



# 08 Insects in winter: cold case files

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## Abstract

Larvae of two goldenrod gall formers have been extensively analysed by our laboratory to compare and contrast the biochemical adaptations needed for two winter cold hardiness strategies: freeze avoidance (*Epiblema scudderiana*) and freeze tolerance (*Eurosta solidaginis*). New directions in our recent research have evaluated three phenomena that are discussed in this chapter: (1) cellular protection provided by the seasonal accumulation of chaperone proteins, (2) the responses of mitochondria in winter, and (3) the expression of the hypoxia-inducible transcription factor and its role in the preparation for freeze-induced ischaemia.

## Introduction

Winter survival for many insect species requires biochemical and physiological adaptations that preserve life at temperatures far below 0°C. Two basic strategies for subzero survival occur: freeze avoidance and freeze tolerance (Storey & Storey 1992). Freeze-avoiding insects achieve deep supercooling, using mechanisms that include the production of antifreeze proteins and the accumulation of high concentrations of polyhydric alcohols as protectants. Freeze-tolerant insects typically endure the conversion of about 65% of total body water into extracellular ice using ice nucleators to trigger and direct ice formation and employing carbohydrate protectants to preserve the liquid state of the cytoplasm. Research in our laboratory uses as models two insect species that overwinter as last instar larvae inside stem galls on Goldenrod (*Solidago* spp.). Caterpillars of the moth *Epiblema scudderiana* (Lepidoptera, Olethreutidae) use the freeze-avoidance strategy, remaining liquid down to -38°C in the Ottawa area and accumulating huge amounts of glycerol (as much as 18% of total body mass) as their protectant. Larvae of the fly *Eurosta solidaginis* (Diptera, Tephritidae) are freeze-tolerant, freezing at temperatures below about -8°C to -10°C and accumulating a mixture of glycerol and sorbitol as protectants. (See [www.carleton.ca/~kbstorey](http://www.carleton.ca/~kbstorey) for photos and life history information on these species.)

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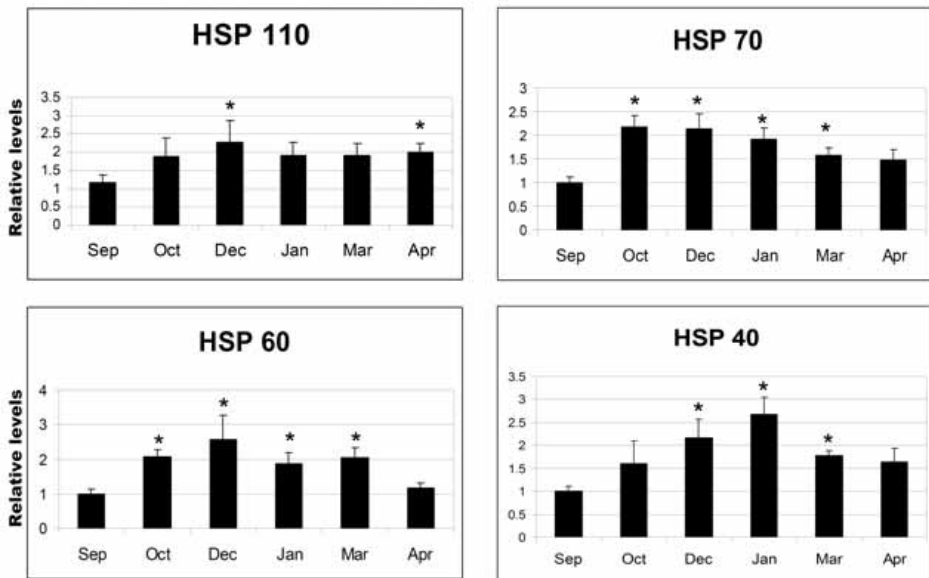
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A major focus of our laboratory is metabolic rate depression – the mechanisms that allow organisms to enter a state of hypometabolism in response to environmental stresses, including low temperature, low oxygen or low water availability (Storey & Storey 2007). Collectively, our studies on multiple systems have identified a conserved suite of adaptive mechanisms that regulate entry into or exit from hypometabolism and provide stress resistance, maintain viability, and ensure life extension over extended periods of hypometabolism. Common themes that we have found across phylogeny include (a) a central role for reversible protein phosphorylation in regulating transitions to/from hypometabolic states by suppressing the activities of key enzymes and functional proteins and reprioritising energy use by different cellular processes; (b) selective up-regulation of gene expression and protein synthesis against a background of overall suppression of transcription and translation; (c) enhancement of cell preservation mechanisms, including antioxidant defences, protein chaperones, and low molecular weight protectants; and (d) appropriate adjustments to central fuel metabolism, including, as needed, provisions to deal with end-product accumulation and buffering. To some extent, studies with cold hardy organisms, both invertebrate and vertebrate, have been slower to explore many of these themes of hypometabolism and have focused more extensively instead on the mechanisms of cryoprotection needed for survival below 0°C, primarily low molecular weight protectants, antifreeze proteins and ice nucleators. This is not surprising since understanding the unique natural mechanisms of cryoprotection is both a fascinating topic itself and also has a huge range of practical applications (Margesin *et al.* 2007). Nonetheless, it is now well-appreciated that many cold-hardy organisms use hypometabolism as an integral part of their winter survival strategy. Many insect species, including the two that we work on, are in diapause for several months over the winter. Hence, in our recent studies we have been broadening our approach to insect cold hardiness to explore several themes in hypometabolism and stress resistance, comparing and contrasting the freeze-avoiding and freeze-tolerant species. This article briefly summarises some of our findings in three areas.

