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Cold induced changes in lipid, protein and carbohydrate levels in the tropical insect *Gromphadorhina coquereliana*

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ABSTRACT

Insects cope with thermal stressors using mechanisms such as rapid cold hardening and acclimation. These mechanisms have been studied in temperate insects, but little is known about their use by tropical insects in response to cold stress. Here, we investigated whether cold stress (1×8 h and 3×8 h at 4 °C) triggers a metabolic response in the Madagascar cockroach *Gromphadorhina coquereliana*. We examined the effects of cold on the levels of selected metabolites in the fat body tissue of *G. coquereliana*. After cold exposure, we found that the quantity of total protein increased significantly in the insect fat body, whereas glycogen decreased slightly. Using antibodies, we observed upregulation of AQP-like proteins and changes in the HSP70 levels in the fat body of *G. coquereliana* when exposed to cold. We also examined the content and nature of the free sugars in the *G. coquereliana* hemolymph and discovered an increase in the levels of polyols and glucose in response to cold stress. These results suggest an important role of the fat body tissue of tropical insects upon cold exposure.

1. Introduction

Insects are widely distributed in different geographical environments; thus their development and survival are intimately linked to the environmental parameters, such as temperature (Zachariassen, 1985; Kellermann et al., 2012; Araujo et al., 2013). Although most insects are active at moderately high temperatures, some exhibit normal behavior at temperatures close to freezing, and many survive low temperatures during winter. Thus, during evolution, insects exposed to cold have developed diverse morphological, physiological and behavioral adaptations; for review see Bale (2002) and Clark and Worland (2008). At the cellular level, these mechanisms protect cells from sudden environmental changes or frequent fluctuations (Kultz, 2003). Additionally, they are often associated with the production of protective compounds, such as heat shock proteins (HSPs) (Kelty and Lee, 2001; Rinehart et al., 2007; Li and Denlinger, 2008), aquaporins (AQPs) (Nielsen et al., 2005; Philip et al., 2008; Clark et al., 2009; Goto et al., 2011; Yi et al., 2011) and polyols (Zachariassen, 1985; Koštal et al., 2007). The response to stress at the cellular level also involves cell cycle control and some aspects of energy metabolism (Kultz, 2003).

Rapid cold hardening (RCH) is a mechanism similar to those found during seasonal cold acclimation in insects (Teets and Denlinger, 2013a), and both chill-susceptibility and chill-tolerance (Michaud and Denlinger, 2006; Overgaard et al., 2007b) are types of rapid cold hardening (RCH). This form of thermal acclimation refers to a brief exposure to mild cold stress over a period of minutes or a few hours. The most profound changes following RCH of *Drosophila* were elevated levels of glucose and trehalose, which correlated with the improved chill tolerance of insects (Overgaard et al., 2007a) and increased levels of glycolytic metabolites in *Sarcophaga crassipalpis* (Michaud and Denlinger, 2006). Overgaard et al. (2014) found that the quantity (abundance) of a few proteins, such as glycogen phosphorylase (GlyP), had changed significantly during the RCH treatment. Moreover, RCH has been associated with changes in the expression of HSPs (Kelty and Lee, 2001; Nielsen et al., 2005; Li and Denlinger, 2008), cold-induced apoptosis (Yi et al., 2007), membrane composition or fluidity and cryoprotectant concentrations (Overgaard et al., 2005). HSPs, which act as chaperones, are induced in response to cold stress in most insects studied (Chen et al., 2005; Rinehart et al., 2007). Interestingly, not only is HSP70 implicated in cold hardening, but changes in Hsp70 gene expression have been observed during the recovery from cold stress in *Drosophila*, (Goto and Kimura, 1998) the parasitic wasp *Aphidius colemani* (Colinet et al., 2007) and the mosquito *Culex pipiens* (Rinehart et al., 2006). Recent studies suggest the role of aquaporins (AQPs) in response to cold stress (Philip et al., 2008; Philip and Lee, 2010). These proteins form water channels that facilitate the movement of water across the cell membrane and play an integral role in cell physiology under stress conditions such as cold (Goto et al., 2011). Macmillan and Sinclair

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(2011) have recently shown the loss of hemolymph homeostasis during cold exposure in the chill susceptible cricket *Gryllus pennsylvanicus*. Colinet et al. (2006) observed the consumption of the lipid reserves in *A. colemani* during cold stress.

