Insect cold hardiness: Metabolic, gene, and protein adaptation 1

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REVIEW / SYNTHÈSE

Insect cold hardiness: metabolic, gene, and protein adaptation[1]

Kenneth B. Storey and Janet M. Storey

Abstract: Winter survival for thousands of species of insects relies on adaptive strategies for cold hardiness. Two basic mechanisms are widely used (freeze avoidance by deep supercooling and freeze tolerance where insects endure ice formation in extracellular fluid spaces), whereas additional strategies (cryoprotective dehydration, vitrification) are also used by some polar species in extreme environments. This review assesses recent research on the biochemical adaptations that support insect cold hardiness. We examine new information about the regulation of cryoprotectant biosynthesis, mechanisms of metabolic rate depression, role of aquaporins in water and glycerol movement, and cell preservation strategies (chaperones, antioxidant defenses and metal binding proteins, mitochondrial suppression) for survival over the winter. We also review the new information coming from the use of genomic and proteomic screening methods that are greatly widening the scope for discovery of genes and proteins that support winter survival.

Key words: freeze tolerance, freeze avoidance, insect winter survival, cryoprotectant metabolism, gene expression, metabolic rate depression, chaperones, antioxidants, genomics, proteomics, signal transduction.

Résumé : La survie à l'hiver chez des milliers d'espèces d'insectes dépend de stratégies adaptatives pour la résistance au froid. Les insectes utilisent généralement deux mécanismes fondamentaux (l'évitement du gel par la surfusion profonde et la tolérance au gel dans laquelle les insectes endurent la formation de glace dans les espaces des fluides extracellulaires), bien que des stratégies additionnelles (déshydratation de cryoprotection, vitrification) soient aussi employées par quelques insectes polaires dans des milieux extrêmes. Notre rétrospective évalue les recherches récentes sur les adaptations biochimiques qui permettent la résistance au froid. Nous examinons de nouvelles données sur la régulation de la biosynthèse des cryoprotecteurs, les mécanismes de dépression du taux métabolique, le rôle des aquaporines dans le mouvement de l'eau et du glycérol et les stratégies de préservation des cellules (chaperons moléculaires, défenses antioxydantes et protéines liant les métaux, suppression mitochondrienne) pour la survie pendant l'hiver. Nous résumons les nouvelles informations provenant de l'emploi de méthodes de génomique et de criblage protéomique qui élargissent la scène pour la découverte de gènes et protéines qui favorisent la survie en hiver.

Mots-clés : tolérance au gel, évitement du gel, survie des insectes à l'hiver, métabolisme des cryoprotecteurs, expression génétique, dépression du taux métabolique, chaperons moléculaires, antioxydants, génomique, protéomique, transduction du signal.

[Traduit par la Rédaction]

Introduction

Strategies for enduring the cold temperatures of winter are needed by all Canadian animals including thousands of ectothermic species, both vertebrate and invertebrate, that need effective mechanisms to endure low body temperatures that no endotherm ever could. The dominant group of cold-hardy terrestrial ectotherms is insects and across most of temperate Canada, huge numbers of species naturally survive at temperatures of –15 to –30 °C, whereas Arctic species often endure –50 to –70 °C. Canada has a strong history of research on insect cold tolerance beginning with the pioneering work of R.W. Salt (University of Lethbridge) who discovered the use of glycerol as a cryoprotectant by insects and identified many parameters of insect cold hardiness (Salt 1957, 1961). Cold survival by Arctic insects has been extensively explored by H.V. Danks (Biological Survey of Canada, Ottawa, Ontario) and R.A. Ring (University of Victoria) and Danks has also contributed much to our understanding of use of diapause (developmental arrest) as an insect survival strategy (Ring

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[1]This review is part of a virtual symposium on recent advances in understanding a variety of complex regulatory processes in insect physiology and endocrinology, including development, metabolism, cold hardiness, food intake and digestion, and diuresis, through the use of omics technologies in the postgenomic era.

and Tesar 1981; Danks 1987, 2004; Danks et al. 1994). Much knowledge about insect antifreeze proteins has also come from the work of P.L. Davies, V.K. Walker, and colleagues (Queen's University) (Davies et al. 2002; Graham et al. 2007).

Interest in insect cold hardiness is not just an academic pastime but has major economic implications as well. It is no surprise therefore that several of the main model species that are used in cold hardiness research are major agricultural or forest pests including the rice stem borer (*Chilo suppressalis* (Walker, 1863)), onion maggot (*Delia antiqua* (Meigen, 1826)), and spruce budworm (*Choristoneura fumiferana* (Clemens, 1865)). Cold hardiness attributes have a major impact on the distribution of pest species and therefore major effects on crop yields (Bale 2002). Species that are disease vectors for both humans and livestock have also received attention (e.g., Mogi 2011). Most recently, issues of cold hardiness or its lack have come to the forefront in the climate change debate because changing weather patterns will clearly have a strong impact on the distribution of all insect species including both pest and beneficial species (Morin and Comrie 2010; Bale and Hayward 2010; Chown 2011; Rose and Wall 2011). An understanding of the interactions of cold and diapause on silkworm (*Bombyx mori* (L., 1758)) eggs has also been critical to the silk industry, allowing the development of simple controls to maintain egg stocks in long-term storage and activate hatching at will. Diapause eggs can be maintained in stasis for >400 days if held at 1 °C but break diapause and begin to develop when moved to 5 °C (Furusawa et al. 1982)[2], suggesting highly precise temperature sensing. Interestingly, a novel offshoot of this research has been the development of silkworm diapause eggs as a bioassay for cosmic radiation on the International Space Station. Diapause eggs are kept in cold storage aboard the station and upon return to earth, they are hatched and assessed for the development of characteristic somatic mutations on the larval integument (white spots) caused by radiation (Furusawa et al. 2009).

Numerous insect species have strategies that allow them to avoid exposure to winter temperatures below 0 °C; for example, honey bees thermoregulate to keep their hives warm, various butterflies and dragonflies migrate south, some insect species spend the winter as aquatic larvae in thermally buffered water (often 0–4 °C), and others burrow underground below the frost line (Storey and Storey 2010*a*). For those that need to endure subzero temperatures in terrestrial environments, two main forms of cold hardiness have traditionally been defined: freeze avoidance and freeze tolerance (Storey and Storey 1988; Lee and Denlinger 1991; Clark and Worland 2008; Denlinger and Lee 2010). Freeze avoidance involves deep supercooling of body fluids to temperatures well below those normally encountered in the microenvironment of overwintering insects. This is potentiated by primary adaptations including the synthesis of antifreeze proteins (AFPs) and the accumulation of extremely high levels of carbohydrate cryoprotectants (most often glycerol) (Fig. 1), as well as other features such as partial dehydration of body fluids and the use of water impermeable cocoons (Storey and Storey 1988). Freeze tolerance involves the regulated freezing of up to about 65% of total body water in extracellular spaces, although values of 74%–82% ice have been reported in some cases (Ramlov and Westh 1993; Block et al. 1998). Ice formation is often triggered by the action of specific ice nucleating agents or proteins (INAs, INPs), whereas low molecular weight cryoprotectants are used to maintain a liquid intracellular space and protect membrane bilayer structure in shrunken cells (Storey and Storey 1991; Zachariassen and Kristiansen 2000; Duman 2001). Although there is evidence for survival of intracellular freezing in the Antarctic nematode *Panagrolaimus davida* Trimm, 1971 and in isolated fat body or other tissue types of some insects (Lee et al. 1993; Wharton and Ferns 1995; Worland et al. 2004; Sinclair and Renault 2010), the phenomenon is still understudied and there is no consensus as to whether intracellular freezing is a normal part of natural freeze tolerance or whether all or only some cell types exhibit the phenomenon naturally.

