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PII: S1095-6433(19)30048-0

DOI: <https://doi.org/10.1016/j.cbpa.2019.04.007>

Reference: CBA 10480

To appear in: *Comparative Biochemistry and Physiology, Part A*

Received date: 22 January 2019

Revised date: 9 April 2019

Accepted date: 11 April 2019

Please cite this article as: P. Srithiphaphirom, S. Lavalley and R.M. Robertson, Rapid cold hardening and octopamine modulate chill tolerance in *Locusta migratoria*, *Comparative Biochemistry and Physiology, Part A*, <https://doi.org/10.1016/j.cbpa.2019.04.007>

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**Rapid cold hardening and octopamine modulate chill tolerance in
*Locusta migratoria***

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ABSTRACT

Temperature has profound effects on the neural function and behaviour of insects. When exposed to low temperature, chill-susceptible insects enter chill coma, a reversible state of neuromuscular paralysis. Despite the popularity of studying the effects of low temperature on insects, we know little about the physiological mechanisms controlling the entry to, and recovery from, chill coma. Spreading depolarization (SD) is a phenomenon that causes a neural shutdown in the central nervous system (CNS) and it is associated with a loss of K^+ homeostasis in the CNS. Here, we investigated the effects of rapid cold hardening (RCH) on chill tolerance of the migratory locust. With an implanted thermocouple in the thorax, we determined the temperature associated with a loss of responsiveness (i.e. the critical thermal minimum - CT_{min}) in intact male adult locusts. In parallel experiments, we recorded field potential (FP) in the metathoracic ganglion (MTG) of semi-intact preparations to determine the temperature that would induce neural shutdown. We found that SD in the CNS causes a loss of coordinated movement immediately prior to chill coma and RCH reduces the temperature that evokes neural shutdown. Additionally, we investigated a role for octopamine (OA) in the locust chill tolerance and found that OA reduces the CT_{min} and mimics the effects of prior stress (anoxia) in locust.

KEYWORDS: Insect; chill coma; cold tolerance; RCH; nervous system; spreading depolarization; SD; epinastine

INTRODUCTION

Many organisms are exposed to and challenged by natural environmental stresses, such as extreme temperatures, and they have adapted mechanisms of tolerance that allow them to survive and thrive. As poikilotherms, insects have limited means to regulate their internal body temperature and are instead dependent on their surrounding temperatures. Therefore, temperature is one of the main factors that can determine insect abundances, distributions and life histories. Most insects are chill-susceptible meaning that they cannot tolerate cold environments and die at temperatures above their freezing points. The critical thermal minimum (CT_{min}) can be defined as the low temperature at which performance drops to zero and animals lose the ability to stand and maintain equilibrium (MacMillan, 2019). When exposed to temperatures below their CT_{min} , chill-susceptible insects enter a chill coma, which is characterized by complete neuromuscular paralysis (Findsen et al., 2014; MacMillan et al., 2014). Chill coma is reversible if the cold exposure is mild or brief and the time required for an insect to recover from chill coma is termed chill coma recovery time (CCRT) (Findsen et al., 2014; MacMillan et al., 2014).

Prior exposure to a non-lethal low temperature can improve insect chill tolerance by increasing the capacity to avoid chilling injury (Wang et al., 2003; Williams et al., 2004; Shintani and Ishikawa, 2007; MacMillan et al., 2015). Rapid cold hardening (RCH), a short-term cold hardening, is a process that allows organisms to survive normally lethal low temperatures following pre-exposure to a less severe low temperature. RCH can be distinguished from thermal acclimation, which refers to the phenotypic responses to prolonged changes in temperature. For flesh flies (*Sarcophaga crassipalpis* and *Sarcophaga bullata*), 91.1 % of flies survive a 2-hour exposure to -10°C if they are pre-exposed to 0°C for 2 hours (Lee et al., 1987). Without RCH, most larvae and adults of flesh flies do not survive at -10°C (Lee et al., 1987). RCH involves the accumulation of low molecular weight compounds (e.g. glycerol, sorbitol) (Chen et al., 1987; Lee et al., 1987; Michaud and Denlinger, 2007) and cell membrane modification to maintain membrane fluidity (Overgaard et al., 2005; Michaud and Denlinger, 2006), but has little impact on gene expression, likely due to the short time frame (Sinclair et al., 2007). RCH enhances insects' ability to deal with daily fluctuation in daily temperature and thus is widespread among species as a mechanism to protect against a sudden drop of environmental temperature, especially for insect species that do not possess an overwintering strategy (e.g. diapause, migration).