Responses to cold stress have been well documented for many species from the temperate (Chen et al., 1987; Lee et al., 1987; Czajka and Lee, 1990) and subarctic zones (Montiel et al., 1998; Clark et al., 2009), but the physiological mechanisms of resistance to low temperatures in tropical species are still poorly understood. The studies of Chen et al. (1990) revealed that the temperate flesh flies from the Sarcophagidae family are better adapted to low temperature extremes than are their counterparts from the tropical lowlands. The survival rate and ability to rapidly respond to cold stress were consistently lower for the tropical insects than the temperate insects. Two subtropical species, *Drosophila watanabei* and *Drosophila trapezifrons*, were less tolerant to the cold than the cool-temperate species *Drosophila triauraria* (Goto and Kimura, 1998). Kostal et al. (2006) showed chilling injury accompanied by the disturbance of ion homeostasis in the coxal muscle of tropical cockroach *Nauphoeta cinerea*.

An important defense against cold stress is the accumulation of cryoprotectant low molecular weight polyhydric alcohols, such as glycerol which is one of the most important cryoprotectants (Storey and Storey, 1986). Glycerol is used by temperate flesh flies in response to short- and long-term acclimation (Lee et al., 1987) and was not detected in tropical flies (Chen et al., 1990). Other results indicated the synthesis of protective compounds, such as free amino acids (FAAs) and polyols, in the tropical beetle *Alphitobius diaperinus* when it is exposed to a fluctuating thermal regime (FTR) (Lalouette et al., 2007).

The aim of our study was to determine whether the cold stress exposure of chill sensitive tropical insects triggers a metabolic response involving fluctuations in carbohydrate and lipid levels and the synthesis of protective compounds, such as HSPs, aquaporins or polyols. The Madagascar hissing cockroach, *G. coquereliana*, was chosen as the experimental model. The natural environment for this large insect is the highly diverse subtropical climate of Madagascar, where it is colder in the mountains and particularly dry in the southern and western regions. The hottest time of the year is December, with average daily highs of 28 °C and lows of 17 °C. July is the coolest month, with average daily temperatures ranging between 21 °C and 9 °C (Britannica.com Inc., 2001; Vences et al., 2009). The average minimum temperature in this month is approximately 10 °C and a record low of 1 °C. Nevertheless, during the last 10 years, the temperature has dropped below 5 °C approximately 100 times (in the capital Antananarivo) and it took place only during night for maximum 3–4 h (WheaterSpark, 2015). Seasonal changes in temperatures and daily fluctuations between day and night occur in most ecosystems and force organism to employ mechanisms to ensure survival in stressful conditions. Thus, do tropical insects have an ecological need for cold tolerance, and if yes, what type of tolerance is the most advantageous? For example, are the behavioral mechanisms sufficient to survive cool periods, or is it better to possess physiological mechanisms of cold hardiness? Recent studies have shown that the responses of insects to repeated cold stresses are distinct from those of a single cold exposure and increase cold hardiness relative to insects receiving a single prolonged cold exposure (Marshall and Sinclair, 2012). Multiple cold exposures significantly decreased the mortality rate compared with the same length of exposure in a single sustained bout, but fecundity significantly decreased. This change was reflected in a long-term decrease in the glycogen stores of flies with multiple exposures, whereas a minor effect on triglyceride stores was observed (Marshall and Sinclair, 2010). We hypothesize that the chill sensitive cockroach *G. coquereliana*, when exposed to seasonal and daily temperature changes, has evolved some adaptive mechanisms. In our study, we examine the effect of a single cold exposure (1 × 8 h) or repeated (3 × 8 h) cold exposures at 4 °C on the thermal acclimation of *G. coquereliana*. The changes arising from the single and multiple chilling treatments were analyzed using *G. coquereliana* haemolymph and fat body tissue. The fat body is a physiological analog of the mammalian liver and plays a crucial role in intermediary metabolism of this insect; thus, the cold-induced changes may be tissue-specific. We tested the influence of low temperature on the levels of specific metabolites in the fat body tissue, such as glycogen, lipids and proteins. Moreover, we evaluated HSP (Petersen et al., 1990; Rinehart et al., 2006) and AQP expression patterns, which may play an integral role in the cold tolerance of the insect (Yi et al., 2011). We also measured the level and quality of carbohydrates in the haemolymph.