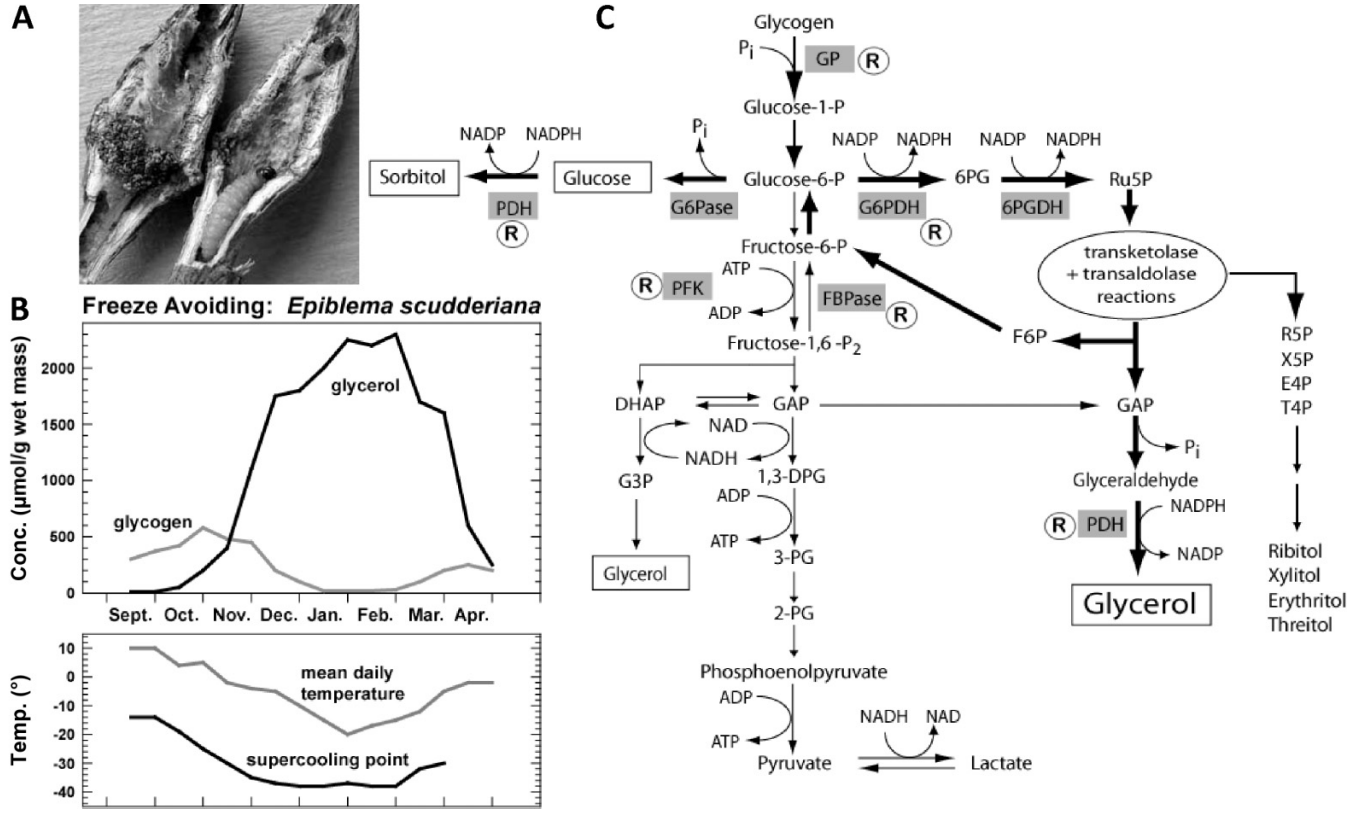
More recently, a third category of cold hardiness—cryoprotective dehydration—was defined for a number of soil invertebrates including a collembolan (Holmstrup and Westh 1994; Holmstrup et al. 2002; Wharton 2003). This strategy combines extreme dehydration (losing virtually all freezable water) with high cryoprotectant levels that stabilize macromolecules. To date, this strategy has only been described for a number of polar species, including several collembolan species (Sørensen and Holmstrup 2011) and a chironomid (Elnitsky et al. 2008), but essentially mirrors strategies used by highly desiccation tolerant insects in other parts of the world (Cornette and Kikawada 2011). The cryoprotective dehydration strategy has the advantage of bringing remaining body fluids into vapour pressure equilibrium with ice (Elnitsky et al. 2008).

Finally, a fourth option for winter survival by an insect has recently been reported for the Alaskan bark beetle (*Cucujus clavipes puniceus* Mannerheim, 1843) (Sformo et al. 2010). Although previously known to supercool to at least –40 °C, Sformo et al. (2010) used differential scanning calorimetry to show that at lower temperatures the larvae do not freeze but transition into a vitrified state and can survive temperatures as low as –100 °C. Vitrification is favoured by extensive dehydration (to about 20% of summer values) and accumulation of AFPs and high concentrations of polyols.

The best known and extensively researched mechanisms of insect cold hardiness are carbohydrate cryoprotectants, AFPs and INAs or INPs. All contribute protective mechanisms that deal with problems of water and ice at subzero temperatures and all have been extensively reviewed in the past including two books (Lee and Denlinger 1991; Denlinger and Lee 2010) and many review articles (some examples: Storey and Storey 1988, 1991; Zachariassen and Kristiansen 2000; Duman 2001; Davies et al. 2002; Block 2003; Graham et al. 2007; Clark and Worland 2008; Doucet et al. 2009). Briefly, both freeze-avoiding and freeze-tolerant species accumulate polyol cryoprotectants; in freeze-avoiding species polyols permit colligative suppression of supercooling point to prevent body freezing, whereas in freeze-tolerant species polyols offer protection against intracellular freezing when ice accu-

[2]We dedicate this review to Toshiharu Furusawa, colleague and friend, whose research has made many significant contributions to silkworm (*Bombyx mori*) metabolism and cold hardiness and enriched our lives with many stimulating interactions.

Fig. 1. (A) Caterpillars of the goldenrod gall moth (*Epiblema scudderiana*) live inside elliptical galls on the stems of goldenrod and use a freeze avoidance strategy of winter survival. (B) Triggered by cooling autumn temperatures, the larvae convert huge stores of glycogen to glycerol, which rises to over 2 mol/L or ~19% of larval body mass. High glycerol plus antifreeze proteins suppress larval supercooling point (SCP) from –14 °C in September to –38 °C by December, values well below environmental temperature extremes. (C) Biosynthetic pathways for glycerol, sorbitol, and other polyol cryoprotectants. Glycogen is the carbon source and most carbon cycles through the pentose phosphate cycle (PPP) to produce NADPH-reducing power and sugar phosphates that are converted to sugars and then reduced by polyol dehydrogenase (PDH) to polyols (see dark thick arrows). Glycerol is the polyol used by most insect species and is made mainly from the glyceraldehyde-3-phosphate (GAP) output of the PPP, although an alternative output via glycerol-3-phosphate (G3P) may also be used. Multiple enzymes in the pathways are regulated (®) by reversible protein phosphorylation. Other enzymes are as follows: GP, glycogen phosphorylase; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphgluconate dehydrogenase; PFK, 6-phosphofructo-6-kinase; FBPase; fructose-1,6-bisphosphatase. Graphical data are redrawn from Rickards et al. (1987), with permission of J. Insect Physiol., vol. 33, issue 6, ©1987 Elsevier Limited. Biosynthetic pathway from Storey and Storey (2010*a*) and reproduced with permission of ©2010 Cambridge University Press. Photograph by J.M. Storey.



mulates in extracellular compartments. In cryoprotective dehydration, the disaccharide trehalose plays the major role in macromolecular protection. AFPs are critical to supercooling point suppression in freeze-avoiding species but can also be found in freeze-tolerant species where they appear to function in inhibition of ice recrystallization. Interestingly, very new research has made a novel discovery of a new class of biological antifreezes—xylomannan glycolipids (Walters et al. 2009). Walters et al. (2011) demonstrated their presence and antifreeze action in six insect species, as well as in one plant and one frog species; much characterization of their action remains to be done. Ice nucleators act in freeze-tolerant species to trigger ice formation at high subzero temperatures and manage ice growth in extracellular compartments; insect INPs are often lipoproteins (Trautsch et al. 2011).

The present review focuses mainly on metabolic concepts as they pertain to insect cold hardiness. We will examine topics including metabolic regulation by reversible protein phosphorylation, mechanisms of global metabolic rate depression, cell preservation mechanisms (e.g., antioxidants, chaperone proteins), and resculpting of the genome and proteome to optimize insects for life in the cold. We acknowledge our very limited treatment of the large literature on AFPs and INPs in cold-hardy species that has been reviewed much more expertly by others (e.g., Zachariassen and Kristiansen 2000; Duman 2001; Davies et al. 2002; Graham et al. 2007; Doucet et al. 2009), the phenomena of chill coma and rapid cold hardening (e.g., Czajka and Lee 1990; Bale 2002; Teets et al. 2008; Clark and Worland 2008; Doucet et al. 2009; Hazell and Bale 2011; Macmillan and Sinclair 2011), as well as many studies of physiological and ecological aspects of insect winter hardiness (e.g., Bale and Hayward 2010; Chown 2011).