To date, much research has focused on the neuromuscular mechanisms underlying chill coma in insects. Anderson and Mutchmor (1968) studied the effect of temperature acclimation on the nerve cord and ganglia of three species of cockroaches and found that both nerve cord and ganglia continue to support electrical signals at temperatures below the chill coma temperatures of all three species. They concluded that failure of the nervous system is not the direct cause of chill coma. These findings have led researchers to focus more on muscle function during chill coma. However, locust comas induced by environmental stressors (e.g. anoxia, hyperthermia) are associated with the sudden loss of potassium ion (K^+) homeostasis and temporary electrical silence in the central nervous system (CNS), which is a hallmark of the phenomenon known as spreading depolarization (SD) (Newman et al., 2003; Rodgers et al., 2007; Armstrong et al., 2009; Rodgers et al., 2009; Rodgers et al., 2010; Robertson et al., 2017). Recent research shows that a loss of coordinated movement immediately prior to chill coma in locusts is associated with SD in the CNS (Robertson et al., 2017) and the temperature of CNS failure correlates closely with CT_{min} (loss of coordination) in five *Drosophila* species adapted to different thermal environments (Andersen et al., 2018).

The goal of this study was to investigate the effects of RCH on chill tolerance of the African migratory locust (*Locusta migratoria migratorioides*), a chill-susceptible insect. In haemolymph and muscle tissues, RCH locusts recover extracellular K^+ concentration ($[K^+]_o$) faster than non-RCH locusts (Findsen et al., 2013). During cold exposure, we recorded electromyographic activity (EMG) in intact locusts and CNS extracellular field potential (FP) in semi-intact locusts. The locusts were either control or pre-exposed to 4 °C for 2, 4 or 6 hours to induce RCH. Additionally, we suspected that octopamine (OA), a biogenic amine that is analogous to norepinephrine, may be involved in mediating changes to chill tolerance. OA activates excitatory responses (e.g. flight or fight response) in invertebrates and its concentration increases in response to stress (Davenport and Evans, 1984; Armstrong and Robertson, 2006). OA decreases the time for the CNS to recover from stress in heat-shocked locusts (Armstrong and Robertson, 2006; Money et al., 2016). We monitored the coordinated movement of intact locusts that were injected with saline (control), OA or epinastine (EP - an OA receptor antagonist) during cold exposure. We also determined the effect of OA and EP on pre-stressed locusts by exposing the locusts to anoxia prior to cold exposure. Then we determined and compared the CT_{min} and the CCRT of intact locusts and the low temperatures that induced neural

shutdown (SD temperatures) in semi-intact preparations. In this study, we defined CT_{min} as the temperature associated with the loss of responsiveness and CCRT as the time required for an animal to recover its coordination. We found that in locust, (1) pre-exposure to cold improves the resistance to chill coma; (2) RCH that affects chill coma onset also affects neural shutdown in a parallel fashion; (3) OA reduces CT_{min} and CCRT; and (4) OA mimics, but EP reverses the effects of prior stress (anoxia).

METHODS

Animals and experimental treatments

Mature male locusts, 4 – 5 weeks past imaginal ecdysis, were chosen randomly from a crowded colony maintained in the Department of Biology at Queen's University. Only male locusts were used. Locusts were reared in large, well-ventilated cages under a 12 h:12 h light:dark cycle and fed daily with fresh wheat seedlings and a dry mixture of skim milk powder, torula yeast, and wheat bran. Temperature inside the cages was 25 ± 1 °C during light hours and 21 ± 1 °C during dark hours. Locusts were distributed between two experimental treatments: RCH and drug injection.

Rapid cold hardening

Separate groups of experimental locusts were kept in ventilated plastic containers (~750 mL; 1 locust per container) and maintained at approximately 4 °C for 2, 4 or 6 h in a cold room. They were then allowed to recover for 1 h at room temperature (22 ± 2 °C) before being subjected to a decreasing temperature ramp. Control locusts were kept in similar containers (1 locust per container) at room temperature for 1 h before the experiment.

Drug injection

OA and EP (from Sigma-Aldrich, St. Louis, MO, USA) solutions were prepared on the same day as the experiment. Standard locust saline containing (in mM): 147 NaCl, 10 KCl, 4 CaCl₂, 3 NaOH, and 10 HEPES buffer (pH 7.2) (all chemicals from Sigma-Aldrich) was used to prepare 10^{-2} M OA and EP. Each locust was loosely attached with tape to a small piece of cork and was abdominally injected with 10 μ L of 10^{-2} M OA (OA group), 10^{-2} M EP (EP group) or

standard locust saline (control group) (Money et al., 2016). The haemolymph volume of *L. migratoria* is approximately 200 μL (Ayali and Pener, 1992). Therefore, the final concentration in the haemolymph of each treatment was around 5×10^{-5} M. The locusts were left for 10 mins before being subjected to cold stress. We decided on a 10 min-period because the half-life of OA in locust haemolymph is approximately 15 mins (Goosey and Candy, 1982).

