen, Folkersen, et al., 2017; Bayley et al., 2018; Boutilier, 2001;
392 MacMillan, Baatrup, et al., 2015).

393 We noted that the majority of *Aedes* larvae tend to sink upon entering chill coma. Mosquito
394 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 a cold stress and cause systemic hypoxia. Like cold stress, anoxia has been demonstrated to cause disruptions of ion homeostasis leading to hyperkalemia in *Drosophila* (Campbell, Andersen, Overgaard, & Harrison, 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 supressed 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 mosquitos displayed a very strong aversion to blood feeding when the opportunity was presented (Fig. 3). As a tropical and subtropical species, *Aedes 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 mosquitos. We will not speculate on whether or not some manner of dormancy is taking place in cold-acclimated *Aedes aegypti* but argue that this subject is worthy of further investigation, particularly given the importance of this species to human health. Despite not feeding, chill coma recovery times of female mosquitos that were simply in the presence of blood increased (became worse) over the 3 h following its 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 mosquitos. Drinking a blood meal induces an adaptive heat shock response in *Ae. 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

418 females fed on blood within 20 mins of its presentation (Fig. 3). Although we hypothesized that
419 the salt load associated with a blood meal would alter ionoregulatory homeostasis and thereby
420 alter chill tolerance, there was no effect of blood feeding on CCRT in warm-acclimated
421 mosquitos. There was a tendency for the CCRT of warm-acclimated mosquitos to increase over
422 time following presentation of the blood (as was seen in cold-acclimated adults), this trend was
423 not statistically significant, possibly because the acclimation temperature (25°C) was closer to
424 the temperature of the blood.

425 The adult chill coma onset temperature (CCO) of *Ae. aegypti* appears highly plastic (Fig. 1B), as
426 cold acclimation reduced the CCO of female and male mosquitoes by approximately 6.4 and
427 3°C, respectively. In stark contrast to adults, however, larvae acclimated to 15°C had the same
428 CCO as those acclimated to 25°C (~6°C; Fig. 1A), despite being more tolerant of chilling by
429 every other measure. The CCO, CCRT, and chilling injury are all thought to be related to the
430 capacity to maintain ion and water balance, but are mediated by different specific physiological
431 mechanisms of failure occurring in different organs and across different time scales (MacMillan,
432 2019; Overgaard & MacMillan, 2017; Robertson et al., 2017). Our results in the present study
433 thus suggest that acclimation alters mechanisms underlying CCRT and the development of
434 chilling injury without impacting the temperature that causes paralysis. Further, this result
435 suggests that for larvae, measuring the CT_{min} or CCO alone may strongly underestimate variation
436 in cold tolerance in this species. We thus strongly recommend that other measures of cold
437 tolerance (e.g. survival following cold stress) be included in future comparisons of thermal
438 tolerance among or within populations, particularly in the study of larval thermal tolerance.

439 Winter temperatures appear to be a critically important predictor of the suitability of
440 environments for the persistence of *Ae. aegypti* and *Ae. albopictus* (Johnson et al., 2017) and

441 both species are spreading into habitats that have been previously considered too cold for their
442 permanent establishment. Recent studies in *Drosophila* and other insects have suggested that
443 range limits are closely associated with the frequency and severity of temperatures crossing
444 critical physiological thresholds that mark the boundaries of activity (e.g. CT_{min}, CCO) or
445 survival (J. L. Andersen et al., 2015; Bozinovic, Calosi, & Spicer, 2011; Calosi, Bilton, Spicer,
446 Votier, & Atfield, 2010; Overgaard et al., 2014). Although typically considered a tropical species
447 with little capacity for overwintering, *Ae. aegypti* appears to have a substantial thermal
448 acclimation capacity, and this ability is at least partly associated with an improved ability to
449 prevent cold-induced hyperkalemia. Given that acclimation can alter thermal limits, thermal
450 plasticity is likely to be an important factor governing the ability for invasive species like *Ae.*
451 *aegypti* to survive in new environments and respond to the effects of climate change on the mean
452 and variance of environmental temperatures. The experimental population used in the present
453 study is derived from strains held in a laboratory environment for decades, and acclimation
454 capacity can vary widely in insects in the wild. Eggs of *Ae. aegypti* at the southern end of their
455 American range appear to have evolved greater cold tolerance than populations previously
456 studied (De Majo et al., 2017), so a careful analysis of population-level variation in thermal
457 tolerance plasticity in *Ae. aegypti* is overdue, and would serve to inform future models of the
458 distribution of this dangerous disease vector.
459