2. Materials and methods

2.1. Insects

Cockroaches (*Gromphadorhina coquereliana*) were reared under laboratory conditions at 28 °C and approximately 65% relative humidity under a 12 h light/12 h dark cycle. The animals (6 to 8 individuals) were kept in plastic boxes (15 × 30 × 20 cm), and food (lettuce, carrots, and powdered milk) and water were provided *ad libitum*. Only adult male individuals of approximately 6 cm (±0,35 cm) in size and a weight of 5.5 g (±0,59 g) were used for experiments. In the experimental conditions, the insects were placed into a cold room with a stable temperature of 4 °C and approximately 65% humidity.

In the first variant (Fig. 1), only one repeat of the cold treatment was applied; in the second variant, the insects were treated with cold three times during the three-day experimental period (8 h of cold treatment per day). The samples were collected in the morning of the next day after the last cold treatment. In all experiments, the *G. coquereliana* were anesthetized by submerging them under water for 30 min. The *G. coquereliana* haemolymph was not initially collected because of its rapid coagulation; thus, after anesthesia, insects were injected with 300 μl of anticoagulant buffer (AC) prepared according to the modified procedure of Garcia-Garcia et al. (2009), containing 69 mM KCl, 27 mM NaCl, 2 mM $NaHCO_3$, 30 mM sodium citrate, 26 mM citric acid and 10 mM EDTA, pH 7.0. *G. coquereliana* were injected under the last pair of legs using a Hamilton syringe and left for 5 min to allow the AC to spread throughout the insect body.

2.2. Analysis of glycogen content in the fat body

To isolate the glycogen, the tissue was hydrolyzed in 30% KOH for 15 min at 90 °C, according to the procedure Van Handel (1965). After isolation, the tissue was dried to a stable weight at 60 °C under vacuum (−0.9 atm), the dry mass of samples was measured and then the sample was lysed. After tissue lysis, a saturated solution of Na_2SO_4 and 70% ethanol was added to precipitate the glycogen. Next, the sample was centrifuged at 10,000 ×*g* for 5 min and washed twice with 70% ethanol. The resulting pellet was dissolved in water and shaken for 10 min at 80 °C. This solution was used to determine the glycogen content using the phenol-sulphuric acid method of DuBois et al. (1956). Oyster glycogen (Sigma-Aldrich) was used as a standard.

2.3. Evaluation of total lipids in the fat body

For control or tested insects, 3–5 mg of the fat body tissue was collected in Eppendorf tubes. After drying to a stable mass at 60 °C under vacuum (−0.9 atm), the dry weight of sample was measured. The isolation of lipids from the fat body tissue was performed according to the method of Folch et al. (1957). The tissue was homogenized in a mixture of chloroform and methanol (2:1, *v:v*) and centrifuged at 10,000 ×*g* for 10 min. The supernatant was washed three times with 0.29% NaCl, and finally, the solvent was evaporated at 30 °C under vacuum (30 °C, −0.9 atm). The pellet was dissolved again in a chloroform and methanol mixture. Aliquots of the mixture were taken, and lipid content was measured gravimetrically after evaporation of the solvent.