Anoxia pre-treatment

We also tested the effect of anoxic prior stress by submerging locusts in de-chlorinated tap water at room temperature for 2 h and allowing them to recover at room temperature for 1 h (e.g. Hou et al., 2014). In separate experiments, the first set was categorized into 3 groups: control, anoxia and Anoxia + OA (OA injection after 1 h recovery from anoxia). The second set was categorized into 3 groups: control, anoxia and Anoxia + EP (EP injection after 1 h recovery from anoxia).

Rapid cold hardening experiments

Whole animal preparation

After the recovery period at room temperature, we recorded the electrical activity in hindwing muscles of locusts (either control or experimental) while they were subjected to cold stress. For temperature measurements, a thermocouple probe (Type T copper/constantan; time constant 0.1 s; Physitemp Instruments Inc., Clifton, NJ, USA) recorded room temperature for ~ 15 – 20 s before it was inserted into the thoracic cavity through a hole under the anterior dorsal edge of the pronotum to a distance of ~1 cm. For EMG recordings, a copper wire electrode (50 μm diameter, insulated except at the cut end) was inserted through a small hole made in the ventral cuticle and secured with a drop of molten wax. The locust was grounded with a chlorided silver wire inserted into the thorax under the anterior lateral edge of the pronotum. Locusts were lightly restrained on a 0.6 x 3.5 cm cork platform and held in the body of a 10 mL plastic syringe. Each locust was placed in a 500 mL container partially submerged in liquid antifreeze (Windex Auto Windshield Wash; Recochem Inc., Montreal, QC, Canada) contained inside a refrigerated circulator (Julabo GmbH, Seelbach, Germany). The temperature of this refrigerated circulator was set at -3°C . The thermocouple probe was connected to a BAT-12 microprobe thermometer (Physitemp Instruments Inc.). The signals from the EMG electrodes were amplified

using a Grass P15 amplifier (low frequency cut off at 3 Hz and high frequency cut off at 3 kHz) and digitized using a MiniDigi 1A digitizer (1 kHz sampling rate; Molecular Devices Inc., Sunnyvale, CA, USA). Both temperature and EMG recordings were displayed and recorded for later analysis using AxoScope 10.3 (Molecular Devices Inc.) (Fig. 1). After the body temperature reached $\sim -1^{\circ}\text{C}$, the locust was removed from the chilling apparatus and returned to room temperature. Each recording took approximately 40 mins. Intermittent mechanical stimuli were applied to the recovering locust to induce a response.

Semi-intact preparation

Dissection

To expose the thoracic ganglia for measuring electrical activity, the legs, wings and pronotum were removed as previously described (Robertson and Pearson, 1982). A dorsal midline incision was made, and the locust was pinned to a cork board. The gut, air sacs and fat bodies were removed to expose the thoracic ganglia. Standard locust saline was perfused into the thoracic and abdominal cavities. The tissue covering the thoracic ganglia as well as the cuticle and attached muscle tissue located between the meso-metathoracic connectives were removed. A metal plate was inserted below the ganglia to stabilize them. A chlorided silver wire was placed in the posterior tip of the abdomen to ground the preparation (Fig. 2A).

Measurement of electrical activity

To determine the timing of chilling-induced SD, FP was measured extracellularly from the metathoracic ganglion (MTG) as temperature progressively decreased. Measuring the FP is a less invasive way to determine the timing of SD because it requires only one electrode rather than the two electrodes required to directly measure $[\text{K}^+]_o$. SD is characterized by an abrupt development of a negative FP that is coincident with the surge in $[\text{K}^+]_o$. The microelectrodes were pulled from 1 mm diameter, filamented borosilicate glass capillary tubes (World Precision Instruments; WPI, Sarasota, FL, USA) using a model P-87 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA). These microelectrodes were backfilled with 500 mM KCl before each experiment. The signals were amplified and digitized using a model 1600 Neuroprobe amplifier (model 1600; A-M Systems Inc., Sequim, WA, USA) and a Digidata

1322A (100 kHz sampling rate; Molecular Devices Inc.), displayed and saved for later analysis using AxoScope 10.3 (Fig. 2B).

Temperature control

While measuring the electrical activity, standard locust saline was perfused into the thoracic and abdominal cavities using gravity flow from a reservoir suspended above the preparation. The saline was chilled by directing the saline line (plastic tubing) through a container containing a slurry of ice, water, and salt. The temperature of icy water was -3 ± 1 °C. A temperature ramp was generated by controlling the flow rate of saline using a stopper on the saline line. The flow rate was slow enough to cool down the saline during its passage through the icy water, and fast enough to avoid freezing in the icy water and warming between the icy water and the preparation. By decreasing the saline temperature close to 0 °C, SD was induced in the semi-intact preparation. The thermocouple probe was placed near the MTG to record the saline temperature. This thermocouple probe was connected to a BAT-12 microprobe thermometer. The signals were amplified and digitized using the Neuroprobe amplifier and a Digidata 1322A, displayed and saved for later analysis using AxoScope 10.3 (Fig. 2B).