## Case 1: Chaperone proteins

Chaperone proteins have extremely important roles, including folding of nascent proteins, refolding of misfolded proteins, preventing or correcting inappropriate aggregation of proteins, and aiding the movement of proteins to their correct subcellular locations. The literature on chaperone proteins is massive and it is well-known that the expression of several kinds of chaperones, including heat-shock proteins (HSPs) and glucose-regulated proteins (GRPs), is not only constitutive but strongly enhanced under a variety of stress conditions. In studying species that naturally experience stresses, including anoxia, dehydration, cold and freezing, it is becoming increasingly apparent to us that these systems naturally enhance a variety of defensive actions to protect cells and provide long-term viability until the stress is removed. Two of the most prominent and widespread defensive actions are enhanced antioxidant defences and elevated levels of chaperone proteins (Storey & Storey 2007). Indeed, when stress tolerance also includes entry into a hypometabolic state, it appears that these defensive actions are critical across phylogeny as mechanisms of preserving viability and life extension. In insects, the heat-shock response is well known in *Drosophila* and many other species, but more physiologically relevant studies, including those by the Denlinger laboratory, show that

HSP accumulation is a widespread component of diapause in at least four orders of insects (Rinehart *et al.* 2007). Sonoda *et al.* (2006) also found that HSP90 was upregulated by cold acclimation in non-diapausing larvae of the Rice Stem Borer *Chilo suppressalis*, but not in diapausing larvae that already had elevated levels of HSP90. Similar results were found when expression of the inducible HSP70 was assessed in the onion maggot (Chen *et al.* 2006). We predicted then that chaperone proteins should have a role to play in the winter survival of our



