Chill-tolerant *Gryllus* crickets maintain ion balance at low temperatures

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Litza E. Coello Alvarado, Heath A. MacMillan [1], Brent J. Sinclair *

Department of Biology, University of Western Ontario, London, ON, Canada

ABSTRACT

Insect cold tolerance is both phenotypically-plastic and evolutionarily labile, but the mechanisms underlying this variation are uncertain. Chill-susceptible insects lose ion and water homeostasis in the cold, which contributes to the development of injuries and eventually death. We thus hypothesized that more cold-tolerant insects will better maintain ion and water balance at low temperatures. We used rapid cold-hardening (RCH) and cold acclimation to improve cold tolerance of male *Gryllus pennsylvanicus*, and also compared this species to its cold-tolerant relative (*Gryllus veletis*). Cold acclimation and RCH decreased the critical thermal minimum (CT_{min}) and chill coma recovery time (CCR) in *G. pennsylvanicus*, but while cold acclimation improved survival of 0 °C, RCH did not; *G. veletis* was consistently more cold-tolerant (and had lower CCR and CT_{min}) than *G. pennsylvanicus*. During cold exposure, hemolymph water and Na^+ migrated to the gut of warm-acclimated *G. pennsylvanicus*, which increased hemolymph $[K^+]$ and decreased muscle K^+ equilibrium potentials. By contrast, cold-acclimated *G. pennsylvanicus* suffered a smaller loss of ion and water homeostasis during cold exposure, and this redistribution did not occur at all in cold-exposed *G. veletis*. The loss of ion and water balance was similar between RCH and warm-acclimated *G. pennsylvanicus*, suggesting that different mechanisms underlie decreased CCR and CT_{min} compared to increased survival at 0 °C. We conclude that increased tolerance of chilling is associated with improved maintenance of ion and water homeostasis in the cold, and that this is consistent for both phenotypic plasticity and evolved cold tolerance.

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1. Introduction

At low temperatures, most insects enter chill coma (characterized by a loss of muscle function and lack of response to external stimuli) at a species-specific temperature, the critical thermal minimum (CT_{min}; MacMillan and Sinclair, 2011a; Mellanby, 1939). Insects in chill coma can recover with minimal evidence of permanent injury; recovery is typically measured as chill coma recovery time (CCR; e.g. David et al. 1998; MacMillan et al., 2012a). However, in many insects, prolonged exposure or exposure to temperatures below lethal limits can result in the accumulation of chilling injuries, and eventually death, even in the absence of ice formation (Koštál et al., 2006; MacMillan and Sinclair, 2011b). Mortality from chilling injuries, CCR, and CT_{min} are phenotypically plastic and can evolve, leading to variation within and among species.

Understanding the mechanisms that underlie variation in thermal tolerance is essential in comparing and predicting species' thermal tolerances (Gaston et al., 2009; Somero, 2010). The CT_{min}, CCR, and susceptibility to chilling injury all vary among insect species and populations, with insects from colder environments generally having a lower CT_{min}, faster CCR, and lower incidence of injury or death following a cold stress (David et al., 2003; Hallas et al., 2002; Hoffmann et al., 2001). In addition, all three traits are phenotypically plastic; rearing temperature, hardening treatments of minutes to hours (termed rapid cold-hardening, RCH), acclimation over days or weeks, and different rates of cooling can all affect susceptibility to injury, as well as the CT_{min} and CCR (e.g. Colinet and Hoffmann, 2012; Findsen et al., 2013; Gibert and Huey, 2001; Gilchrist and Huey, 2001; Hoffmann et al., 2003; Koštál et al., 2004, 2006; Rajamohan and Sinclair, 2008; Ransberry et al., 2011; Sinclair and Roberts, 2005).

Insect mortality in the cold can be caused by a range of phenomena, including apoptosis, protein misfolding, membrane phase transitions, and loss of ion balance (Teets and Denlinger, 2013). A wide variety of insects, including cockroaches (Koštál et al., 2006), true bugs (Koštál et al., 2004), locusts (Findsen et al., 2013), crickets (MacMillan and Sinclair 2011b), and *Drosophila*

(MacMillan et al., 2015, in press) lose ion and water balance during exposure to the temperatures that cause chilling injury. The current model proposes that, at low temperatures, thermally-sensitive ion pumps lose activity and can no longer balance relatively thermally-insensitive passive leak.

This model of ion balance disruption is based, in part, on the fall field cricket (*Gryllus pennsylvanicus* Burmeister [Orthoptera: Gryllidae]). In *G. pennsylvanicus* adults maintained at 25 °C, low temperature exposure leads to a net leak of Na^+ down its concentration gradient from the hemolymph to the gut lumen. Water from the hemocoel also moves into the gut; and the resulting reduction in estimated hemolymph volume concentrates the K^+ remaining in the hemolymph. Insect muscle resting potential is heavily dependent on extracellular $[K^+]$ (Djamgoz, 1987; Hoyle, 1954), and thus the rise in $[K^+]$ in the hemolymph depolarizes muscle cells (MacMillan and Sinclair, 2011b). With time spent at low temperature, continued depolarization of the potassium equilibrium potential (E_K) leads to slower CCR and correlates with the onset of chilling injury and death in this species (MacMillan and Sinclair, 2011b; MacMillan et al., 2012a). Although this loss of ion balance appears to be closely tied to CCR (MacMillan et al., 2012a) and chilling injury (MacMillan and Sinclair, 2011a), it does not appear to be directly responsible for the onset of chill coma (Findsen et al., 2014; MacMillan et al., 2014).