Octopamine and epinastine experiments

Each locust was abdominally injected with 10 μ L of 10^{-2} M OA (OA group), 10^{-2} M EP (EP group) or standard locust saline (control group) and was left for 10 mins before being subjected to cold stress (see above: *Drug injection*). Pre-injected locusts were placed in a 500 mL container partially submerged in liquid antifreeze contained inside a refrigerated circulator. The procedure was similar to the RCH experiments (see above: *Whole animal preparation*), but instead of recording the electrical activity in hindwing muscles of locusts, we focused on whole animal observations. The locust movements were monitored as temperature decreased.

Data analysis

Data were collected from 153 intact locusts and 40 semi-intact preparations. The recordings were analyzed using Clampfit 9.0 (Molecular Devices Inc.). For the whole animal preparations, the CT_{min} was determined at the beginning of the first obvious burst of electrical activity from hindwing muscles (Robertson et al., 2017) (Fig. 1) in the RCH experiments, whereas in the OA and EP experiments, the CT_{min} was determined when the locust lost the ability

for coordinated movement. CCRT was recorded as the time taken for the locust to start coordinated walking. For the semi-intact preparations, the SD temperature was determined at the half-maximal amplitude of the negative shifts of FP (Fig. 2B). The amplitude of FP shift, time to SD and time to SD recovery were also determined by measuring the difference between the baseline level and the lowest point of FP, as the time taken from the beginning of recording to SD temperature, and as the time taken between the point where the saline temperature rose back and at the half-maximal amplitude of FP shift when returning to the baseline level, respectively (Fig. 2B). Data were plotted as the means and standard error (SE) or as the medians and interquartile ranges (IQR) using SigmaPlot 12.5 (Systat Software Inc., Chicago, IL, USA) and were reported in Table 1 and 2. Statistical analyses were performed using SigmaPlot 12.5. Statistical significance among groups was assessed using a one-way ANOVA. $P < 0.05$ is indicated by letter codes. Statistics are reported in the Results text.

RESULTS

Rapid cold hardening

Behaviour

The CT_{min} of control locusts ($7.5^{\circ}C$) was significantly higher than the CT_{min} of locusts that were exposed for 4 hours (4hRCH) ($4.8^{\circ}C$; $p < 0.001$) and locusts that were exposed for 6 hours (6hRCH) ($5.9^{\circ}C$; $p = 0.009$), but not the CT_{min} of locusts that were exposed for 2 hours (2hRCH) ($6.9^{\circ}C$; $p = 0.518$) (Fig. 3A). The CT_{min} of 2hRCH locusts was significantly different from the CT_{min} of 4hRCH locusts ($p < 0.001$), but not the CT_{min} of 6hRCH locusts ($p = 0.287$) (Fig. 3A). Lastly, the CT_{min} of 4hRCH locusts and 6hRCH locusts were not significantly different ($p = 0.189$) (Fig. 3A). Except for the 6hRCH locusts, longer durations of RCH reduced CT_{min} more. The CCRT of 6hRCH locusts (7.2 minutes) was significantly shorter than the CCRT of control locusts (11.2 minutes), 2hRCH locusts (9.9 minutes) and 4hRCH locusts (8.2 minutes) ($p < 0.001$) (Fig. 3B).

Spreading depolarization in the CNS

SD events were recorded from the MTG as abrupt negative shifts of FP while the saline temperature progressively decreased. After RCH treatment at 4 °C, the SD of locusts that were exposed for 4 hours (4hRCH) was induced at 7.8 °C, which was significantly lower than the SD temperatures of control locusts (10.8 °C), 2hRCH locusts (9.6 °C) and 6hRCH locusts (9.9 °C) ($p < 0.050$) (Fig. 4A). Similarly, the time from the start of the temperature ramp to SD event of 4hRCH locusts was 6.0 minutes, which was significantly longer than the times to SD of control locusts (3.2 minutes), 2hRCH locusts (4.1 minutes) and 6hRCH locusts (3.7 minutes) ($p < 0.050$) (Fig. 4B). The amplitude of the FP shift was not significantly different among the 4 groups ($p = 0.089$). The time to SD recovery of control locusts (2.2 minutes) was significantly different from the time to SD recovery of 4hRCH locusts (2.9 minutes; $p = 0.037$), but not the times to SD recovery of 2hRCH locusts (2.5 minutes; $p = 0.234$) and 6hRCH locusts (1.7 minutes; $p = 0.197$) (Fig. 5). The time to SD recovery of 2hRCH locusts was significantly different from the time to SD recovery of 6hRCH locusts ($p = 0.020$), but not the time to SD recovery of 4hRCH locusts ($p = 0.249$) (Fig. 5). The time to SD recovery of 4hRCH locusts was significantly different from the time to SD recovery of 6hRCH locusts ($p < 0.001$) (Fig. 5).