460 **Data Accessibility**

461 All data is provided as a supplementary file for review and the same file will be uploaded to a
462 data repository should the manuscript be accepted for publication.
463

Author Contributions

All authors contributed to the conception and design of the study, A.J. H.D. and G.Y. conducted the experiments. A.J., G.Y., H.D. and H.M. analyzed the data, H.M. drafted the manuscript, and all authors edited the manuscript.

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681 ## Figures

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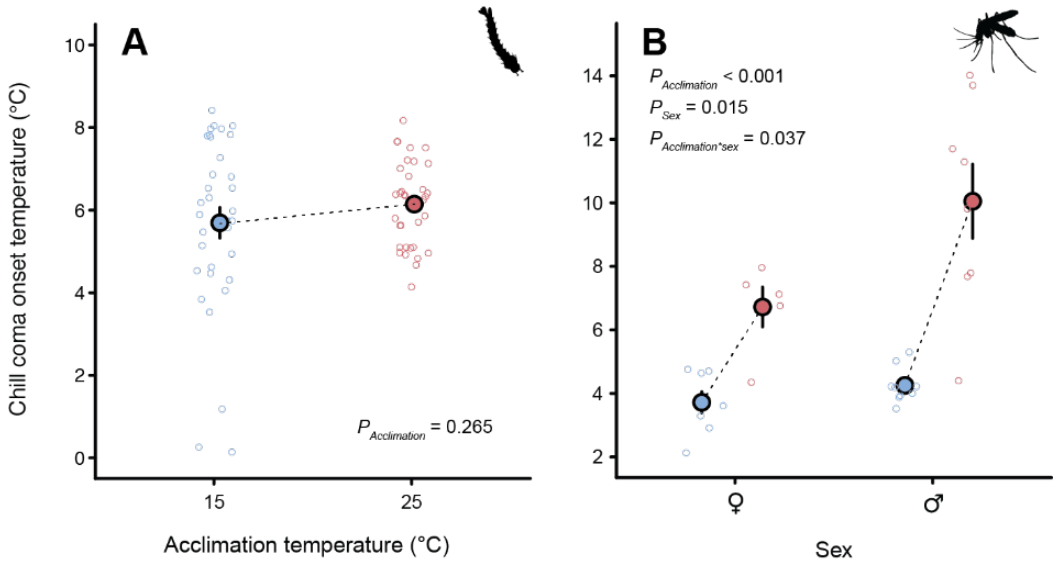

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685 **Figure 1:** Chill coma onset temperatures of larval (A) and adult (B) *Aedes aegypti* acclimated to
686 warm (25°C; red) and cool (15°C; blue) conditions. Open circles represent individual mosquitoes
687 and closed circles represent the mean (± sem) for each acclimation group and life stage. Error
688 bars that are not visible are obscured by the symbols.

