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Physiological and molecular mechanisms associated with cross tolerance between hypoxia and low temperature in *Thaumatotibia leucotreta*

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ABSTRACT

Biochemical adaptations allow insects to withstand exposures to hypoxia and/or hypothermia. Exposure to hypoxia may interact either synergistically or antagonistically with standard low temperature stress responses yet this has not been systematically researched and no clear mechanism has been identified to date. Using larvae of false codling moth *Thaumatotibia leucotreta*, a pest of southern Africa, we investigated the physiological and molecular responses to hypoxia or temperature stress pre-treatments, followed by a standard low temperature exposure. Survival rates were significantly influenced by pre-treatment conditions, although *T. leucotreta* shows relatively high basal resistance to various stressors (4% variation in larval survival across all pre-treatments). Results showed that mild pre-treatments with chilling and hypoxia increased resistance to low temperatures and that these responses were correlated with increased membrane fluidity (increased UFA:SFA) and/or alterations in heat shock protein 70 (HSP70); while general mechanical stress (shaking) and heat (2 h at 35 °C) do not elicit cross tolerance (no change in survival or molecular responses). We therefore found support for some limited cold hardening and cross tolerance responses. Given that combined exposure to hypoxia and low temperature is used to sterilize commodities in post-harvest pest management programs, researchers can now exploit these mechanisms involved in cross tolerance to develop more targeted control methods.

1. Introduction

Insects typically encounter a wide array of atmospheric gas conditions in their natural environments, including hypoxia at altitude, and hypoxia and hypercapnia underground, inside plant tissues or within dung pats (e.g. Frazier et al., 2006; Holter and Spangenberg, 1997). Artificially-manipulated atmosphere treatments can be used in combination with temperature stress to increase the success of postharvest disinfestation (Hallman and Denlinger, 1998; Saha et al., 2015) and form a more environmentally-friendly option to chemical disinfestation. However, exposure to hypoxia (or indeed any other stressor) may interact either synergistically or antagonistically with standard low temperature stress responses (Boardman et al., 2011) resulting in either cross tolerance and increased pest survival, or increased pest mortality – the desired outcome. Despite the potential efficacy of combined controlled atmosphere and temperature treatments for some species, the underlying mechanisms are not well understood, and it is unclear how the proposed interactions among multiple stressors might work (Mitcham et al., 2006; Boardman et al., 2011). Stress responses to temperature can be maintained over long periods of time (as adaptation/acclimation responses) or for short periods after acute exposures (reviewed in Clark and Worland, 2008; Doucet et al., 2009; Lee, 2010) directly affecting the outcome and efficacy of such treatments. While cellular mechanisms of hypoxia and anoxia survival of animals are increasingly well studied (e.g. Boutilier, 2001; Harrison and Haddad, 2011; Galli and Richards, 2014), the underlying molecular mechanisms that ensure insect survival are not especially well documented but may be inter-linked with low temperature responses to enhance survival over a broader range of environmental conditions (e.g. Morin et al., 2005).

Survival after low temperature exposure is commonly mediated by responses that alter the membrane phospholipid composition (e.g. an increase in the ratio of unsaturated to saturated fatty acids, UFA:SFA) to maintain cell membrane fluidity over a larger temperature range (Overgaard et al., 2005; Lee et al., 2006; Murray et al.,

Abbreviations: HSP, heat shock protein; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

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2007), cryoprotective low molecular weight sugars and polyols that prevent mechanical damage from ice and stabilise biological membranes (Košťál et al., 2007; Overgaard et al., 2007) and molecular chaperones from the heat shock protein family, e.g. heat shock protein 70 (HSP70), which may stabilise proteins affected by thermal stress and prevent caspase-mediated apoptosis (Jäättelä et al., 1998; Yi and Lee, 2003) and increase the efficiency of repair of chilling injury (e.g. Košťál and Tollarová-Borovanská, 2009). Although recent work has increasingly implicated ion homeostasis as a mechanism underlying recovery from chill coma stress (e.g. MacMillan et al., 2012), it remains unclear if this is a universal mechanism across insect taxa for three primary reasons. First, insects possess a wide variety of active and passive ion channels, ionoregulatory and water homeostasis mechanisms (Nation, 2002). Secondly, cause and effect of chilling is difficult to distinguish in these types of experimental assays, and thirdly, it is unclear how an ionoregulatory mechanism underlying chill coma recovery may be linked with long-term survival and fitness, or other measures of cold stress resistance. Therefore it remains reasonable to consider a diverse array of mechanisms underlying physiological responses to gas stress on low temperature survival (reviewed in Boardman et al., 2011). Exposure to anoxia affects low temperature responses in unpredictable ways (Coulson and Bale, 1991; Yocum and Denlinger, 1994; Nilson et al., 2006; Cui et al., 2014) possibly through a generalised stress response, alterations to metabolic rate or the additional effects of desiccation imposed by anoxia (e.g. Levis et al., 2012). Nevertheless, the interactions between hypoxia and low temperature stresses are not well understood from a biochemical perspective and remain largely hypothetical for many non-model taxa.