Metabolic regulation in cold-hardy insects

Control of polyol metabolism

The seasonal acquisition of high concentrations of carbohydrate cryoprotectants has been well characterized in many insect species. Glycerol is by far the most common protectant

but pairing of two polyols (often glycerol and sorbitol) occurs frequently and some species have multiple components in their protectant mix (Storey and Storey 1988). For example, the bark beetle (*Ips typographus* (L., 1758)) shows five major components (glucose, trehalose, sorbitol, mannitol, erythritol) and several minor components (Koštál et al. 2007). High polyol levels are key to deep supercooling in freeze-avoiding insects or to preventing intracellular ice formation in freeze-tolerant species. Trehalose as a minor component of the cryoprotectant mix is widespread and contributes to stabilization of the lipid bilayer of membranes particularly during cell volume shrinkage in freeze-tolerant animals (Crowe et al. 1992). During cryoproective dehydration, extremely high levels of trehalose effectively replace water in the protection of cellular macromolecules. Cryoprotectant synthesis is supported by the accumulation of high concentrations of glycogen in cells (mainly fat body) during summer–autumn feeding and enhanced activities of enzymes in the biosynthetic pathways, particularly the sugar phosphatases and polyol dehydrogenase that are needed for the two-step conversion of intermediates of glycolysis or the pentose phosphate cycle into polyols (Fig. 1) (Storey and Storey 1991; Joanisse and Storey 1994*a*, 1994*b*). Similarly, polyol catabolism at winter's end begins when the catabolic enzymes appear, particularly glycerol or glyceraldehyde kinase and sorbitol dehydrogenase. Indeed, a definitive marker of diapause termination in eggs of *B. mori* is the induction of glycerol kinase and sorbitol dehydrogenase (Kihara et al. 2009).

Cryoprotectant synthesis involves a massive conversion of glycogen to polyols or sugars; for example, in freeze-avoiding larvae of the goldenrod gall moth (*Epiblema scudderiana* (Clemens, 1860)) (Lepidoptera, Olethreutidae), glycerol levels reach over 2 mol/L and account for nearly 20% of the body mass of the insect in midwinter (Fig. 1) (Rickards et al. 1987). Such a massive undertaking needs close regulation both during autumn synthesis and spring degradation. Cold activation of glycogen phosphorylase (GP) is well known to initiate cryoprotectant synthesis. The underlying mechanism is phosphorylation to convert inactive GP b to the active GP a form and, at the time, this was one of the earliest demonstrations of the use of reversible protein phosphorylation (RPP) to mediate a stress response (Ziegler et al. 1979). However, GP activation cannot determine the fate of the glucose-1-phosphate units that are cleaved off glycogen and so controls on other enzymes are needed. It now appears that RPP of selected enzymes is intimately involved in guiding the flow of carbon into the appropriate polyol product.

Because of the high demand for NADPH-reducing power for the synthesis of polyols, almost all carbon must be pushed through the pentose phosphate pathway during the production of polyols resulting in a close match between the outputs of the cycle: NADPH and various sugar phosphate precursors—glyceraldehyde-3-phosphate (GAP) in the case of glycerol synthesis (Storey and Storey 1991). Regulation is needed to control glycolytic vs. pentose phosphate cycle flux, prevent GAP (or other intermediates) recycling back to glycogen, and promote production of the polyol product. Previous studies have shown that dephosphorylation-mediated inhibition of the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) plus phosphorylation inhibition of glycogen synthase (GS) are key to preventing GAP recycling back into glycogen (Fig. 1) (Muise and Storey 1997, 1999). Temperature-dependent control of phosphofructokinase (PFK) that determines the synthesis of glycerol vs. sorbitol in *E. solidaginis* (Storey 1982) is probably also due to RPP control, as also occurs for this enzyme in *B. mori* (Furusawa et al. 1999). These enzymes (GP, GS, FBPase, PFK) have long been known to be regulated by RPP. However, new studies have discovered that other key enzymes of polyol synthesis are also regulated by RPP—the novel feature being that these are dehydrogenases, a class of enzyme that has almost always been viewed as being nonregulatory equilibrium enzymes that are not known as targets for RPP control.

A new study of polyol dehydrogenase (PDH) indicates RPP control of this enzyme. PDH catalyzes the NADPH-dependent conversion of glyceraldehyde to glycerol and the *E. scudderiana* enzyme is regulated by RPP (Holden and Storey 2011). Maximum activity of PDH was the same in larvae acclimated at +5 °C (a temperature of active glycerol synthesis) and –15 °C (below the range of major glycerol accumulation), but enzyme substrate affinity (K_m) for glyceraldehyde differed significantly between the two groups, being higher (K_m 2-fold lower) for the 5 °C larvae (all assays were done at a constant 25 °C). However, K_m values were interconvertible when extracts from +5 and –15 °C larvae were incubated under conditions that promoted the actions of protein kinases (raised K_m) vs. protein phosphatases (lowered K_m). Furthermore, analysis of purified PDH using both phosphoprotein staining and immunoblot detection of phosphoserine residues showed a much higher phosphate content of the enzyme from –15 °C larvae (Holden and Storey 2011). Hence, the PDH reaction is promoted by a low phosphate form of the enzyme that has a low K_m for glyceraldehyde and dominates at temperatures that are permissive for the rapid accumulation of glycerol pools. However, at very low temperatures that are characteristic of midwinter, the enzyme is phosphorylated and has a high K_m that is not conducive to glycerol synthesis. Furthermore, given that a main route of glycerol catabolism begins with a reversal of the PDH reaction, high phosphorylation of PDH over the midwinter months may also extend inhibitory control over the reverse reaction and thereby contribute to maintaining a stable glycerol pool.

There is also good evidence that glucose-6-phosphate dehydrogenase (G6PDH), the initial enzyme of the pentose phosphate pathway (Fig. 1), is also regulated by RPP in *E. scudderiana*. Two forms of G6PDH are separable on ion-exchange chromatography, they show significantly different affinities for glucose-6-phosphate, and are interconvertible by treatments with protein kinases vs. phosphatases (K. Abnous and K.B. Storey, unpublished data); all of these are characteristics of enzymes that are regulated by RPP (Storey 2002; Storey and Storey 1990, 2004). Overall, then, these new findings give strong evidence that glycerol synthesis is a highly regulated event in cold-hardy insects. Future research needs to focus on some remaining questions such as whether RPP or other regulatory controls on PDH or sugar phosphate phosphatases are responsible for the biphasic synthesis of glycerol and sorbitol in freeze-tolerant species that produce both polyols, whether synthesis of 4- and 5-carbon polyols by some species results from the same control processes but perhaps with sugar phosphate phosphatase and polyol dehy-

drogenase enzymes with modified substrate affinities, and how RPP or other regulatory mechanisms are utilized to manage the spring clearance of polyol pools and the distribution of this huge carbon reserve into different metabolic fates.

Metabolic rate depression

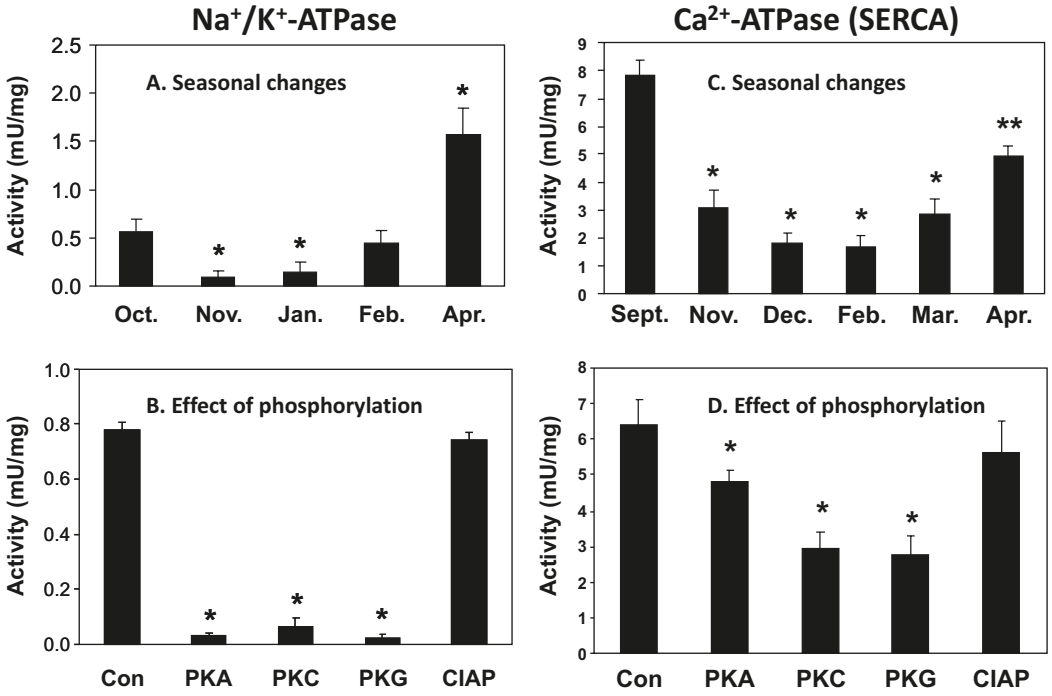
To survive in the face of severe environmental stress (extreme heat or cold, oxygen deprivation, water limitation, food scarcity), organisms often transition into hypometabolic states. By reducing metabolic rate, animals gain a comparable extension of the time that they can "wait out" the stress and survive via the slow catabolism of stored body-fuel reserves. Strong metabolic rate depression underlies hypometabolism in multiple forms including diapause, dauer, hibernation, estivation, anaerobiosis, and anhydrobiosis (Denlinger 2002; Storey and Storey 2004, 2007). Among insects, for example, metabolic rate depression to <10% of aerobic values supports anoxia tolerance by various terrestrial species (Hoback and Stanley 2001). Diapause is widely used among insects, may occur at one or more developmental stage in each species, may be facultative or obligate depending on the species and its poly-, bi-, or uni-voltine status, and can contribute not just to stress resistance (e.g., summer heat, winter cold) but also to synchronize populations for events such as adult hatching (Danks 1987; Denlinger 2002). A winter diapause is quite common among cold-hardy species and, indeed, strategies that could disrupt winter diapause such as by using agonists or antagonists of diapause hormone may be valuable tools for management of insect pests of agricultural crops (Zhang et al. 2011*a*). For example, larvae of two goldenrod gall formers (*E. scudderiana* and the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis* (Fitch, 1855) (Diptera, Tephritidae)) have an obligate diapause of about 3–3.5 months that ends in about mid-February in the Ottawa (Ontario, Canada) region and is followed by a period of cold quiescence until warming temperatures in the spring initiate pupation. The metabolic rate of diapausing *E. solidaginis* larvae in midwinter was just 35%–40% of the early-autumn value at the same temperature, confirming the occurrence of metabolic rate suppression (Irwin and Lee 2002; Levin et al. 2003).