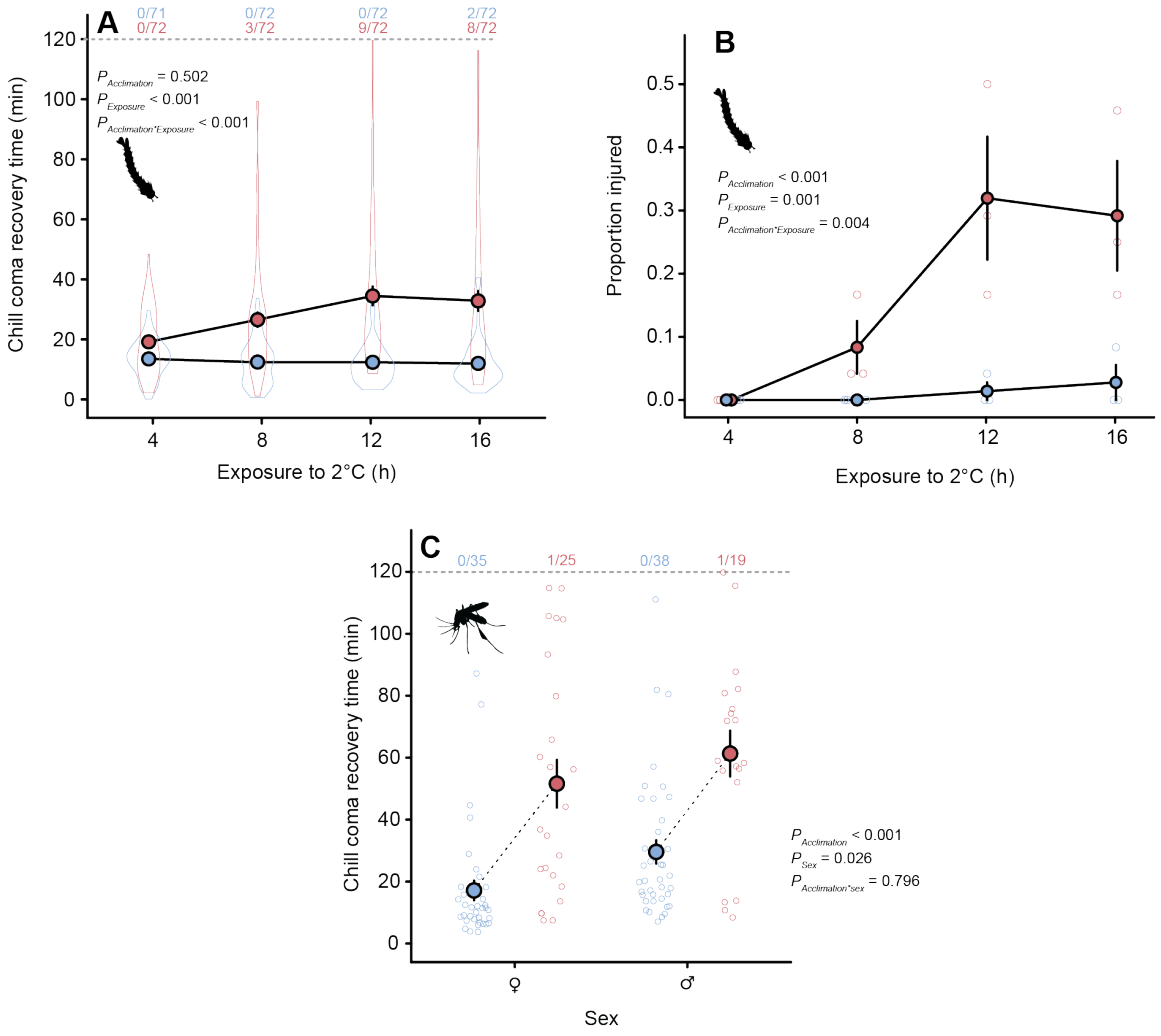


Figure 2: Chill coma recovery times (CCRT) of larval (A) and adult (B) warm- (25°C) and cold (15°C) *Aedes aegypti*. Larvae were exposed to 2°C for one of four different durations (x-axis) and adult CCRT was recorded following 6 h at 2°C. Violin plots in panel A represent sample distributions (owing to large sample size). Open circles in panel B represent proportion of mosquitoes injured (unable to resume siphon use within 2 h) in three independent trials. Open circles in panel C represent individual adult mosquitoes. Ratios above the dashed lines in panels A and C represent the number of individuals that did not recover from chill coma within the observation period (120 min). In all panels closed circles represent the mean (± sem) of each group. Error bars that are not visible are obscured by the symbols.