Octopamine and epinastine

The CT_{min} of OA locusts (1.7 °C) was significantly lower than the CT_{min} of control locusts (4.0 °C) and EP locusts (3.5 °C) ($p < 0.050$) (Fig. 6A). However, the CT_{min} of control locusts and EP locusts were not significantly different ($p > 0.05$) (Fig. 6A). Similarly, the CCRT of OA locusts (5.2 minutes) was significantly shorter than the CCRT of control locusts (7.8 minutes) and EP locusts (8.3 minutes) ($p < 0.050$) (Fig. 6B). The CCRT of control locusts and EP locusts were not significantly different ($p = 0.490$) (Fig. 6B).

The CT_{min} of locusts that were pre-exposed to anoxia and injected with OA (Anoxia + OA; 3.9 °C) and locusts that were pre-exposed to anoxia (4.2 °C) were significantly lower than the CT_{min} of control locusts (6.3 °C) ($p < 0.001$) (Fig. 7A). However, the CT_{min} of Anoxia + OA and anoxia locusts were not significantly different ($p = 0.660$) (Fig. 7A). Similarly, the CCRT of Anoxia + OA (5.6 minutes) and anoxic (6.4 minutes) locusts were significantly shorter than the CCRT of control locusts (8.3 minutes) ($p < 0.050$) (Fig. 7B). The CCRT of Anoxia + OA and anoxia locusts were not significantly different ($p = 0.660$) (Fig. 7B).

The CT_{min} of locusts that were pre-exposed to anoxia and injected with EP (Anoxia + EP; 7.1 °C) was significantly higher than the CT_{min} of control (4.8 °C) and anoxia locusts (4.2 °C) ($p < 0.001$) (Fig. 8A). The CT_{min} of control and anoxia locusts were not significantly different ($p = 0.070$) (Fig. 8A). Similarly, the CCRT of Anoxia + EP (8.5 minutes) was significantly longer than the CCRT of control (6.5 minutes) and anoxia locusts (4.9 minutes) ($p < 0.001$) (Fig. 8B). The CCRT of anoxia locusts was significantly shorter than the CCRT of control locusts ($p < 0.001$) (Fig. 8B).

DISCUSSION

Low temperature during winter is one of the greatest challenges for insects living in temperate regions. When exposed to temperatures below their CT_{min} , locusts, like other chill-susceptible insects, enter chill coma (Findsen et al., 2014; MacMillan et al., 2014). This state is reversible, but it takes time (CCRT) to recover from the paralysis (Findsen et al., 2014; MacMillan et al., 2014). Temperature sensitivity is a powerful factor that can determine the geographic limits of chill-susceptible insects. Despite the importance of temperature effects, much remains to be learned regarding the chill coma mechanisms in insects. Here, we conducted two parallel experiments using the migratory locust to investigate the effects of RCH on chill tolerance. Our primary finding is that RCH affects the locust chill tolerance by reducing CT_{min} , CCRT and SD temperature. Then we conducted the OA experiment to investigate whether OA, the invertebrate stress hormone, plays a role in the locust chill tolerance. We found that OA reduces the CT_{min} in locust. Additionally, we found that OA mimics the effects of prior anoxia and EP reverses these effects.

Cold hardening is an important process that prevents chilling injury by prior exposure to non-lethal low temperatures. Pre-exposure to mild cold, either chronically or acutely, has been found to reduce the sensitivity to cold exposure of insects (Wang et al., 2003; Williams et al., 2004; Shintani and Ishikawa, 2007; Armstrong et al., 2012; MacMillan et al., 2015). We know that if *Drosophila* are pre-exposed to a change in long-term acclimation temperature of 1 °C, their CT_{min} changes approximately 0.4 °C (Overgaard et al., 2011; Sørensen et al., 2016; Andersen et al., 2018), however, the effect of RCH on CT_{min} is still unclear. EMG recordings demonstrated that locusts had a characteristic burst of muscular activity during chilling that had a

neural origin and preceded behavioural impairment and chill coma paralysis (Robertson et al., 2017). Our findings showed that RCH at 4 °C reduced the temperature that the first obvious burst of electrical activity from the hindwing muscles occurred (CT_{min}). After returning to room temperature, the locusts recovered. As we determined CT_{min} as a temperature inducing loss of coordination (Hazell and Bale, 2011; Andersen et al., 2018), we used the time it took for the locusts to start walking as the CCRT. The RCH locusts took a shorter time to be able to walk after being exposed to cold stress.