Contributions to global metabolic suppression in overwintering insects could come from several sources. Cessation of feeding saves energy expenditures on digestion, may also cause gut regression, and would reduce or halt biosynthesis of storage fuels (e.g., glycogen, triglycerides). The diapause program of developmental arrest coordinates the shutdown of major energy expenditures associated with cell division and tissue differentiation and growth. However, not all species enter diapause while overwintering. Low environmental temperatures directly suppress the rates of all metabolic reactions but, as a regulatory mechanism, temperature itself is likely not very effective for accomplishing differential regulation of metabolic functions. However, temperature (and (or) thermoperiod) is clearly a triggering mechanism for various features related to cold hardiness including stimulating cryoprotectant synthesis and degradation (Ziegler et al. 1979; Furusawa et al. 1982; Storey and Storey 1991). Cold exposure and diapause also interact in upregulating aquaporins to permit water and glycerol transport (Izumi et al. 2007) and the process of rapid cold hardening (a short cold exposure prior to rewarming) is also key to upregulating a variety of genes and proteins to quickly enhance cold tolerance during the next bout of cold exposure (Li and Denlinger 2008). In all of these cases, however, temperature is likely just acting as the trigger to set off intracellular signaling cascades that ultimately coordinate responses by multiple cell functions. Central to this signaling is RPP that manipulates responses by protein kinases, protein phosphatases, target proteins and transcription factors to control and coordinate both intermediary metabolism and the expression of genes.

Although coarse controls on protein synthesis or degradation can be associated with hypometabolism, for example, leading to altered patterns of enzymes of polyol metabolism, or cold induction of desaturase enzymes to restructure lipids for function in the cold (Kayukawa et al. 2007), the transition into a hypometabolic state primarily involves reversible controls that can provide both global suppression of metabolic functions and differential regulation of cell processes. Energy expensive yet "optional" cell functions are frequent targets; for example, transcription and translation may be largely shut off by the selective use of RPP (Storey and Storey 2004, 2007). Via the addition of covalently bound phosphate by protein kinases or phosphate removal by protein phosphatases, target proteins undergo conformational changes that can have consequences including altered activity or kinetic properties, association or dissociation of protein subunits, altered binding interactions with other proteins, and changes in subcellular compartmentation.

A prime example of RPP-mediated metabolic suppression in cold-hardy insects is the control of ion-motive ATPases. The plasma membrane Na^+/K^+-ATPase is often the greatest single consumer of ATP in cells and hence, a major reduction in cellular ATP turnover can be achieved by the coordinated suppression of Na^+/K^+-ATPase ion pumps and the oppositely directed ion channels that allow facilitated diffusion of Na^+ and K^+ ions down their concentration gradients. Figure 2 shows the seasonal patterns of change in Na^+/K^+-ATPase and the sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) activities in freeze-tolerant *E. solidaginis* larvae. Activities of both ion pumps were highest in early autumn and strongly reduced by 80%–85% in midwinter (Figs. 2A, 2C) (McMullen and Storey 2008*a*, 2010). Similar midwinter suppression of both pumps by 65%–75% occurred in freeze-avoiding *E. scudderiana* larvae (McMullen et al. 2010) and activities rose again in both species in March–April. Notably, the changes in SERCA activities over the winter months occurred without a change in SERCA protein content in *E. solidaginis* larvae and despite a 6- to 8-fold increase in SERCA protein content in *E. scudderiana* between September to April, as assessed by immunoblotting (McMullen et al. 2010). Hence, a post-translational mechanism of ion pump suppression was clearly indicated and proved to be RPP. In vitro studies with extracts from 15 °C acclimated larvae showed that stimulation by protein kinases A, G, or C reduced Na^+/K^+-ATPase activity by more than 90%, whereas SERCA activity decreased by 25% after treatment with PKA and 50% in response to PKG or PKC (Figs. 2B, 2D) (McMullen and Storey, 2008*a*; McMullen et al. 2010). By contrast, incubations with calf intestinal alkaline phosphatase (CIAP) had no effect. RPP often modifies the kinetic properties of enzymes and a kinetic analysis of *E. scudderiana*

Fig. 2. Na^+/K^+-ATPase and sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) in freeze-tolerant goldenrod gall fly (*Eurosta solidaginis*) larvae. (A, C) Seasonal changes in Na^+/K^+-ATPase and SERCA activities in larvae sampled in the second week of each month. (B, D) Effect of in vitro incubations to stimulate endogenous protein kinases (PKA, PKG, or PKC) or the addition of exogenous calf-intestinal alkaline phosphatase (CIAP) on ATPase activities in extracts from 15 °C acclimated larvae. Data are means ± SE, n = 3–5. *, $P < 0.05$, significantly different from corresponding Sept.–Oct. or control values; **, $P < 0.05$, significantly different from all other months. Modified from McMullen and Storey (2008*a*), with permission of J. Insect Physiol., vol. 54, issue 6, ©2008 Elsevier Limited; modified from McMullen et al. (2010), with permission of Physiol. Biochem. Zool., vol. 83, issue 4, ©2010 The University of Chicago Press.



SERCA showed that this occurred; the enzyme from –20 °C acclimated larvae had a 3.2-fold higher K_m ATP (i.e., much lower substrate affinity) than the enzyme from 15 °C control larvae (McMullen et al. 2010). Comparable regulation of Na^+/K^+-ATPase or SERCA by reversible phosphorylation has been demonstrated in hibernating mammals and estivating snails supporting the universality of the mechanism (MacDonald and Storey 1999; Ramnanan and Storey 2006, 2008).

Finally, SERCA is regulated in mammals by a 52 amino acid integral membrane protein called phospholamban; the dephosphorylated form interacts with SERCA and inhibits its activity (Colyer 1998). The gene for this protein was putatively upregulated when *E. scudderiana* responses to low temperature were assessed by gene screening with a fruit fly (*Drosophila melanogaster* Meigen, 1830) microarray (Storey and McMullen 2004). Subsequent analysis of phospholamban protein levels via immunoblotting were consistent with a strong increase in dephosphorylated phospholamban content over the winter in *E. scudderiana* that was reduced again in the spring (McMullen et al. 2010). Control via this inhibitor protein may be particularly important in *E. scudderiana* because the amount of SERCA protein increased so strongly in this species over the winter months and yet measurable SERCA activity declined.

The identification of PKA as one of the protein kinases that could suppress Na^+/K^+-ATPase and SERCA activities is interesting for two reasons. Firstly, total PKA activity increases over the autumn–winter months in both species, whereas oppositely directed protein phosphatase 1 activity shows an inverse decrease (Pfister and Storey 2006*a*, 2006*b*). Furthermore, low temperature exposure enhances dissociation of the PKA holoenzyme to release the catalytic subunits by strongly reducing the K_a value for cyclic 3′,5′-monophosphate (Pfister and Storey 2002). Secondly, PKA is well known for its ability to activate phosphorylase kinase that in turn activates GP so PKA may be a critical protein kinase involved in coordinating the low temperature regulation of both energy-expensive ion pumps and multiple enzymes of polyol metabolism (e.g., GP, FBPase, G6PDH, PDH).