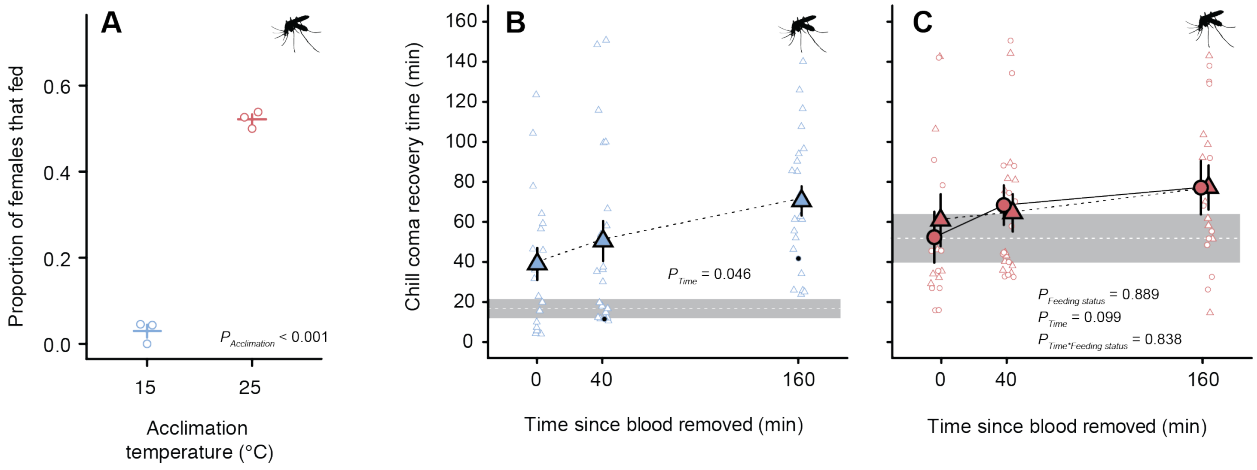


Figure 3: Rates of blood feeding (A) and chill coma recovery times following blood feeding of cold- (B; 15°C; blue) and warm-acclimated (C; 25°C, red) adult *Aedes aegypti* females. Mosquitoes of both acclimation groups were offered warm sheep's blood for 20 min before the blood was removed. Individual 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 circles in panel A represent the proportion of mosquitoes that fed within each treatment group. Open symbols in panels B and C represent CCRT values of individual adult mosquitoes. The two closed circles in panel B represent the two cold-acclimated mosquitos that took a bloodmeal (see Results text). In all cases, closed blue and red symbols represent the mean (± sem). Error bars that are not visible are obscured by the symbols.