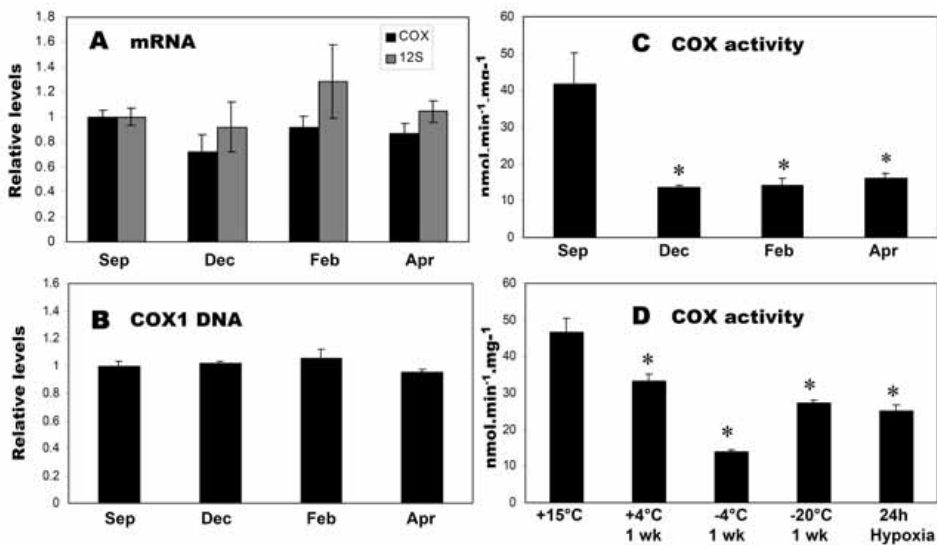
**Figure 1.** Seasonal changes in the levels of four heat-shock proteins in larvae of the freeze-avoiding gall moth *E. scudderiana*. Galls were collected in September–October, held outdoors in cloth bags over the winter, and sampled at selected times over the winter. HSPs were detected via immunoblotting using mammalian antibodies. Confirmation that the antibodies cross-reacted with the correct protein came from two tests: (a) 2-D gel electrophoresis demonstrated that each antibody cross-reacted with only a single protein, and (b) competition experiments with duplicate blots incubated with or without added antigen peptide (also called immunising peptide) showed that in the presence of the peptide the HSP band disappeared on the gel. Bands were visualised by chemiluminescence and band intensities were quantified using a Chemi-Genius (Syngene, Frederick, MD). Gels were then stained with Coomassie blue and the intensity of a strong protein band (distant from the molecular mass of the HSP being assessed) that did not change among experimental groups was quantified. To correct for minor differences in protein loading, HSP band intensities were then normalised against the corresponding intensity of the Coomassie band in the same lane. Data show the means  $\pm$  SEM ( $n = 4$  independent trials) of normalised band intensities for each experimental group. \* Significantly different from the September value as determined by Student's *t*-test,  $p < 0.05$ . Modified from Zhang (2006).

two cold-hardy insect species and in recent studies we have analysed HSPs in both our freeze-avoiding and freeze-tolerant model animals (Zhang 2006). Figure 1 shows the levels of four HSPs in the freeze-avoiding larvae of *E. scudderiana* over the winter months. All four proteins were significantly elevated by 2–2.5-fold over September values for several months during midwinter. HSP110 is an abundant chaperone; it is not well-characterised but is known to have a role in preventing thermal aggregation of proteins. Inducible ATP-dependent HSP70 and its partner protein HSP40 were both elevated in concert. The mitochondrial ATP-dependent chaperone HSP60 also increased in *E. scudderiana*. Analysis of the same four proteins in the freeze-tolerant larvae of *E. solidaginis* showed similar increases of 40–70% for HSP110, HSP70 and HSP40 over the midwinter months, but the mitochondrial HSP60 responded differently. The levels of this chaperone were reduced by 50–60% between December and March. This correlates with the changes in mitochondrial abundance in the two species, the freeze-avoiding insect apparently maintaining its mitochondria over the winter, whereas the freeze-tolerant species reduces mitochondrial content by about 50%. We further analysed the response of the heat-shock transcription factor (HSF1). The active trimeric phosphorylated form of HSF1 is translocated to the nucleus and can bind to the heat-shock element in the promoter regions of heat-shock genes. Total HSF1 protein content was increased by about 2-fold in mid-winter in the freeze-avoiding larvae of *E. scudderiana* but was relatively unchanged in freeze-tolerant *E. solidaginis* larvae. However, the amount of active HSF1 was elevated in early autumn in both species, rising by about 1.5-fold in *E. solidaginis* in October and 3–4-fold in *E. scudderiana* in October and December, as compared with September values. Active HSF1 also responded strongly to both anoxia and freezing stresses in *E. solidaginis*. These data suggest that the enhanced production of HSPs is a component of cold hardening and/or entry into diapause in the two goldenrod gall formers mediated by an early increase in HSF1 levels in mid-autumn.