AMPK and metabolic poise over the winter

Cold-hardy species go through major metabolic transitions as they acquire cold hardiness. Metabolism shifts from an anabolic focus over the summer and early autumn (eating, growing, storing fuels) to both a catabolic focus (cryoprotectant synthesis, consumption of stored fuel reserves for energy) and a hypometabolic focus (energy-saving mode associated with diapause entry or triggered by freezing, hypoxia, etc.) over the late autumn and winter. Multiple signaling pathways are undoubtedly involved. In freeze-tolerant *E. solidaginis* larvae, for example, signaling via the hypoxia-inducible transcription factor (HIF-1) is clearly one of the mechanisms involved, responding to oxygen restriction in the frozen state (Morin et al. 2005). The alpha subunit of HIF-1 is stabilized under low oxygen conditions, whereas under normoxic conditions HIF-1α is rapidly destroyed after oxygen-mediated hydroxylation of key proline residues (Semenza 2007). *Eurosta solidaginis* larvae responded to anoxia exposure, as expected, with a strong 2.7-fold increase in *hif-*

1α transcript levels. Cold exposure also had the same effect, transcript levels increased by 1.6-fold in response to an acute temperature decrease from 15 to 3 °C and remained high when larvae were frozen at –16 °C. HIF-1α protein levels also increased sharply by 2.3-fold when larvae were transferred from 15° to 3 °C. In addition, analysis of HIF-1α protein seasonal patterns showed a progressive increase over the winter months to peak in February. Hence, the data suggest a significant role for this transcription factor in mediating metabolic responses to freeze-induced ischemia or hypoxia. HIF-1 mediates responses to oxygen restriction, whereas other protein kinases and transcription factors mediate responses to other limiting factors including low energy and low nutritents.

The AMP-activated protein kinase (AMPK) is proving to be a major regulator of catabolic vs. anabolic poise in cells, its actions favoring the former and inhibiting the latter. AMPK was first discovered as a protein kinase that was allosterically activated by AMP accumulation under low energy conditions (e.g., hypoxia) and it is often called the energy sensor or the fuel gauge of the cell (Hardie 2007; Hue and Rider 2007). In addition, AMPK is now known to respond to various upstream kinases that allow other signals to have input on AMPK targets. The best known action of AMPK is phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), which inhibits lipogenesis and promotes fatty acid oxidation under energy-limiting conditions. AMPK activation also exerts inhibitory control over carbohydrate storage (by inhibiting glycogen synthase) and protein synthesis (by activating the protein kinase that inactivates the ribosomal eukaryotic elongation factor-2 (eEF2). A series of recent studies has consistently shown AMPK activation in animals transitioning into hypometabolic states (e.g., frog freeze tolerance, turtle and fish anaerobiosis, nematode dauer) (summarized in Rider et al. 2011).

A new study suggests that AMPK has a comparable role in insect cold hardiness and diapause. AMPK was analyzed in larvae of the two goldenrod gall formers collected in September (prior to substantial cold hardening) and February (midwinter fully cold hardened) (Rider et al. 2011). AMPK activity was strongly elevated by 70%–90% in February larvae compared with September and this was supported for *E. scudderiana* by a 3-fold greater amount of Thr172 phosphorylation on the catalytic α-subunit that is known to activate AMPK. Downstream targets of AMPK were also significantly affected. Freeze-avoiding *E. scudderiana* larvae showed 2-fold higher levels of phosphorylation of ACC (Ser79) in February vs. September indicating inhibition of lipid synthesis but the antibody did not cross-react with *E. solidaginis*. This correlates well with the previously measured suppression of lipogenic enzyme activities (e.g., ATP-citrate lyase, malic enzyme) seen over the winter in both species (Joanisse and Storey 1996*a*). Inactivation of ACC also leads to reduced malonyl-CoA levels that in turn stimulates mitochondrial long-chain fatty acid oxidation. Together with the observed elevation of activities of fatty acid oxidizing enzymes in *E. scudderiana*, this shows a catabolic poise that supports lipolysis as the fuel for winter survival by the freeze-avoiding species. By contrast, freeze-tolerant *E. solidaginis* showed generally reduced levels of lipolytic enzymes that correlates both with a probable reduced reliance on lipid fuels (larvae cannot oxidize lipids under anoxic conditions when frozen) and with reduced numbers of mitochondria over the winter (see next section).

Mitochondrial metabolism

Cold temperatures, cessation of growth, and entry into diapause or quiescence by cold-hardy insects would all reduce the demand on mitochondrial oxygen-based ATP production over the winter months. Furthermore, as evidenced by glycolytic end product (lactate, alanine) accumulation during freezing, freeze-tolerant species rely on anaerobic metabolism while frozen and so have a reduced need for mitochondria over much of the winter (Storey and Storey 1985). By contrast, there is no evidence of a disruption of aerobic metabolism in freeze-avoiding species. Hence, mitochondrial capacity could be reduced in winter and this could have at least two beneficial functions: (*i*) reduced production of potentially damaging reactive oxygen species (ROS) because superoxide radicals made by complex I of the electron transport chain are the major source of ROS in most cells, and (*ii*) in freeze-tolerant species, reduced potential damage to mitochondrial membranes and macromolecules during freezing episodes as the result of freeze concentration of intracellular and intramitochondrial fluids. Indeed, activities of multiple mitochondrial enzymes are suppressed over the winter months in both freeze-avoiding *E. scudderiana* and freeze-tolerant *E. solidaginis*, except for increased activities of fatty acid oxidizing enzymes in *E. scudderiana*, as mentioned earlier. Activities of citrate synthase, NAD-isocitrate dehydrogenase and glutamate dehydrogenase were all reduced by about 50% between about November and March (compared with September) in both species and rose again in the spring (Joanisse and Storey 1994*c*). Rider et al. (2011) also reported reduced GDH activity (by ~50%) in both species between September and February and further showed a strong decrease in the activity of the pyruvate dehydrogenase complex (PDC), the rate-limiting enzyme for carbohydrate oxidation, by 50% in *E. solidaginis* and 80% in *E. scudderiana*. Immunoblotting also showed ~75% reduction in the total amount of the E1 enzyme in the complex during winter in *E. solidaginis*, as well as a more than 2-fold increase in S293 phosphorylation of E1 that is known to inhibit PDC activity. Neither of these parameters changed significantly in *E. scudderiana* larvae, but the strong decrease in overall PDC activity suggests an alternate mode of inhibitory control.

Lowered mitochondrial enzyme activities could come from metabolic regulation or from a decrease in total mitochondrial numbers over the winter months. Accumulating evidence suggests that it is the former for freeze-avoiding and the latter for freeze-tolerant insects. An initial report by Kukal et al. (1989) on the high Arctic wooly bear caterpillar (*Gynaephora groenlandica* (Wocke, 1874)), a freeze-tolerant species, indicated a huge reduction in mitochondrial numbers in –15 °C acclimated caterpillars vs. 15 °C controls. However, a second study using different methods reached a less dramatic conclusion (Levin et al. 2003) showing that, compared with summer active caterpillars, the mitochondrial DNA (mtDNA) content decreased by about one-half when the larvae entered late summer diapause and stayed low over months of winter freezing. This was correlated with rates of respiration measured at 15 °C for winter caterpillars that were

only about one-third of the summer active value. In addition, the authors showed a similar ~50% reduction in mtDNA content of winter- vs. summer-collected *E. solidaginis*. The activity of cytochrome *c* oxidase (COX), the terminal enzyme of the electron transport chain, also decreased by 30%–50% over the winter months in *E. solidaginis* (McMullen and Storey 2008*b*). Hence, there is a preponderance of evidence for a reduction in mitochondria numbers in freeze-tolerant insects while overwintering.

The situation in freeze-avoiding *E. scudderiana* larvae is different. Figure 3 shows transcript levels for two mitochondrially encoded genes—COX subunit 1 mRNA and 12S rRNA—along with COX 1 genomic DNA content, and COX activity over the winter (McMullen and Storey 2008*b*). COX maximal activity decreased to a midwinter low of about one-third of the September value, which agrees with responses by other mitochondrial enzymes (discussed above). However, analysis of other markers of mitochondrial function showed no change over the winter in COX1 genomic DNA content or transcript levels of COX 1 mRNA and 12S rRNA (genes encoded by the heavy and light chains of the mitochondrial genome, respectively). This indicates that *E. scudderiana* maintain mitochondrial numbers over the winter and implies that the changes in mitochondrial enzyme activities (most down but lipolytic enzymes up) comes from control over individual enzymes via altered protein levels and (or) post-translational regulation. Hence, freeze-avoiding species demonstrate selective reorganization of mitochondrial metabolism during the winter but do not appear to undergo mitochondrial degradation.

Cell preservation strategies

Hypometabolism in nature has two main components: (1) regulatory mechanisms that control the reversible suppression of metabolic rate and reprioritize metabolic functions for maximal energy savings and (2) preservation mechanisms that stabilize and (or) protect cell macromolecules to allow for long-term life extension (Storey and Storey 2004, 2007). This latter is critical because strong suppression of metabolically expensive functions such as transcription and translation during hypometabolism (as well as restrictive physical circumstance such as being frozen or dehydrated or oxygen deprived) means that organisms do not have the normal ability to respond to environmental insults (e.g., heat, cold, UV radiation, osmotic challenge, toxins) by degrading damaged macromolecules and resynthesizing new ones. Hence, preservation strategies are needed. Two in particular are at the forefront of current study: chaperone proteins and antioxidant defenses.