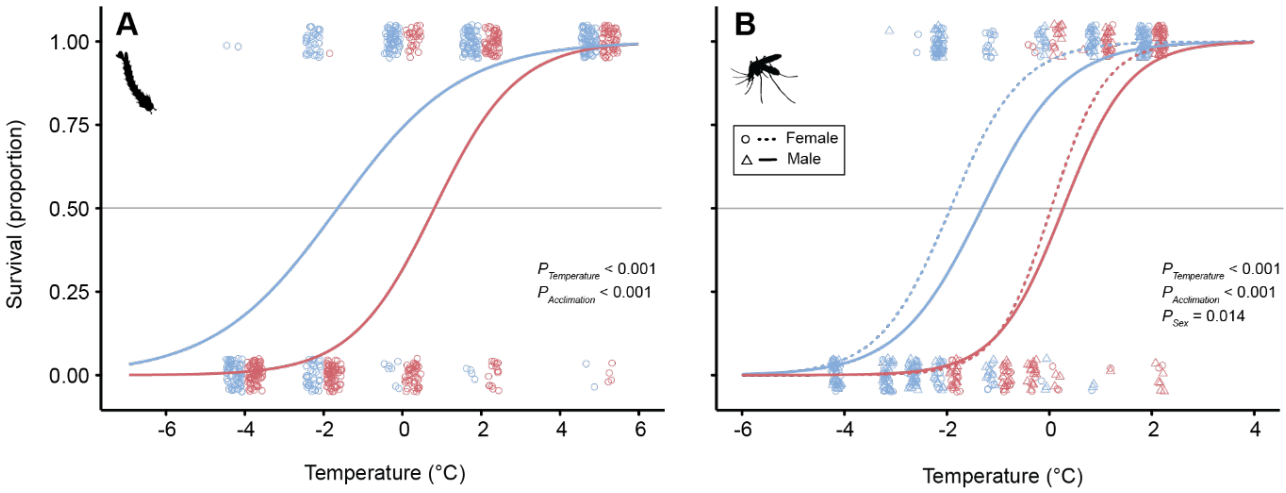


Figure 4: Rates of survival following cold exposure in larval (A) and adult (B) warm- (25°C; red) and cold-acclimated (15°C; blue) *Aedes aegypti*. Open symbols represent individual mosquitoes and are slightly shifted (both vertically and horizontally) for visual clarity. Lines represent models of best fit. Larvae were exposed to treatment temperatures for 24 h and adults for 6 h.