Extreme temperatures, either high or low, can impair neural performance, leading to the failure of properly coordinated behaviour and of the generation of vital motor patterns (Robertson, 2004; Robertson and Money, 2012). In the parallel experiment, we measured FP to monitor the occurrence of SD because FP represents mass depolarization in the CNS associated with a surge of extracellular K^+ in the CNS. Low temperature causes a disturbance of K^+ homeostasis in the muscular system of insects, leading to chill coma paralysis (Esch, 1988; Goller and Esch, 1990; Hosler et al., 2000; Findsen et al., 2014). In this study, we confirmed that, in addition to the muscular system, a disturbance of K^+ homeostasis caused by low temperature also occurs in the CNS (Robertson et al., 2017). The disturbance of K^+ homeostasis in the CNS is a mechanism underlying chill coma during cold exposure. We exposed locusts to 4 °C for 2, 4 and 6 hours. We were predicting that the locusts that were exposed for 6 hours would have the lowest failing temperature, but we found that the locusts that were exposed for 4 hours entered chill coma at a significantly lower temperature than the control locusts and the locusts that were exposed for 2 hours and 6 hours. Likewise, the time to SD of locusts that were exposed for 4 hours was significantly longer than the times to SD of the other three groups. In other words, the locusts that were exposed to 4 °C for 4 hours tolerated a longer exposure to cold and entered chill coma at a lower temperature. We did not observe any significant difference in the locusts that were exposed for 2 hours because pre-exposure to 4 °C for 2 hours might not be long enough to have an effect that we could measure. There are at least two reasons why the results of the locusts that were exposed for 6 hours were different from our prediction: (1) the locusts were permanently impaired after being exposed to low temperature for 6 hours and (2) the locusts that were exposed for 6 hours needed more than 1 hour to recover from chilling. We measured the amplitude of the FP shift to examine whether RCH affected the magnitude of ion disturbance. The amplitudes of FP shift were not significantly different regardless of the different periods of

pre-exposure to cold. It should be noted that we measured the amplitude at a single point where the microelectrode was inserted and thus it may not accurately represent the overall ion disturbance. For technical reasons, we could not measure CCRT in this semi-intact preparation, but we measured the time to SD recovery among control, 2-hour-, 4-hour- and 6-hour-RCH locusts and found that the 4-hour-RCH locusts had the longest time to SD recovery. The time to SD recovery did not correlate directly with the CCRT because their preparations were different. While we measured the CCRT in intact locusts, the time to SD recovery was measured in semi-intact preparation, which locusts were cut open and exposed to their surrounding environment. Recovery from chill coma involves the re-establishment of ion homeostasis in the nervous system (Robertson et al., 2017). Therefore, a possible reason to explain the longest time to SD recovery of 4-hour-RCH locusts is that they tolerate cold exposure for longer, causing larger ion concentrations to build up. With larger CNS ion disturbances, they take longer times to restore ion concentrations to the baseline levels.

The sudden loss in K^+ homeostasis and the electrical silence in the CNS are triggered by cellular stressors, such as hyperthermia, anoxia, ATP depletion, and Na^+/K^+ -ATPase impairment by drugs (e.g. ouabain) (Newman et al., 2003; Rodgers et al., 2007; Armstrong et al., 2009; Rodgers et al., 2009; Rodgers et al., 2010). The present study is consistent with the hypothesis from previous studies that the rapid surges of $[K^+]_o$ are the results of an imbalance in processes of K^+ accumulation and clearance and impairment of Na^+/K^+ -ATPase operation may cause this imbalance. During cold exposure, the Na^+/K^+ -ATPase may be modulated by temperature or OA. The Na^+/K^+ -ATPase has a temperature coefficient (Q_{10}) around 1.5 – 3 (Leong and Manahan, 1997; Nakamura et al., 1999; MacMillan and Sinclair, 2011; DiFranco et al., 2015). Low temperature reduces Na^+/K^+ -ATPase activity, leading to excess K^+ in the extracellular space because K^+ clearance cannot keep up with K^+ accumulation (Rodgers et al., 2010; Andersen et al., 2018). Neuronal membranes are depolarized when $[K^+]_o$ increases and a neuron discharges when its membrane potential reaches the threshold for action potential generation (Somjen, 2002). The abnormal rise in $[K^+]_o$ creates a positive feedback loop and causes further depolarization (Somjen, 2002; Armstrong et al., 2009; Andersen et al., 2018). This depolarization can depress neuron excitability and spread across the CNS, leading to the silence of CNS (Armstrong et al., 2009; Andersen et al., 2018). Although we do not provide the evidence to suggest that RCH can increase the Na^+/K^+ -ATPase activity, heat-shock pretreatment

has been found to increase the abundance of Na^+/K^+ -ATPase in neuronal membrane which enhances the ability to manage high level of $[\text{K}^+]_o$ and restores ion homeostasis (Hou et al., 2014). RCH may operate via a similar mechanism; however, this remains to be discovered.