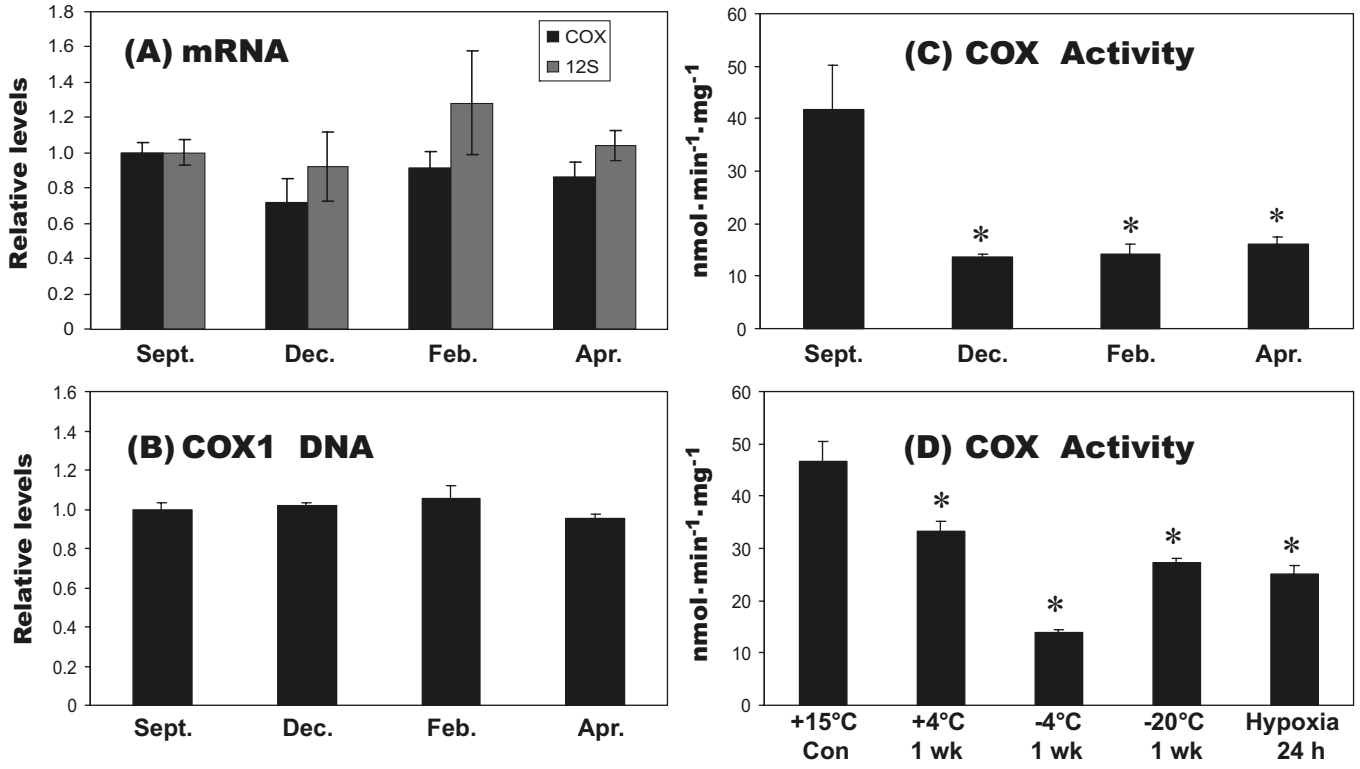
Chaperone proteins

Chaperone proteins have critical roles in maintaining the "health" of the intracellular proteome. Either alone or in conjunction with partner proteins, they act to prevent the aggregation of unfolded proteins (either naïve or denatured), facilitate folding of naïve proteins or re-folding of malfolded proteins, and direct protein trafficking and assembly (Gething and Sambrook 1992; Feder and Hofmann 1999). The best known chaperones are the heat shock proteins (HSPs), named because they were first discovered as responders to acute heat stress in *D. melanogaster*, but other types of chaperones are also known such as some crystallins and the glucose-regulated proteins (GRPs) that have localized chaperone functions in the endoplasmic reticulum. HSP families were first defined by their molecular masses: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsps (sHsps) (sizes >30 kDa) (Gething and Sambrook 1992; Feder and Hofmann 1999), but a revised nomenclature for the mammalian families has recently been designed that calls these HSPH, HSPC, HSPA, HSPD, DNAJ, and HSPB, respectively, and incorporates GRPs and other types of chaperones (Kampinga et al. 2009). Whether this is adopted in the insect literature remains to be seen. Chaperones are ubiquitous in animal cells, some are constitutive and others are inducible in response to many kinds of stress (e.g., heat, low oxygen, UV radiation, heavy metals) that can perturb the folding and functional conformation of proteins. There have been relatively few studies of HSP involvement in animal responses to cold temperatures, and most of those focused on species that are not naturally cold hardy. For example, *D. melanogaster* has been a frequent target for studies of cold effects on HSP gene and protein expression (e.g., Burton et al. 1988; Yiangou et al. 1997; Goto et al. 1998; Nielsen et al. 2005; Qin et al. 2005; Sinclair et al. 2007; Udaka et al. 2010; Colinet et al. 2010) .

Studies of chaperone involvement in cold tolerance of cold-hardy insects are beginning to accumulate (for review: Clark and Worland 2008; Storey and Storey 2011) and show a definite role for HSPs and other chaperones as part of the cell preservation strategy for winter survival. An initial lack of recognition of the possible importance of chaperone action in cold hardiness probably stemmed from several realities: (*i*) most cold-hardy species go through a prolonged seasonal cold acclimation process that can include chaperone accumulation but masks a response to acute cold shock, (*ii*) chaperones may be put in place as part of diapause entry in some species and therefore show little or no direct response to cold, and (*iii*) cold denaturation of many proteins and enzymes, unlike heat effects, is often reversible, so the need for chaperones may have been underappreciated. However, significant examples of chaperone expression supporting cold hardiness are now accumulating. For example, cold acclimation triggered enhanced expression of Hsp90 in nondiapausing larvae of *C. suppressalis*, but not in diapausing larvae that already had elevated Hsp90 (Sonoda et al. 2006). Transcripts of *hsp90* were also upregulated during the winter in larvae of the ghost moth (*Thitarodes pui* Zhang, 2007) (Zou et al. 2011). Upregulation of *hsp70*, *hsp60* (chaperonin), and *tcp1* (t complex polypeptide-1) genes occurs in cold-hardy pupae of *D. antiqua* (Kayukawa et al. 2005; Chen et al. 2006; Kayukawa and Ishikawa 2009). Continuous expression of *hsp70*, *hsp90*, and small *hsps* was reported in cold-hardy larvae of the Antarctic flightless midge (*Belgica antarctica* Jacobs, 1900), but not in the short-lived summer adult stage (Rinehart et al. 2006). These studies underscore what is now becoming recognized as a common theme among organisms that naturally enter prolonged periods of hypometabolism—that stress-responsive upregulation of HSPs is quite consistently observed (Storey and Storey 2011).

The upregulation of HSPs also occurs during diapause in insects (Rinehart et al. 2007, 2010; Clark and Worland

Fig. 3. Mitochondrial status in freeze-avoiding goldenrod gall moth (*Epiblema scudderiana*) caterpillars. Winter profiles of (A) transcript levels of two mitochondrially encoded genes, cytochrome oxidase (COX) subunit 1 mRNA and 12S rRNA, (B) COX 1 genomic DNA content, and (C) COX enzymatic activity. (D) COX activity as a function of decreasing acclimation temperature (1 week (wk) at each temperature) or hypoxia exposure for 24 h at 15 °C, compared with controls held at 15 °C. Data are means ± SE, $n = 3$. *, $P < 0.05$, significantly different from the September or 15 °C control values. Modified from McMullen and Storey (2008*b*), with permission of Insect Biochem. Mol. Biol., vol. 38, issue 3, ©2008 Elsevier Limited.