If ion and water balance disruption are a primary cause of chilling injury in *Gryllus*, variation in survival at low temperatures could arise through variation in the ability to maintain ion and water homeostasis in the cold. In *Drosophila*, decreased hemolymph $[Na^+]$ accompanies lower CT_{min} (both within and among species), which appears to be associated with suppression of Na^+–K^+-ATPase activity (MacMillan et al., 2015). In locusts, RCH decreases chill coma recovery time, apparently by increasing the rate at which homeostasis is re-established (Findsen et al., 2013). Together, these imply that both among-species variation in cold tolerance and phenotypic plasticity are tied to modulation of mechanisms of ion and water balance. However, it is not clear whether modified ion and water balance accompanies increased cold tolerance in other genera (such as *Gryllus*), nor is it clear whether RCH and (longer-term) cold acclimation operate through similar ionoregulatory mechanisms within a species.

Here, we explore within-species variation in the ability to maintain ion and water balance at low temperatures. We do this by comparing low temperature maintenance of ion and water homeostasis among individuals of the fall field cricket, *G. pennsylvanicus* that experienced warm-acclimation (WA), rapid cold-hardening (RCH), or cold-acclimation (CA). Because even cold-acclimated *G. pennsylvanicus* still enter chill coma and are killed by long exposures to cold, we also compare low temperature ion and water homeostasis of *G. pennsylvanicus* to that of a more cold-hardy congener, the spring field cricket *Gryllus veletis* (Alexander and Bigelow, 1960). Whereas *G. pennsylvanicus* overwinters as a diapausing egg (which we do not examine here), *G. veletis* overwinters as a late-instar nymph (Alexander and Bigelow, 1960), and as a consequence we expect it to have evolved considerably greater cold tolerance than *G. pennsylvanicus*. This comparison allows us to determine whether loss of ion and water balance is specifically associated with the mortality we observe in *G. pennsylvanicus*, or is simply a property of *Gryllus* crickets when exposed to low temperatures. Although we are cognizant that a two species comparison does not allow us to draw specific conclusions about the adaptive nature of any variation we see (Garland and Adolph, 1994), it does give some insight into whether low temperature ion and water balance follow similar patterns in cold-adapted and non-adapted species.

We hypothesize that cold tolerance is improved (lower CT_{min}, faster CCR, and increased survival of prolonged cold exposure)

through the maintenance of water and ion homeostasis at low temperatures, regardless of whether it arises from phenotypic plasticity or adaptive evolution. Thus, we predict that improvements in cold tolerance achieved through different forms of plasticity (RCH and acclimation) will be consistently associated with an improved ability to maintain ion and water balance within *G. pennsylvanicus*, and that the more cold-tolerant species (*G. veletis*) will similarly better maintain ion and water homeostasis in the cold.

2. Materials and methods

Laboratory colonies of *G. pennsylvanicus* (originally collected on the University of Toronto at Mississauga campus in 2004) and *G. veletis* (Collected on the University of Lethbridge campus in 2010) were maintained as previously described by Judge et al. (2010) and reared under constant-temperature summer conditions (25 °C, 14L:10D photoperiod, 70% relative humidity). *G. pennsylvanicus* eggs required chilling a 4 °C for three months due to obligate diapause, but *G. veletis* eggs (which hatch in the summer, soon after being laid) did not. All experiments were conducted on adult male *G. pennsylvanicus* approximately seven weeks post final molt, and third and fourth instar male *G. veletis* (the overwintering stages; Alexander and Bigelow, 1960) at least 180 mg in body mass. We chose males because previous work (MacMillan and Sinclair, 2011b; MacMillan et al., 2012a,b) had been conducted on females, and we wished to expand our understanding of the responses to cold; also, because we were comparing mature (*G. pennsylvanicus*) and immature (*G. veletis*) individuals, we wished to reduce the physiological differences caused by significant resources devoted to egg production.

2.1. Acclimation treatments

G. pennsylvanicus were divided into three groups: warm-acclimated (WA, standard rearing conditions), rapid cold-hardened (RCH) and cold-acclimated (CA). Crickets were transferred from their rearing bin into individual 177 mL translucent plastic cups (Polar Plastics, Summit Food Distributors Inc., London, ON, Canada) and provided food, water and a piece of egg carton as shelter. The RCH crickets were transferred from their rearing temperature to a cold room at +4 °C for four hours and given one hour to recover at 25 °C before being cold-exposed. Note that although the RCH pre-treatment is consistent with some RCH protocols (e.g. Nunamaker, 1993; Sinclair and Chown, 2003; Ransberry et al., 2011; Armstrong et al., 2012), it differs from those which utilise slow cooling to the pre-treatment temperature (e.g. Overgaard et al., 2005, 2007), and from others that do not include a recovery period (e.g. Lee et al., 1987; Rajamohan and Sinclair, 2008). Note that our RCH pre-treatment did not change all measures of low temperature performance (see results). Three week post-eclosion individuals in the CA treatment were cooled in an incubator (Sanyo MIR 154, Sanyo Scientific, Bensenville, Illinois) from 25 °C to 12 °C in 2.5 °C day^{-1} steps, and kept at 12 °C under a 10L: 14D photoperiod, 70% RH, and *ad libitum* food and water for the subsequent three weeks (the entire acclimation therefore took four weeks). *G. veletis* and WA *G. pennsylvanicus* were kept under standard rearing conditions for the duration of the acclimation period.

2.2. Low temperature responses

We measured critical thermal minima (CT_{min}) using a method modified from Klok and Chown (1997) and described by MacMillan and Sinclair (2011b). In keeping with these previous studies, crickets were not fasted prior to experiments. Briefly, we