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Elevated chaperone proteins are a feature of winter freeze avoidance by larvae of the goldenrod gall moth, *Epiblema scudderiana*

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ABSTRACT

Winter survival for many insect species includes a need to maintain metabolic homeostasis and structural/functional integrity of macromolecules not only over a wide range of cold temperatures but also in response to rapid temperature change. Chaperones are well-known to protect/stabilize protein structure with regard to heat stress but less is known about their potential involvement in long-term protection of the proteome at subzero temperatures. The present study assessed the participation of chaperone proteins in the cold hardiness of larvae of the goldenrod gall moth, *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae), monitoring changes in nine proteins over the winter months as well as their responses to laboratory cold acclimation or anoxia exposure. Four heat shock proteins (HSPs: Hsp110, Hsp70, Hsp60, Hsp40), three glucose-regulated proteins (GRPs: Grp78, Grp 94, Grp170) and the tailless complex polypeptide 1 (TCP-1) as well as the heat shock transcription factor (HSF1) were investigated. In general, all were significantly elevated in larvae collected from an outdoor site between October and March, as compared with September values, and chaperone levels were reduced again in April. The October to March interval also includes the period of diapause followed by cold quiescence in the species. Relative expression of Hsp70, Hsp60 and Hsp40 rose by 2–3-fold, GRPs increased 1.5–3-fold, and levels of active (hyperphosphorylated) HSF1 increased by 4–4.8-fold over the midwinter months. Chilling from 15 °C to 4 °C in the laboratory upregulated Grp78 protein content that remained high as temperature was further reduced to −4 °C and then −20 °C whereas Hsp110, Hsp70 and HSF1 levels increased when larvae were exposed to −4 °C and −20 °C. Grp170 (also known as oxygen-regulated protein 150) was the only chaperone that increased significantly in the larvae in response to anoxia exposure at 4 °C. The data also indicated that multiple subcellular compartments received enhanced protection for their proteome since upregulation of chaperones included proteins known to occur in cytosolic (Hsp40, Hsp70), mitochondrial (Hsp60) and endoplasmic reticulum (Grp170) locations. Overall, the data indicate that chaperones have a significant role to play in the winter cold hardiness of *E. scudderiana* and identify declining temperatures (and perhaps also oxygen restriction) as potential modulators of chaperone production. The data add support to a relatively understudied area of insect cold hardiness – the long-term protection and stabilization of the proteome over the winter months.

1. Introduction

Larvae of the goldenrod gall moth larvae, *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae) grow within elliptical stem galls on goldenrod plants and the fifth instar of this univoltine species overwinters (Miller, 1976). Pupation and adult emergence occur in the spring. Locked in the woody stalk of the plant (that may grow to > 1 meter tall), the larvae are often exposed above the snowpack to low subzero temperatures. This species uses the freeze avoidance strategy of winter hardiness and has been well-studied for investigations into the metabolic and enzymatic adaptations that support this strategy (for review: Storey and Storey, 1991, 2012, 2015). In southeastern Ontario (Canada) autumn cold hardening includes suppression of larval supercooling point to about −38 °C and the accumulation of glycerol as a cryoprotectant with levels rising to well over 2 M, representing as much as 18% of total body mass (Rickards et al., 1987). Two other features that contribute to freeze avoidance by *E. scudderiana* larvae are a reduction in body water content over the autumn months to minimize

Abbreviations: HSP, heat shock protein; HSF1, heat shock factor 1; GRP, glucose-regulated protein; TCP, tailless complex polypeptide; CCT, chaperonin containing t-complex polypeptide-1; HRE, hypoxia response element; Orp150, oxygen-regulated protein; PVDF, polyvinylidene difluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UPR, unfolded protein response

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the amount of freezable water remaining in the larvae and lining the gall with silk that can help to prevent nucleation of body fluids via contact with environmental ice (Rickards et al., 1987; Storey and Storey, 2012). Winter survival by insects is often complemented by diapause that allows for fuel/energy savings via a regulated suppression of growth and development over the midwinter months (Denlinger, 2002; Hahn and Denlinger, 2011) and *E. scudderiana* has a 3–4 month diapause that lasts until about mid- to late February in southeastern Ontario and is followed by quiescence until temperatures warm sufficiently in the spring to allow pupation (Storey and Storey, 2012).

A common animal response to environmental stress is the production of chaperone proteins that help to protect the cellular proteome through actions that include: (a) inhibiting aggregation of unfolded proteins, (b) guiding folding of naïve proteins or re-folding of malfolded proteins, and (c) aiding intracellular trafficking and assembly of proteins (Gething and Sambrook, 1992; Feder and Hofmann, 1999). For cold-hardy species that overwinter, a critical issue is long term viability over many months, including survival using only "on board" fuel reserves, minimizing energy expenditures on ATP-expensive activities such as protein turnover (i.e. both synthesis and degradation), and stabilizing the cellular proteome against environmental insults (Hahn and Denlinger, 2011; Storey and Storey, 2012, 2015). The strong metabolic rate depression afforded by diapause is a crucial contributor to long term viability whereas chaperones and other protectants (e.g. glycerol) help to stabilize and preserve the functionality of cellular macromolecules. The best known chaperones are the heat shock proteins (HSPs) that respond to many different types of stress and are an integral part of the broader cell stress response (Kültz, 2005). Both constitutive and stress-inducible HSPs occur and well-known groups include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsps with sizes < 30 kDa. Various other chaperones exist such as glucose-regulated proteins (GRPs) (e.g. Grp170, Grp95, Grp78) that were originally named due to their strong up-regulation in cells cultured in glucose-free medium (Shiu et al., 1977). These GRPs respond to stresses that disrupt protein synthesis and processing in the endoplasmic reticulum (ER) and are components of the unfolded protein response (UPR), a concerted response to ER stress. GRP170, that is also known as oxygen-regulated protein 150, is of additional interest as a potential indicator of low oxygen stress in the larvae at low temperature. Another chaperone, tailless complex polypeptide 1 (TCP-1) is related to Hsp60 and participates in folding a wide range of proteins, particularly those associated with the cytoskeleton (e.g. actin, tubulin) (Kayukawa et al., 2005; Kayukawa and Ishikawa, 2009).