## Case 2: Mitochondria in winter

Entry into a hypometabolic state such as diapause, as well as the effects of winter cold on ectotherms, both strongly reduce the rate of oxygen consumption by organisms. Furthermore, freezing imposes anoxic and ischaemic stresses on cells that make oxygen consumption impossible. The question then is whether cold-hardy insects make adjustments to their mitochondrial capacity in response to the reduced demands for oxygen-based metabolism. Do they degrade a significant portion of their mitochondria over the winter by a process such as autophagy or do they maintain the organelles but suppress their activity by either passive (e.g. temperature-dependent) or active (e.g. reduced amounts of functional proteins, reversible protein phosphorylation of key enzymes) methods? Kukul *et al.* (1989) reported an extreme reduction in mitochondrial numbers in the high Arctic caterpillar *Gynaephora groenlandica* when comparing -15°C-acclimated animals vs 15°C controls, but a more recent analysis by Levin *et al.* (2003), which measured 16S rRNA levels, concluded that hibernating caterpillars had about half the mitochondrial DNA of summer-active animals. Levin *et al.* (2003) similarly quantified mitochondrial DNA content of summer- and winter-collected *E. solidaginis* and also found a reduction by about half in winter larvae. To examine this more closely, we

evaluated multiple parameters of mitochondrial function in both of our model species (McMullen & Storey 2007). Figure 2 shows data for the freeze-avoiding species *E. scudderiana*. The maximal activity of the key mitochondrial enzyme, cytochrome c oxidase (COX), decreased over the winter in the larvae to levels about one-third of September values. However, COX activity can be controlled by translational and post-translational means, so we also evaluated two more “permanent” markers of mitochondrial numbers and found that both of these were unchanged in this species over the winter months. Three of the subunits of COX are encoded by the mitochondrial genome and an evaluation of the genomic DNA content of one of these showed no change in COX 1 genomic DNA content over the winter (data normalised against nuclear genomic  $\alpha$ -tubulin DNA content). Furthermore, the transcript levels of two mitochondrially encoded genes, COX 1 mRNA and 12S rRNA, were also unchanged over the winter; these represent genes encoded by the heavy and light chains of the mitochondrial genome, respectively. This strongly suggests, therefore, that the freeze-avoiding species does not decrease its content of mitochondria over the winter. This seems reasonable when we consider that a



**Figure 2.** Evaluation of mitochondrial status in larvae of the freeze-avoiding gall moth *E. scudderiana* over the winter months. A. Transcript levels for mitochondrially encoded genes, cytochrome oxidase (COX) subunit 1 mRNA and 12 S rRNA. Levels were normalised against the mRNA transcript levels of  $\alpha$ -tubulin, a constitutively-expressed nuclear gene. B. COX 1 genomic DNA content, normalised against  $\alpha$ -tubulin genomic DNA content. C. Maximal enzymatic activity of COX in the larvae. D. COX activity as a function of acclimation temperature or anoxia exposure. Data are means  $\pm$  SEM,  $n = 3$  independent samples. \* Significantly different from the September or 15°C control values as determined by Student's  $t$ -test,  $p < 0.05$ . Modified from McMullen and Storey (2008).

freeze-avoiding species can use aerobic lipid oxidation by mitochondria to fuel metabolism throughout the winter months, even at deep subzero temperatures, because the insect remains in a liquid (supercooled) state. Indeed, the activities of selected mitochondrial enzymes involved in fatty acid oxidation (3-hydroxyacyl-CoA dehydrogenase, thiolase) increased 2–4-fold over the winter in *E. scudderiana* (Joanisse & Storey 1996) whereas activities of several other mitochondrial enzymes (citrate synthase, glutamate dehydrogenase, NAD-isocitrate dehydrogenase, carnitine palmitoyl transferase, malic enzyme) decreased by about 50%, similar to the effects on COX 1 activity. Hence, it appears that the freeze-avoiding species resculpts its mitochondrial enzyme profile over the winter, perhaps by a combination of changes in protein levels and post-translational regulation of enzyme activities, but does not undergo mitochondrial degradation.