Exposure to hypoxia may either elicit or block standard stress responses which can amplify or reduce low temperature survival more than expected for a particular temperature treatment alone (reviewed in Boardman et al., 2011). Based on the key stress responses whereby temperature, mechanical stress (non-thermal, non-gas stress) and hypoxia may influence low temperature tolerance, we tested four major mechanistic hypotheses using a strong inference approach. First, a brief exposure to any stressful environment (including low or high temperature) may increase protection against subsequent low temperature by reducing water loss rates, and/or increasing the proportion of unsaturated fatty acids and heat shock protein amount – all of which are candidate mechanisms for low temperature survival or recovery. Therefore, the first two hypotheses considered are the *cold hardening hypothesis*, stating that a low temperature pre-treatment elicits protection against additional low temperature exposure (Lee et al., 1987), and is distinct from the *cross tolerance hypothesis*, which posits that mechanical stress or high temperature exposure may increase low temperature tolerance through overlapping molecular mechanisms (e.g. Bubliy et al., 2012). A third major mechanism which is proposed for cross tolerance between gas and temperature stress is that hypoxia may result in a switch to anaerobic metabolism, which reduces the amount of adenosine triphosphate (ATP) production and initiates apoptosis (e.g. Orrenius et al., 2003; Teets et al., 2008; Chiappini et al., 2009). Therefore, we consider this as the *hypoxia-induced apoptosis hypothesis*, in which hypoxia, but not low temperature stress or variation in ambient moisture, is expected to cause tissue damage and apoptosis that cannot be repaired during subsequent low temperature exposure. The fourth and final hypothesis that can be distinguished using the pre-treatments employed here is that hypoxia causes insects to open their spiracles for longer periods in order to meet their oxygen demands (e.g. Hetz and Bradley, 2005; Alonso et al., 2005) which leads to an increased respiratory water loss rate. The insect must thus actively regulate water loss or face dehydration and/or loss of ion homeostasis (e.g. Groenewald et al., 2014) which, over prolonged periods, may result in death. Indeed, regulation of body water content and tissue ion concentrations are essential during low temperature exposures and recovery (e.g. MacMillan and Sinclair, 2011; MacMillan et al., 2012) and therefore any irreparable damage to these regulatory mechanisms may be fatal to insects exposed to additional low temperature stress. Hypoxia may cause insects to keep their spiracles open for longer periods in order to meet necessary oxygen demand (Hetz and Bradley, 2005), which would also increase respiratory water loss. Therefore, we also considered and tested a *respiratory water loss hypothesis*, stating that hypoxia increases whole-animal water loss rates, which in turn disrupt ion- and osmoregulation. Depending on the organism's specific physiological responses to desiccation, this may lead to reduced low temperature tolerance.

In order to distinguish among these hypotheses, we developed a series of experimental predictions to differentiate among them using a suite of physiological and biochemical assays (Fig. 1). In all cases, predictions for the anticipated result 2 h after pre-treatment were outlined and the four hypotheses were differentiated using a strong-inference approach (Table 1). Here, we report on the results of these cross tolerance experiments using larvae of the false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), which is a major pest of quarantine importance globally, and a pest that directly affects agricultural trade and food security in sub-Saharan Africa.

2. Materials and methods

2.1. Animals

Larvae of false codling moth *T. leucotreta* (Meyrick) (Lepidoptera, Tortricidae) were obtained from Cedar Biocontrol Insectary, XSIT (Pty) Ltd, Citrusdal, South Africa and maintained in the laboratory in 5 L containers in an incubator (±25 °C, L:D 12:12 h; YIH DER growth chamber, model LE-539, SCILAB instrument CO Ltd., Taiwan OR) (Boardman et al., 2012). Fourth to fifth instar larvae were placed individually in 1.5 mL tubes with air holes for the duration of the experiment, and were fasted for 24 h prior to experimental pre-treatment in an attempt to remove the confounding effects of gut contents. Individual experiments were performed over five weeks with $n = 600$ per experimental pre-treatment (at least 100 individuals were removed at each timepoint – details in Section 2.2 Experiments, Fig. 1b), and individuals from this set were used to measure survival, HSP70 and body water content and body lipid content. Experiments were repeated with $n = 100$ per pre-treatment group to obtain fresh samples for cell viability (total $n = 54$) and freshly frozen samples for cryoprotectant and membrane phospholipid composition analyses (total $n = 99$ and 108 respectively), as well as to increase sample sizes for survival (minimum $n = 182$) and assess pupation (minimum $n = 72$) and emergence. Although *T. leucotreta* has high basal tolerance to the pre-treatments employed (i.e. relatively high average survival), the experimental pre-treatments showed small but highly significant effects on larval survival.

2.2. Experiments

After 24 h fasting, larvae were subjected to one of seven experiments (Fig. 1a). Each experiment consisted of one of seven pre-treatments, followed by 2 h recovery at 25 °C, a standard low temperature exposure of −1 °C for 10 h and 26 h recovery period at 25 °C (Fig. 1b). The pre-treatments were: handling control (2 h at 25 °C, relative humidity (RH) > 40%), mechanical stress (shaking for 4 h at 25 °C; RH > 40%), low temperature (2 h at 0 °C; RH > 50%), high temperature (2 h at 35 °C; RH > 25%), acute