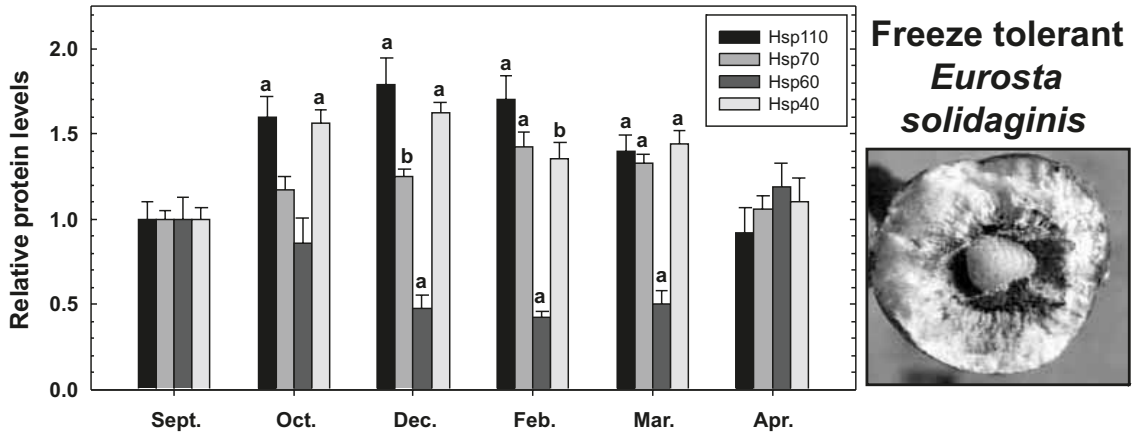


2008). Transcript levels of *hsp70a* and *hsp70b*, an *hsp60*, *hsp23*, and some other small *hsps* were upregulated during pupal diapause in the flesh fly (*Sarcophaga crassipalpis* Macquart, 1839), although *hsp90* was suppressed (Hayward et al. 2005; Rinehart et al. 2007, 2010). Transcript levels of *hsp70* were also upregulated in five other species during diapause (Rinehart et al. 2007) and *hsp70a* transcripts also rose over time in diapause eggs of *B. mori* (Moribe et al. 2010). HSPs are also differentially expressed in poly-, bi-, and univoltine *B. mori* strains (Manjunatha et al. 2010).

Both goldenrod gall formers, *E. scudderiana* and *E. solidaginis,* show elevated HSPs over the winter. Protein levels of Hsp110, Hsp70, Hsp60, and Hsp40 all rose by 2- to 2.5-fold in *E. scudderiana* larvae over the midwinter months, compared with September values (Storey and Storey 2008). A similar response occurred in *E. solidaginis* larvae; Hsp110, Hsp70, and Hsp40 all increased by 1.5- to 2.0-fold in late autumn and winter (Fig. 4) (Zhang et al. 2011*b*). It should be noted that proteins in these three families work in partnership; Hsp70 family proteins conduct the actual ATP-dependent folding of proteins, Hsp40 works by stimulating the ATPase activity and activating the substrate binding of Hsp70, and Hsp110 catalyzes nucleotide exchange on Hsp70 (Dragovic et al. 2006). However, unlike the response in freeze-avoiding *E. scudderiana*, levels of the mitochondrial Hsp60 decreased by about 50% over the winter in freeze-tolerant *E. solidaginis* larvae (Zhang et al. 2011*b*). This correlates with comparable reductions in mitochondrial DNA content and activities of mitochondrial enzymes in this freeze-tolerant species that indicate winter degradation of these organelles, as discussed earlier. The months of October through March when HSPs are high in both goldenrod gall formers are also those with high levels of carbohydrate cryoprotectants. However, diapause lasts for a shorter 3–3.5 months beginning in late October and is followed by cold quiescence until warming temperatures in the spring initiate pupation. Hence, high HSPs correlate with the full season of cold hardiness and not just the shorter diapause period; this suggests a targeted benefit of chaperones for cold hardiness. Studies of pupal diapause in *S. crassipalpis* came to a similar conclusion. Transcript levels of *hsp70* and *hsp23* genes rose when diapause began, but the use of RNAi knockdown to suppress expression of these genes reduced the cold tolerance of diapausing larvae but did not affect diapause capacity (Rinehart et al. 2007).

How could high HSPs contribute to winter survival of cold-hardy insects? Winter may present multiple challenges to the cellular proteome that require enhanced HSP action to fold or refold proteins. Given that winter temperatures for many species can vary from above 0 to –50 °C, well-developed constitutive chaperone defenses could help to stabilize protein conformation over wide environmental temperatures ranges. For example, nonhardy pupae of *D. antiqua* showed cold-induced depolymerization of actin but cold-hardy pupae did not and this correlated with the upregulation of all subunits of a chaperone called CCT (chaperonin-

Fig. 4. Relative expression levels of heat shock proteins Hsp110, Hsp70, Hsp60, and Hsp40 from September to April in freeze-tolerant goldenrod gall fly (*Eurosta solidaginis*) larvae that live inside round galls on goldenrod stems. Data are means ± SE, $n = 4$. a, $P < 0.01$, significantly different from the corresponding September value; *b*, $P < 0.05$, significantly different from the corresponding September value. Data redrawn from Zhang et al. (2011*b*), with permission of J. Insect Physiol., vol. 57, issue 8, ©2011 Elsevier Limited. Photograph by J.M. Storey.



containing t-complex polypeptide-1) in cold-hardy pupae (Kayukawa and Ishikawa 2009). For freeze-tolerant species, additional functions could include protein stabilization under the anoxic or ischemic conditions in frozen animals where the ATP-limited conditions are not conducive to resynthesis of damaged proteins. Proteins in freeze-tolerant species could also be susceptible to freeze denaturation and (or) aggregation caused by an approximate 3-fold increase in the concentrations of all dissolved molecules (e.g., ions, metabolites, proteins) in intracellular fluids when 65% or more of total body water freezes out as extracellular ice. Chaperones could be key to preventing and (or) reversing such problems over cycles of freezing and thawing.

Antioxidant defenses

Upregulation of antioxidant defenses and of metal-binding proteins (particularly iron-binding proteins) is proving to be a widespread event that contributes to cell preservation in various forms of hypometabolism (Storey and Storey 2007). Enhanced antioxidants provide defense against reactive oxygen species (ROS) damage to macromolecules (e.g., proteins, DNA, membranes, and lipid depots) at a time when the dormant state limits the capacity of the organism to respond to ROS damage by degradation and resynthesis of damaged molecules. Enhanced antioxidants could provide this same action to support winter survival by cold-hardy insects. Furthermore, freeze-tolerant insects may need antioxidants for another reason: freeze and thaw events are functionally equivalent to ischemia and reperfusion events that are well known to trigger ROS formation when oxygen is reintroduced during reperfusion. Freeze-tolerant species also face another stress—freeze concentration of remaining body fluids. Thus, if 65% of total body water is converted to ice, then the concentrations of metal ions and other solutes in remaining fluids are automatically tripled. This could require increased attention to ion sequestering.

Exploration of links between heavy metal binding proteins and insect cold hardiness have received little study to date, but some interesting points can be made. Studies in several animal models show links between metal-binding proteins and anoxia or freeze survival. Both stresses upregulated metallothionein (MT) (that binds metals such as cadmium and copper) in freeze-tolerant intertidal snails (common periwinkle, *Littorina littorea* (L., 1758)) and anoxia also upregulated ferritin (that binds iron) (English and Storey 2003; Larade and Storey 2004). cDNA array screening also indicated their putative upregulation in freeze-tolerant Wood Frogs (*Rana sylvatica* LeConte, 1825) and Painted Turtles (*Chrysemys picta* (Schneider, 1783)) (Storey 2004, 2006). Use of *D. melanogaster* microarrays to screen for freeze- and anoxia-responsive gene upregulation in goldenrod gall formers also consistently showed positive responses by ferritin (Storey and Storey 2010*a*). Acute chilling of 15 °C acclimated *E. solidaginis* larvae at 3 °C for 24 h, –4 °C for 4 h, or frozen at –16 °C for 24 h all led to 4- to 5-fold increases in ferritin heavy-chain expression and anoxia triggered a 2-fold increase. Ferritin heavy-chain mRNA levels also increased ~1.5 fold in *E. scudderiana* larvae after 4 h of cold or anoxia exposure. Upregulation of the transferrin gene by 2- to 4-fold was also reported in response to cold (4 °C), heat (37 °C), and other stresses (iron overload, H_2O_2 or paraquat exposure) in the mulberry longhorn beetle (*Apriona germari* (Hope, 1831)) (Lee et al. 2006). Overall, then, a link between insect cold hardiness and an improved capacity for metal ion binding is indicated. Several reasons can be postulated for this attention to metal-binding proteins in cold hardiness. Firstly, enhanced binding of metals lowers potential damage from free metals (especially oxidizing actions of iron) over the winter months, particularly in freeze-tolerant forms where freeze concentration of body fluids greatly increases free metal concentrations. Secondly, Viarengo et al. (1999) reported that MTs can act directly as antioxidants owing to their high content of oxidizable cysteine residues (~30% of total amino acids) and Zachariassen et al. (2004) also suggested this as a secondary function for the high cysteine AFPs found in many freeze-avoiding insects. The lipoprotein ice nucleators of some insects may also have a potent action in metal binding and contribute to detoxification during freezing bouts (Trautsch et al. 2011). Thirdly, iron sequestering is a known part of innate immunity; by denying a source

of iron, microbial growth is inhibited (Cherayil 2011). Hence, upregulation of ferritin iron-storage protein and (or) other iron-binding or transport proteins (e.g., transferrin) over the winter may lower the risk of bacterial infection. This may be a key reason why iron-binding proteins are enhanced in multiple forms of hypometabolism (e.g., hibernation, estivation, vertebrate freeze tolerance, anaerobiosis) (Storey and Storey 2007) because the dormant state may not be conducive to mounting an effective defense by the adaptive immune system.

