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Ion and water balance in *Gryllus* crickets during the first twelve hours of cold exposure

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ABSTRACT

Insects lose ion and water balance during chilling, but the mechanisms underlying this phenomenon are based on patterns of ion and water balance observed in the later stages of cold exposure (12 or more hours). Here we quantified the distribution of ions and water in the hemolymph, muscle, and gut in adult *Gryllus* field crickets during the first 12 h of cold exposure to test mechanistic hypotheses about why homeostasis is lost in the cold, and how chill-tolerant insects might maintain homeostasis to lower temperatures. Unlike in later chill coma, hemolymph $[Na^+]$ and Na^+ content in the first few hours of chilling actually increased. Patterns of Na^+ balance suggest that Na^+ migrates from the tissues to the gut lumen via the hemolymph. Imbalance of $[K^+]$ progressed gradually over 12 h and could not explain chill coma onset (a finding consistent with recent studies), nor did it predict survival or injury following 48 h of chilling. *Gryllus veletis* avoided shifts in muscle and hemolymph ion content better than *Gryllus pennsylvanicus* (which is less chill-tolerant), however neither species defended water, $[Na^+]$, or $[K^+]$ balance during the first 12 h of chilling. *Gryllus veletis* better maintained balance of Na^+ content and may therefore have greater tissue resistance to ion leak during cold exposure, which could partially explain faster chill coma recovery for that species.

1. Introduction

Because insects are ectotherms, many of their physiological processes are directly influenced by ambient temperature. The mechanisms that underlie thermal physiology will therefore determine how climate change impacts insect performance and, consequently, ecosystem function (Sinclair et al., 2003; Chown and Terblanche, 2006; Somero, 2010; Williams et al., 2015). Insect performance is bounded at low temperatures by the critical thermal minimum (CT_{min}), below which insects enter a reversible paralysis called chill coma. Insects lose ion and water homeostasis when in chill coma and regain homeostasis during recovery (Koštál et al., 2004; MacMillan et al., 2012). The ability to survive and maintain homeostasis in the cold is variable and plastic; cold-acclimated or -adapted insect populations sustain water and ion balance at lower temperatures than their warm-acclimated or -adapted counterparts (Gibert and Huey, 2001; Ayrinhac et al., 2004; Koštál et al., 2004, 2006; Andersen et al., 2014; Coello Alvarado et al., 2015; MacMillan et al., 2015a).

In several insects (including crickets, locusts, and cockroaches), hemolymph Na^+ and water migrate out of the hemolymph during chilling, while hemolymph $[K^+]$ increases (Koštál et al., 2006; MacMillan and Sinclair, 2011; Andersen et al., 2013; Findsen et al., 2014; Coello Alvarado et al., 2015). The migration of Na^+ is likely a result of active ion transport failure and, as water balance is often tightly linked to Na^+ gradients, so too is hemolymph water lost. The decreased hemolymph volume is thought to increase hemolymph $[K^+]$ (MacMillan and Sinclair, 2011). In *Gryllus pennsylvanicus* Burmeister, the largest decrease in hemolymph $[Na^+]$ occurs within the first 12 h of cold exposure (MacMillan and Sinclair, 2011). Chill coma onset occurs rapidly (within minutes of cold exposure) and appears to be mechanistically unrelated to processes underlying loss of water and ion homeostasis (Findsen et al., 2014; MacMillan et al., 2014b; Andersen et al., 2015). In particular, previous authors have not observed a loss of homeostasis associated with chill coma paralysis within the first few minutes of cold exposure (Findsen et al., 2014; MacMillan et al., 2014b; Andersen et al., 2015). However, loss of homeostasis during chilling is readily apparent at longer timescales (hours to days) in the context of studies of chill coma recovery time (CCRT) and chilling injury (*e.g.* Koštál et al. (2006), MacMillan and Sinclair (2011), Findsen et al. (2013)). Thus we do not know how quickly Na^+ or K^+ balance is lost during cold exposure, or whether the patterns of

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homeostasis in the initial cold exposure reflect those observed at longer timescales. Similarly, little is known about how ion and water imbalance during chilling relates to or predicts survival and chilling injury (MacMillan et al., 2014b).

Insects vary in their ability to maintain ion and water balance in the cold (Koštál et al., 2004, 2007; MacMillan et al., 2014a, 2015a; Coello Alvarado et al., 2015). Our understanding about the mechanisms underlying this variation is incomplete (Gibert and Huey, 2001; Ransberry et al., 2011), but recent studies have revealed a potential role for Na^+ balance. Cold-acclimated *Drosophila melanogaster* Meigen maintain low hemolymph [Na^+] (and consequently low [K^+]) in both warm and cold conditions, and may also exhibit lower Na^+-transport capacity (MacMillan et al., 2014a, 2015a). *Gryllus veletis* (Alexander and Bigelow) nymphs maintain Na^+ balance at 0 °C, while *G. pennsylvanicus* adults (which are less chill tolerant) lose Na^+ balance at 0 °C unless they have undergone prior cold acclimation (Coello Alvarado et al., 2015).

Understanding why insects lose water and ion homeostasis during chilling requires an understanding of the short-term movements of water and ions during cold exposure. Here we explore the patterns of water and ion balance during the first 12 h of cold exposure with the aim of testing and generating mechanistic hypotheses for why homeostasis is lost in the cold, and why chill-tolerant insects are better at maintaining homeostasis at low temperatures. We used two species of field cricket: *Gryllus pennsylvanicus* (which was used to develop the initial model of loss of ion and water homeostasis in the cold), and *G. veletis*, the nymphs of which are more chill-tolerant and maintain ion and water balance at lower temperatures (Coello Alvarado et al., 2015).