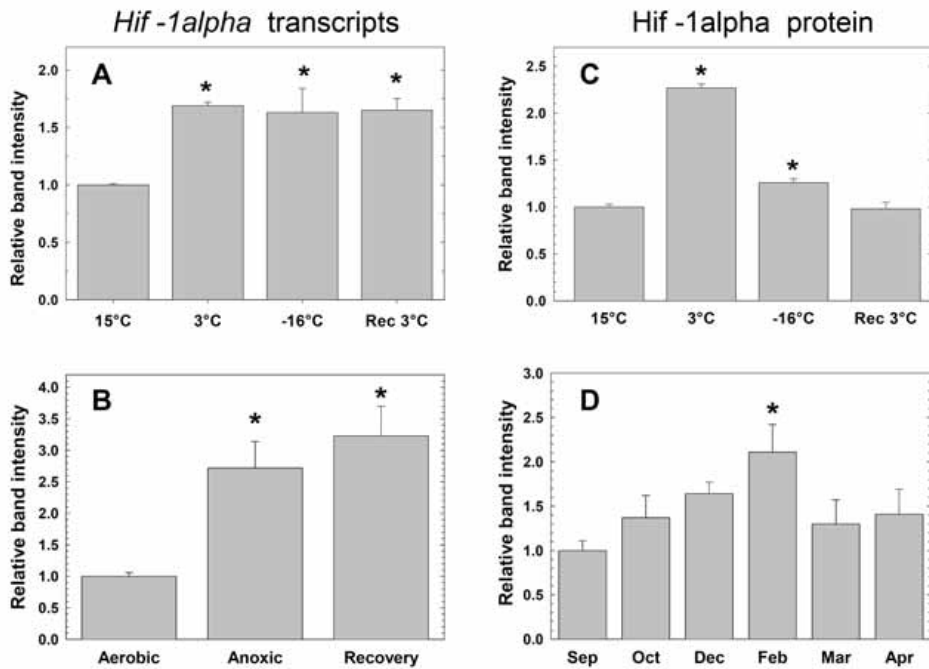
The situation in the freeze-tolerant species *E. solidaginis* is different. Levin *et al.* (2003) found that frozen, winter-collected larvae had only about half of the mitochondrial DNA content of summer-active larvae held at 15°C. Our analysis showed that COX maximal activity was reduced over the winter months to 30–50% of the value seen in September-collected larvae (McMullen & Storey 2008). Furthermore, the maximal activities of all other mitochondrial enzymes that have been assessed were also reduced by ~50% in *E. solidaginis* over the midwinter months, including those involved in fatty acid oxidation (Joanisse & Storey 1994, 1996). Levin *et al.* (2003) also showed that the metabolic rate (oxygen consumption) of the larvae over the autumn/winter months was only 35–40% of the September value (all measured at 15°C). All this evidence suggests that mitochondrial capacity is reduced over the winter months in *E. solidaginis*, probably by a reduction in mitochondrial numbers. For a freeze-tolerant insect this makes sense. While the larvae are frozen for many weeks during the winter, metabolism is anaerobic with lactate and alanine accumulating as end products (Storey & Storey 1985). Lipid fuels cannot be used when oxygen supply is interrupted in frozen animals and, furthermore, mitochondria may be vulnerable to structural or metabolic damage arising from osmotic, volume, oxygen and oxyradical stresses associated with freeze/thaw cycles. Therefore, it may be beneficial for *E. solidaginis* larvae to reduce their number of mitochondria over the winter months.

### Case 3: Hypoxia-inducible factor

Extracellular freezing causes ischaemia and anoxia and over time results in a decrease in ATP content in frozen *E. solidaginis* and an increase in glycolytic end products (Storey & Storey 1985). We wondered then whether freeze-tolerant species enhance their capacity to deal with oxygen limitation as part of cold hardening. The hypoxia-inducible transcription factor (HIF-1) is well-known to coordinate multiple cellular responses to low oxygen, falling into two general categories: those that enhance pathways of non-oxygen dependent ATP production and those that enhance oxygen delivery to tissues. Oxygen delivery cannot be enhanced in a frozen system but glycolytic capacity can and HIF-1 could have this function. Indeed, we have previously shown that cold exposure elevates the activities of glycogen phosphorylase, hexokinase and phosphofructokinase in the larvae (Storey & Storey 1981). This can have two key effects

on the larvae: (1) glycolytic capacity is elevated in an anticipatory way at near 0°C temperatures as a prelude to the need for anaerobic ATP generation in the frozen larvae, and (2) glycolytic capacity is also elevated to support cryoprotectant synthesis near 0°C (sorbitol production is triggered below 5°C). How then does HIF-1 respond in *E. solidaginis*?

HIF-1 is a dimeric protein that is primarily regulated by the availability of its alpha subunit (the beta subunit being constitutive), which is stabilised at low oxygen, but rapidly targeted for destruction by oxygen-dependent proline hydroxylation when oxygen levels are high.