OA is another possible factor that can modulate Na^+/K^+ -ATPase. We found that in intact locusts, OA increased chill tolerance by reducing both $\text{CT}_{\min}$ and CCRT. OA receptors in the locust CNS are coupled with G-proteins (Roeder and Gewecke, 1990; Roeder, 1992; Armstrong and Robertson, 2006). The $\lambda\beta$ subunits of G-protein then interact with adenylyl cyclase to stimulate the production of cyclic adenosine monophosphate (cAMP) and activate cAMP-dependent protein kinase A (PKA) (Roeder and Gewecke, 1990; Roeder, 1992; Armstrong and Robertson, 2006). PKA regulates many cellular processes, including K^+ channels and Na^+/K^+ -ATPase pumps (Feschenko et al., 2000). This indicates that OA modulates K^+ conductance and Na^+/K^+ -ATPase activity, which are necessary for maintaining $[\text{K}^+]_o$ in the locust CNS (Walther and Zittlau, 1998; Armstrong and Robertson, 2006). While OA reduces the locust $\text{CT}_{\min}$, EP (OA antagonist) has similar results as control. We suggest that the OA level is normally low in unstressed locusts and thus EP would not affect the $\text{CT}_{\min}$ of control locusts.

Anoxic pre-treatment has been found to increase heat tolerance in locusts (Wu et al., 2002). Our results showed that OA mimics the effects of anoxia on chill tolerance. The $\text{CT}_{\min}$ and CCRT of anoxic pre-treatment locusts and anoxic pre-treatment with OA injection locusts were not significantly different, so there was no additive effect. Both OA and anoxic pre-treatment reduced $\text{CT}_{\min}$ and CCRT in locusts, suggesting that prior stress increased endogenous OA levels. However, EP reverses the effects of anoxia. The $\text{CT}_{\min}$ and CCRT of anoxic pre-treatment with EP injection locusts were significantly higher than the $\text{CT}_{\min}$ and CCRT of anoxic pre-treatment locusts and control locusts. As mentioned before, EP had no effect on unstressed locusts, however, when locusts were exposed to prior stress (anoxia) which increased OA levels, EP increased both $\text{CT}_{\min}$ and CCRT, supporting the idea that the effect of anoxia is via OA.

Climate change around the globe will affect the distributions of temperature-dependent animals, such as insects. We demonstrated that low temperature (hypothermia), like other environmental stressors, can result in physiological changes consistent with disruptions in ion homeostasis, particularly K^+ , in the locust CNS which lead to the temporary neural shutdown. We provided evidence that pre-exposure to moderately low temperature can improve the locust's

resistance to chill coma although, in the wild, locusts are less likely to experience rapid changes in temperature similar to the temperature ramp used in this study. We also showed that OA, the biogenic amine, increases locust chill tolerance. However, the idea that OA acts through Na^+/K^+ -ATPase during cold exposure remains to be demonstrated. Nevertheless, our findings highlight the important, but relatively neglected, role that the CNS has in mediating and modulating chill tolerance in insects and suggest that future investigations in this area will be fruitful (see for example Andersen and Overgaard, 2019).

ACKNOWLEDGEMENT

This study was funded by a Discovery grant to RMR from the Natural Sciences and Engineering Research Council of Canada (NSERC).

Conflict of Interest:

The authors declare no conflict of interest.

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Figure 1. Sample EMG recording from a locust hindwing muscle during entry to chill coma. As temperature (Temp) decreased, the electrical activity was recorded from a hindwing muscle (EMG). The first burst of EMG (red line) was used to indicate CT_{min} .

Figure 2. Semi-intact preparation of the locust. (A) Schematic diagram of the metathoracic ganglion (Meta) with recording electrodes used to measure FP (red in inset) and recording probe used to measure saline temperature (Temp). (B) Sample extracellular recording of electrical activity in the metathoracic ganglion (FP) as the saline temperature decreased from 26 °C to 4 °C and increased back to 26 °C (Temp). A red line and a blue line in (B) indicate the point where the FP went negative at half-maximal amplitude and the point where the FP returned to the baseline level at half-maximal amplitude, respectively.