2. Materials and methods

Gryllus pennsylvanicus and *G. veletis* colonies originated from individuals collected from the University of Toronto at Mississauga campus, Ontario (2004) and the University of Lethbridge, Alberta (2010), respectively. We reared crickets under constant summer-like conditions (25 °C, 14 L:10 D photoperiod, 70% RH) at the University of Western Ontario Biotron Research Center, as described previously (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). Crickets were housed in transparent plastic containers with stacked cardboard egg cartons for shelter and provided with tap water and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills, Elmira, ON, Canada). We collected eggs in containers of moist vermiculite and sterile sand; *Gryllus veletis* eggs hatched after two weeks, and we placed *G. pennsylvanicus* eggs at 4 °C to accommodate an obligate three month diapause (Rakshpal, 1962) before returning them to 25 °C to hatch. For all experiments we used adult virgin female *G. pennsylvanicus* and *G. veletis* (approximately 1 and 5 weeks post final molt, respectively). The difference in age reflected a longer development time for *G. veletis*. For one week prior to experiments, crickets were held individually in 177 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh fabric lids and containing egg carton shelters, rabbit food, and water. This isolation prevented cannibalism and any associated changes in gut contents.

2.1. Measurements of chill tolerance

We assessed low temperature performance of *G. pennsylvanicus* and *G. veletis* adult females by measuring the CT_{min}, CCRT, and survival following cold exposure. Measurement of the CT_{min} ($N = 20$ per species) was as described by (MacMillan and Sinclair, 2011). Briefly, we cooled crickets from room temperature at 0.25 °C min^{-1} until the CT_{min} was reached. We defined the CT_{min} as the temperature at which physical stimulus with a metal probe elicited no muscular response. We defined CCRT as the time required for the righting response (a coordinated movement) after 48 h of cold exposure. To measure CCRT and survival of cold exposure, we placed crickets ($N = 24$ per species) in 15 mL Falcon tubes immersed in an ice-water slurry at 0 °C (a temperature that induced chill coma in both *G. pennsylvanicus* and *G. veletis* in preliminary experiments). This time period should not induce substantial mortality; *G. veletis* survive at least five days at 0 °C, while *G. pennsylvanicus* suffer approximately 20% mortality after 108 h at 0 °C (Coello Alvarado et al., 2015). After 48 h, we moved the crickets to room temperature, placed them on their dorsum in a 6-well plate, and video recorded their recovery for up to 9 h (Hazell et al., 2008). We extracted twitch and righting response times from the video. Crickets that did not exhibit signs of recovery within 9 h were not included in CCRT analyses. All crickets were then returned to 25 °C in individual cups and provided with food, water, and shelter. After 24 h at 25 °C, we assessed survival and injury (the latter defined as uncoordinated locomotion or inability to jump when stimulated with a probe) (MacMillan and Sinclair, 2011).

2.2. Cold exposure and dissection

We held crickets at 25 °C (control, 0 h) or exposed them to 0 °C for a duration of 0.5, 1, 3, 6, or 12 h ($N = 14$–19 individuals per species per treatment). Size-matching of crickets ensured that mean wet mass did not differ among treatments within each species ($F_{5,83} = 0.30$, $P > 0.9$ and $F_{5,89} = 0.32$, $P > 0.9$ for *G. pennsylvanicus* and *G. veletis*, respectively). We placed cold-exposed crickets individually into loosely-capped 50 mL plastic tubes suspended in a bath of 50% methanol in water, pre-cooled to 0 °C (Lauda Proline RP 3530, Würzburg, Germany). We added a thermocouple in contact with one of the crickets to monitor its body temperature during cold exposure.

Immediately after removal from 0 °C we dissected crickets on a Petri dish surrounded by ice within a large Styrofoam box. We punctured the pronotum with an insect pin and collected hemolymph (5–30 µl) with a micropipette, then opened the body cavity by a mid-dorsal incision and collected as much hemolymph from the body as possible by applying gentle pressure to the abdomen. We approximated hemolymph volume gravimetrically by weighing extracted hemolymph and assuming a density equal to water. This method of hemolymph extraction and approximation correlates linearly with inulin dilution estimates for hemolymph volume in *G. pennsylvanicus* (MacMillan et al., 2012). We pinned open the body cavity and removed the gut (from anterior foregut to rectum) into a pre-weighed microcentrifuge tube. We then severed the hind legs and used forceps to extract femur muscles into pre-weighed 0.2 mL microcentrifuge tubes.

To identify potential reservoirs of Na^+ (as we observed increased hemolymph Na^+ content during chilling), we measured Na^+ in the fat body, head, Malpighian tubules, and ovaries from an additional six control *G. pennsylvanicus* females. We calculated tissue water contents calculated from the difference between the tissue fresh (wet) mass and mass after drying at 70 °C for 24 h (muscle, Malpighian tubules, and fat body) or 48 h (gut, head, and ovaries).

2.3. Ion quantification

We assessed ion homeostasis over 12 h of cold exposure by quantifying the concentration and content of Na^+ and K^+ in the hemolymph and tissues. Ion contents indicate bulk movement of Na^+ or K^+ between body compartments (which in turn affects bulk movement of water), while ion concentrations are important for