Although thousands of studies have evaluated HSP responses to high temperature exposures and many other stresses, few have analyzed HSP responses to low temperature and those that have mainly feature species that are not naturally cold hardy. For example, among insects, studies of HSP responses to cold in *Drosophila melanogaster* dominate and, furthermore, most studies focus on chaperone responses to short-term cold shock (e.g. Burton et al., 1988; Colinet et al., 2010; Stetina et al., 2015; Tungjitwitayakul et al., 2015). Recently, however, various authors have begun to show that chaperones contribute to the natural winter diapause and/or cold hardiness of insects (Sonoda et al., 2006; Kayukawa et al., 2005; Chen et al., 2006; Kayukawa and Ishikawa, 2009; Rinehart et al., 2006, 2007; Yu et al., 2016); for review also see Storey and Storey (2011) and King and MacRae (2015). Indeed, work by our group has shown that larvae of the freeze tolerant goldenrod gall fly, *Eurosta solidaginis*, that are often found in the same fields (or even on the same plants) as *E. scudderiana*, up-regulate HSPs both during the winter season when larvae are in diapause and in response to laboratory cold-acclimation (Zhang et al., 2011).

The present study evaluates the chaperone responses to stress by *E. scudderiana* whose caterpillars utilize the freeze avoidance strategy of winter cold hardiness. The responses of four heat shock proteins (Hsp110, Hsp70, Hsp60, Hsp40) were tracked in outdoor larvae from September to April along with the heat shock transcription factor 1 (HSF1) and four other chaperones (Grp110, Grp95, Grp78, TCP-1). Chaperone and HSF1 responses to controlled low temperature or anoxia exposures in the laboratory were also analyzed.

2. Materials and methods

2.1. Animals

Galls containing the fifth (and final) instar larvae of *E. scudderiana* were collected in mid-September 1999 from goldenrod plants in fields on the outskirts of Ottawa (Ontario, Canada). Both collection sites (approximately latitude N45°17′, longitude W75°40′) and the holding site for larvae that were held outdoors over the winter were less than 10 km from the MacDonald-Cartier International Airport in Ottawa. Mean monthly minimum temperatures and extreme minima were taken from records for the 1999–2000 winter at the airport weather station that are available at http://climate.weather.gc.ca/. Also see Zhang et al. (2011) for daily minimum and maximum temperatures over this period. Half of the galls were placed in cloth bags and hung on a fence where they experienced ambient temperatures over the winter months. These were sampled in the second week of the month from September to April. At each sampling time, a subgroup of galls was brought into the laboratory at ~9:00 a.m. and briefly stored in an incubator set to the current outdoor temperature. Galls were opened as soon as possible and larvae were flash-frozen in liquid nitrogen and then stored at −80 °C.

Other September-collected galls were brought into the laboratory and acclimated to 15 °C in incubators for two weeks. After that time, some galls were opened and larvae were sampled as above. The larvae in remaining galls were then subjected to either cold or anoxia exposures. For cold exposures, larvae in their galls were treated as follows: (a) an acute drop from 15 °C down to 4 °C for 7 days, followed by (b) another acute decrease from 4 °C to −4 °C for 1 or 7 days, and finally (c) an acute decrease from −4 °C to −20 °C for 7 days. At each temperature, insects were sampled after 7 days exposure; the −4 °C group was also sampled after 1 day at this temperature.

For anoxia exposure, 15 °C-acclimated larvae in their galls were acutely cooled to 4 °C and acclimated for 2 weeks. Groups of larvae were then quickly removed from their galls and placed in open petri dishes (held on ice) with a piece of moist filter-paper to prevent desiccation. Open dishes were placed individually in 1.5 L clear plastic containers (one for each sampling time), then lids were screwed on. Each lid had two ports installed, one to introduce nitrogen gas and one to vent the gas. Containers were held on ice and flushed (through the ports) with 100% nitrogen gas for 20 min and then both ports were closed, lids and ports were sealed over with parafilm, and containers were replaced in 4 °C incubators. Larvae were sampled after 1, 4 or 24 h of nitrogen gas exposure by rapidly opening a container, removing the petri plate and immediately dropping the larvae into liquid nitrogen.

2.2. Sample preparation and protein content

Whole frozen larvae (6 per sample) were weighed and quickly homogenized 1:2 w:v in ice-cold buffer that contained inhibitors of endogenous protein phosphatases and kinases: 20 mM HEPES, 400 mM NaCl, 20% v:v glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4. Protease inhibitors were also added: 1 μM each of phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and benzamidine. Samples were centrifuged at 10,000*g* at 4 °C for 10 min and supernatants were transferred to a fresh tube. Soluble protein concentration was determined by the Coomassie blue dye-binding method using the Bio-Rad prepared reagent with bovine serum albumin as the standard. Aliquots of supernatant were then mixed 1:1 (v:v) with 2x sample buffer containing 100 mM Tris-HCl pH 6.8, 4% w:v SDS, 20% v:v glycerol, 5% v:v β-mercaptoethanol and 0.2% w:v bromophenol blue. Samples were boiled for 5 min, chilled and then