Figure 3. RCH reduces CT_{min} and CCRT. (A) CT_{min} and (B) CCRT of locusts after different durations of RCH at 4 °C. Data are medians in A and means ± S.E. in B (n = 12 each group; p-values are reported in section Results). Letter codes indicate statistically significant differences.

Figure 4. RCH reduces SD temperature and increases latency of chill-induced SD. (A) Temperature at negative FP shifts recorded in the MTG and (B) time to FP shift of control and RCH locusts. Data are means $\pm$ S.E. (n = 10 each group; p-values are reported in section Results). Letter codes indicate statistically significant differences.

Figure 5. RCH increases time to SD recovery. Data are means $\pm$ S.E. (n = 10 each group; p-values are reported in Results). Letter codes indicate statistically significant differences.

Figure 6. OA reduces CT_{min} and CCRT. (A) CT_{min} and (B) CCRT of locusts 10 mins after injection of OA and EP. Data are medians ($n = 15$ each group; p-values are reported in Results). Letter codes indicate statistically significant differences.

Figure 7. OA has no additive effect on CT_{min} and CCRT after prior anoxia. (A) CT_{min} and (B) CCRT of locusts that were pre-exposed to anoxia and injected with OA (Anoxia + OA) and were exposed to anoxia without OA injection (Anoxia). Data are medians ($n = 15$ each group; p-values are reported in Results). Letter codes indicate statistically significant differences.

Figure 8. EP reverses the effect of prior anoxia on CT_{min} and CCRT. (A) CT_{min} and (B) CCRT of locusts that were pre-exposed to anoxia and injected with EP (Anoxia + EP) and were exposed to anoxia without EP injection (Anoxia). Data are means $\pm$ S.E. (n = 15 each group; p-values are reported in Results). Letter codes indicate statistically significant difference.

Table 1. Rapid cold hardening experiments. CT_{min} and CCRT in whole animal and SD temperature, time to SD, FP amplitude and time to SD recovery in semi-intact preparation. Parametric data are presented as $x \pm$ Standard Error; nonparametric data are presented as x (Inter Quartile Range).

	Whole animal			Semi-intact preparation				
	n	CT _{min} (°C)	CCRT (min)	n	SD temperature (°C)	Time to SD (min)	FP amplitude (mV)	Time to SD recovery (min)
Control	12	7.5 (7.3 - 9.9)	11.2 $\pm$ 0.3	10	10.8 $\pm$ 0.6	3.2 $\pm$ 0.3	35.5 (27.2 - 40.7)	2.2 $\pm$ 0.2
2hRCH	12	6.9 (6.2 - 7.3)	9.9 $\pm$ 0.2	10	9.6 $\pm$ 0.2	4.1 $\pm$ 0.3	38.9 (31.6 - 41.9)	2.5 $\pm$ 0.2
4hRCH	12	4.8 (4.6 - 5.5)	8.2 $\pm$ 0.3	10	7.8 $\pm$ 0.3	6.0 $\pm$ 0.6	34.0 (27.9 - 40.8)	2.9 $\pm$ 0.2
6hRCH	12	5.9 (5.8 - 6.8)	7.2 $\pm$ 0.3	10	9.9 $\pm$ 0.4	3.7 $\pm$ 0.2	24.4 (22.5 - 29.4)	1.7 $\pm$ 0.1

Table 2. Octopamine and epinastine experiments. CT_{min} and CCRT in whole animal. Parametric data are presented as $x \pm$ Standard Error; nonparametric data are presented as x (Inter Quartile Range).

	n	CT _{min} (°C)	CCRT (min)
Control	15	4.0 (2.2 - 4.5)	7.8 (7.0 - 8.4)
OA	15	1.7 (1.4 - 3.2)	5.2 (4.8 - 6.0)
EP	15	3.5 (2.0 - 6.7)	8.3 (7.6 - 10.7)
Control	10	6.3 (5.6 - 7.9)	8.3 (7.3 - 10.3)
Anoxia	10	4.2 (4.0 - 4.6)	6.4 (5.7 - 7.3)
Anoxia+OA	10	3.9 (3.6 - 4.4)	5.6 (5.3 - 7.3)
Control	10	4.8 $\pm$ 0.2	6.5 $\pm$ 0.2
Anoxia	10	4.2 $\pm$ 0.2	4.9 $\pm$ 0.2
Anoxia+EP	10	7.1 $\pm$ 0.3	8.5 $\pm$ 0.2

Highlights

- Pre-exposure to cold improves the resistance to chill coma of the African migratory locust.
- RCH that affects chill coma onset also affects neural shutdown in a parallel fashion.
- Octopamine reduces the temperature associated with a loss of coordinated movement and the time taken for the locust to start coordinated walking.

Graphical abstract

ACCEPTED MANUSCRIPT

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8