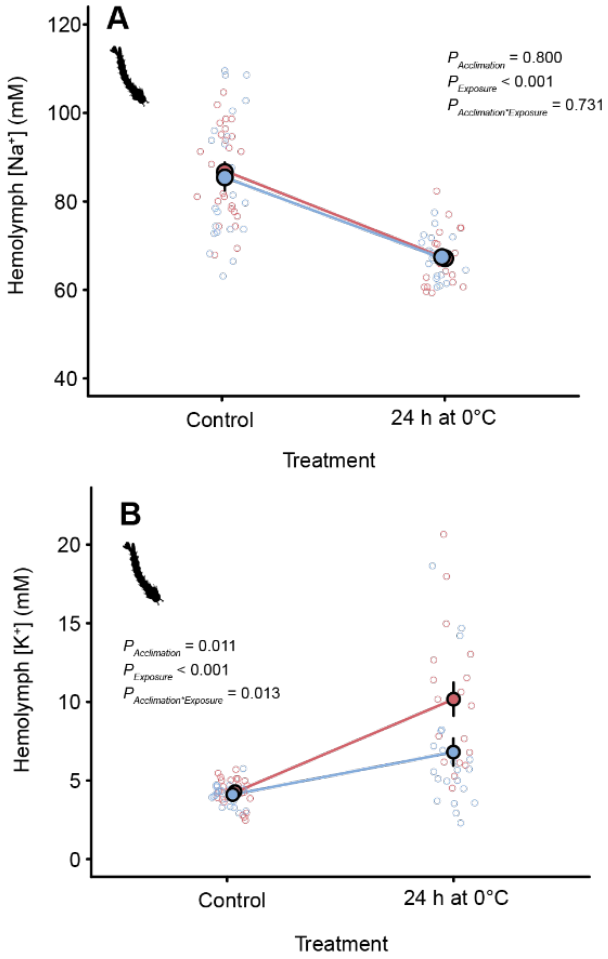
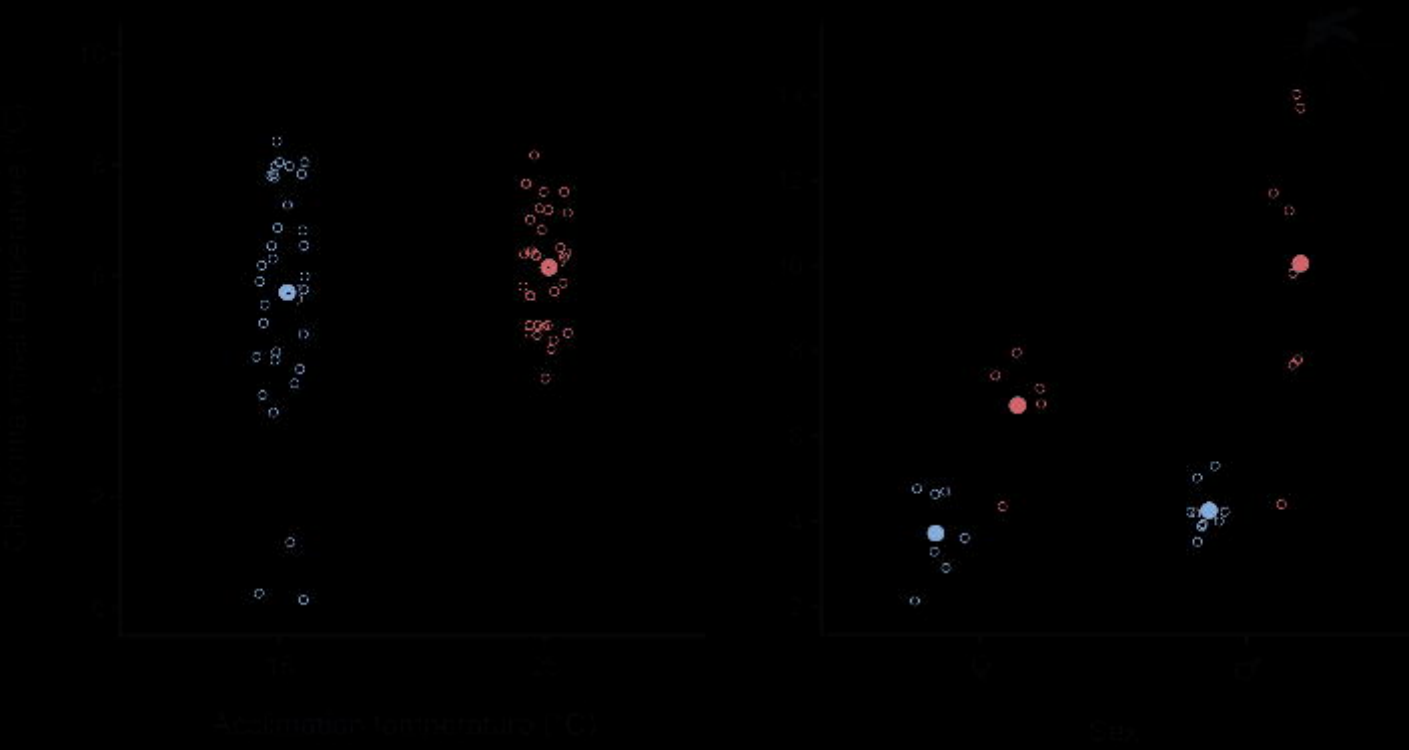
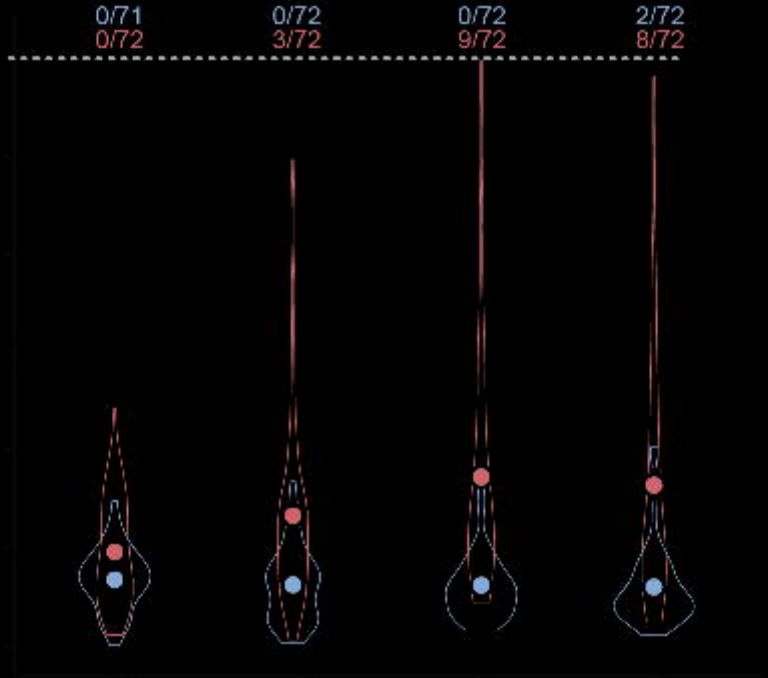