**Figure 3.** Modulation of the hypoxia inducible transcription factor in *Eurosta solidaginis*. A. Changes in *hif-1α* mRNA transcripts in response to temperature change comparing 15°C controls with larvae given serial exposures to chilling at 3°C for 24 hr, freezing at -16°C for 24 hr, and thawing recovery (Rec) at 3°C for 24 hr. Bands for *hif-1α* were normalised against the corresponding band for  $\alpha$ -tubulin band and then intensities were expressed relative to the values in 15°C larvae. B. Effects of anoxia exposure and subsequent aerobic recovery on *hif-1α* transcript levels comparing aerobic controls, anoxia under N<sub>2</sub> gas for 24 hr and aerobic recovery for 24 hr (Rec); all treatments are at 15°C. C. Effects of low temperature and freezing on HIF-1 $\alpha$  protein levels, assessed by immunoblotting, in the same experimental groups as in part A above. D. Seasonal changes in HIF-1 $\alpha$  protein levels as determined by Western blotting. Data are means  $\pm$  SEM, n = 3-5 independent trials. \* Significantly different from the corresponding control or September value, as assessed by Student's *t*-test,  $p < 0.01$ . Modified from Morin *et al.* (2005).

Hence, only under low oxygen conditions can the active dimer persist for long enough to activate gene transcription. In a recent study we analysed HIF-1 $\alpha$  responses to cold hardening, freezing and anoxia in *E. solidaginis* (Morin *et al.* 2005). Cloning and sequencing of gall fly larva *hif- $\alpha$*  showed that it had strongest similarity to *Drosophila* HIF- $\alpha$  and Western blotting also indicated that the full size of the gallfly protein was around 1,500 amino acids, similar to the fruit fly protein (Nambu *et al.* 1996), but about twice as big as vertebrate HIF-1 $\alpha$ . Analysis of HIF-1 $\alpha$  responses to anoxia and cold exposures, as well as seasonal changes, are shown in Figure 3. Anoxia exposure of the larvae at 15°C resulted, not unexpectedly, in a strong increase in *hif-1 $\alpha$*  transcript levels by 2.7-fold. Cold exposure had the same effect; an acute drop from 15°C to 3°C elevated transcripts by 1.6-fold and levels remained high when the larvae were subsequently frozen at -16°C. HIF-1 $\alpha$  protein levels also increased sharply by 2.3-fold when larvae were transferred from 15° to 3°C but were reduced somewhat at -16°C. Furthermore, HIF- $\alpha$  protein in the larvae rose progressively over the winter months to peak in February. Hence, these results give significant support for the idea that HIF-1 mediated gene expression has a role to play in winter cold hardiness. The most interesting result of this analysis is the effect of cold exposure at 3°C (not freezing) on both transcript and protein levels of HIF-1 $\alpha$ . Levels are elevated before the insect actually freezes. This could allow HIF-1 mediated preparatory measures to be put in place before freezing since the energy expenditures of new protein synthesis would likely be prohibitive once the larvae are frozen and oxygen-dependent ATP production is unavailable.

## Concluding remarks

As much as we know about insect cold hardiness, there are still many lessons to be learned. We are currently very interested in the theme of transcription factor control of the metabolic responses to cold and freezing. Transcription factors provide the means to modulate the expression of a suite of genes to achieve balanced and coordinated expression of a diverse group of protein products that are united in achieving a specific goal. Transcription factor control can range from influence over families of proteins (like HSF control of HSPs) to broad-based readjustments of many aspects of metabolism (like the huge umbrella group of genes/proteins, including other transcription factors, which are controlled by HIF-1). Indeed, transcription factor profiling is providing us with many new leads into the broad range of metabolic functions that must be adjusted and regulated to achieve biochemical adaptation; this concept, as applied to frog freeze tolerance, is discussed in detail in another chapter in this volume (Storey 2008). Of current interest to us in our continuing studies of insect cold hardiness are the roles of FOXO (forkhead box class O) transcription factors in mediating cell cycle arrest and stress resistance (potentially analogous to their roles in *Caenorhabditis elegans* dauer formation; Burgering & Kops 2002) and the roles of ATFs (activating transcription factors) in mediating responses that minimise endoplasmic reticulum stress and coordinate protein synthesis and folding (similar to the mammalian situation; Harding *et al.* 2002) with respect to changes in energy availability and temperature in cold-hardy species.

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