Figure 5: Concentrations of Na^+ (A) and K^+ (B) before and after cold stress in larval *Aedes aegypti*. Open circles represent individual samples and closed circles represent the mean (± sem). Error bars that are not visible are obscured by the symbols.

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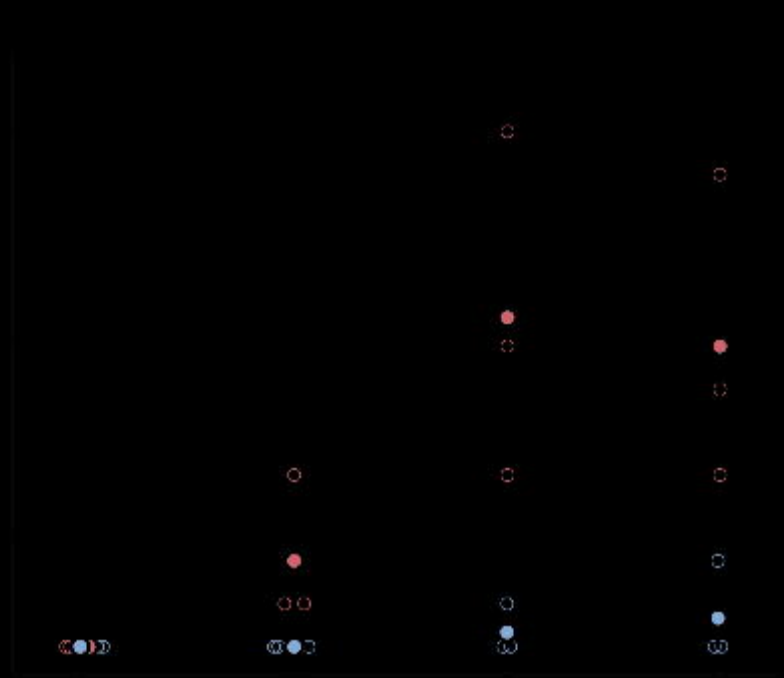

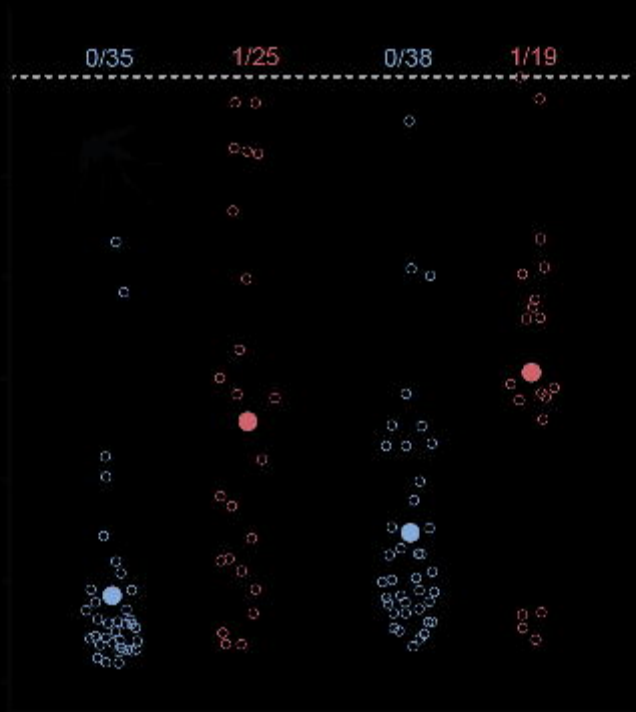
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