The importance of antioxidant defenses to long-term cell preservation during hypometabolism has also been demonstrated in multiple systems (Hermes-Lima and Zenteno-Savín 2002; Storey and Storey 2007). Several gene screening studies have reported upregulation of genes for antioxidant and detoxifying enzymes in cold-hardy insects, particularly glutathione S-transferase (GST) (see section on gene screening that follows). Other information on antioxidant defenses in cold-hardy insects is rather limited and there is certainly scope for greater exploration of this field. Goldenrod gall formers showed opposing responses by antioxidant enzymes over the winter. Activities of five antioxidant enzymes were generally high in September in freeze-tolerant *E. solidaginis* larvae (perhaps needed while larvae were still feeding on plant material), decreased over the midwinter months, and then rose again in the spring prior to pupation (perhaps in preparation for increased metabolic rates associated with renewed development) (Joanisse and Storey 1996*b*). However, in freeze-avoiding *E. scudderiana* activities of catalase (CAT), glutathione peroxidase and GST all increased by 2- to 3-fold in midwinter compared with September (Joanisse and Storey, 1996*b*). This suggests a greater need for antioxidant defenses over the winter in the freeze-avoiding vs. freeze-tolerant species. This may be because the supercooled state allows unimpeded aerobic metabolism over the winter, and therefore there is a continuing potential for ROS production and for ROS damage to macromolecules. To counter potential damage, antioxidant defenses are enhanced and may be particularly important to preventing oxidative damage to polyunsaturated fatty acids in the large lipid storage depots of the insects.

Changes in antioxidant defenses are also implicated in the cold hardiness of other species. CAT and superoxide dismutase (SOD) activities, glutathione (GSH) levels, and lipid peroxidation (LP) products were analyzed in larvae, pupae, and imagoes of the mealworm beetle (*Tenebrio molitor* L., 1758) (Gulevsky et al. 2006). Evidence of oxidative stress was seen at the most cold-sensitive developmental stage (imagoes) with elevated LP products and CAT activity after 2 weeks at 4 °C. Pupae showed a rise in CAT and GSH fell in both larvae and pupae. Jovanović-Galović et al. (2007) analyzed antioxidant enzyme responses in mitochondria of diapausing and nondiapausing larvae and pupae of the European corn borer (*Ostrinia nubilalis* (Hübner, 1796)). The study did not involve cold exposure but showed that CAT and GST activities were significantly lower in diapausing larvae, which correlates with differences in the metabolic rate of diapausing and nondiapausing larvae. In *C. fumiferana*, the gene for the detoxifying enzyme GST was highly expressed before the second instar larval diapause, and persisted throughout diapause (Feng et al. 2001). Lalouette et al. (2011) analyzed antioxidant defenses over fluctuating cold and warm exposures in the lesser mealworm (*Alphitobius diaperinus* (Panzer, 1797)) and found a decrease in glutathione pools during cold exposure that recovered during warming coincident with enhanced SOD activities. In *B. antarctica*, high antioxidant capacity involving SOD and CAT is necessary for protection from intense ultraviolet radiation that can generate high levels of ROS (Lopez-Martinez et al. 2008).

Genome and proteome screening in cold hardiness

Insect genome sequencing

Insects are an ancient, huge, and diverse group and forward progress on cold hardiness using genomic tools is still limited by the relatively few insect genome projects that have been completed. Indeed, of major model species used to study insect cold hardiness, full genome sequencing is available only for *B. mori* (http://www.silkdb.org/silkdb/, accessed 21 March 2012) (Xia et al. 2004; Mita et al. 2004). Expressed sequence tag (EST) libraries are also available for *O. nubilalis* (http://butterflybase.ice.mpg.de/, accessed 21 March 2012), *C. fumiferana* (http://pestgenomics.org/index.cfm, accessed 21 March 2012) (Nisole et al. 2010), the apple maggot (*Rhagoletis pomonella* (Walsh, 1867)) (Schwarz et al. 2009), and *S. crassipalpis* (Hahn et al. 2009). Sequence information is available for several other species that show some cold hardiness traits including genome sequences for the red flour beetle (*Tribolium castaneum* (Herbst, 1797)) and the pea aphid (*Acyrthosiphon pisum* (Harris, 1776)), as well as EST libraries for several other species (summarized by Denlinger and Lee 2010). However, genome sequencing is in a log phase of growth and will soon provide many more resources for insect cold-hardiness research. Indeed, in June 2011, the i5k project was launched that aims to sequence the genomes of 5000 insects and other arthropods (Levine 2011). The Arthropod Genomic Consortium (http://arthropodgenomes.org/wiki/Main_Page, accessed 21 March 2012) provides a growing list of insect and other arthropod species (and links to databases) for whom genomic information is available. Genome sequencing has already been completed for 12 species of fruit flies, three mosquitoes (*Anopheles gambiae* Giles, 1902, *Aedes aegypti* (Linnaeus in Hasselquist, 1762), and *Culex pipiens* L., 1758), and the honey bee (*Apis mellifera* L., 1758), and is underway for several others. Given this wealth of sequence information, a range of gene-based studies with unsequenced species should now be facilitated allowing, for example, heterologous screening of cDNA arrays, primer design for retrieving species-specific cDNAs, investigation of noncoding RNA, and identification of transcription factor binding elements, etc.

Screening for gene discovery in insect cold hardiness

A variety of gene screening tools are now available to investigate differential gene expression including cDNA library screening (Bilgen et al. 2001), suppressive subtractive hybridization (SSH) (Robich et al. 2007; Rinehart et al. 2010), and microarray screening (e.g., Storey and McMullen 2004; Qin et al. 2005; Laayouni et al. 2007; Sørensen et al. 2007; Kankare et al. 2010; Zhang et al. 2011*c*). "Next generation"

technologies for transcriptomic studies are also starting to have a big impact (methods including 454 pyrosequencing or Illumina bead arrays) and are now being put to use to explore a variety of questions in insect biology such as tissue-specific sequencing of a major pest species (Mittapalli et al. 2010), analyzing the immune genome (Vogel et al. 2011), analysis of transcriptome responses involved in pesticide resistance (Carvalho et al. 2010), or studies of stress responses in *S. crassipalpis* (Hahn et al. 2009). To date, direct application of these gene screening tools for specifically analyzing the responses of cold-hardy insects to cold or freeze exposure are quite limited, but a number of studies have investigated related phenomena including (*i*) responses to cold selection by *D. melanogaster* (e.g., Qin et al. 2005; Laayouni et al. 2007; Sørensen et al. 2007), (*ii*) responses to diapause by cold-hardy species (e.g., *S. crassipalpis*) (Hahn et al. 2009), and (*iii*) responses to extreme desiccation.

Transcriptional responses to cold exposure by *D. melanogaster* have been addressed in several studies. For example, Qin et al. (2005) evaluated responses to a 2 h cold shock identifying 36 genes that were differentially expressed and implicating stress proteins (Hsp23, Hsp26, Hsp83, Frost), as well as membrane-associated proteins in the cold-hardening response. Using microarray analysis, Zhang et al. (2011*c*) found differential gene responses to short vs. prolonged and single vs. repeated cold exposures in *D. melanogaster*, identifying cold-responsive genes that were associated with muscle structure and function, the immune response, stress response, carbohydrate metabolism, and egg production. A core group of three genes were upregulated under all cold exposure conditions and significantly, whereas short "cold shock" type exposures upregulated 20 genes, more than 3-fold more genes were upregulated by prolonged or repeated cold exposure which suggests that a different program of responses is unleashed when organisms may be "alerted" to a prolonged or seasonal change in thermal conditions. Selected studies also examined changes in gene expression resulting from multigenerational selection of *D. melanogaster* for environmental stress resistance. A study by Sørensen et al. (2007) showed no substantial change in gene expression patterns in flies that were selected for increased cold tolerance over 10 generations (each generation transferred from 20–25 to 11 °C for 5 days before testing and selection for cold shock resistance at 0.5 °C), compared with controls, whereas flies selected for desiccation and starvation resistance did show significantly divergent patterns from controls. Results were somewhat different, however, when Telonis-Scott et al. (2009) omitted the intermediate acclimation step at 11 °C and directly selected for chill coma resistance in 25 °C reared flies. In this case, selection altered basal patterns of expression of 94 genes including suppression of multiple genes associated with the electron transport chain and proteolysis. Laayouni et al. (2007) also analyzed whole-genome transcriptional responses after 3 years of thermal adaptation in another fruit fly (*Drosophila subobscura* Collin in Gordon, 1936).

With respect to diapause, Robich et al. (2007) analyzed diapause-specific gene expression in the female mosquito *C. pipiens* using SSH to compare nondiapausing adults with both early and late diapause stages (all at 18 °C) and identifying six genes that were upregulated in early diapause, 17 in late diapause, and 2 that were elevated throughout diapause. Among upregulated genes were several ribosomal proteins, actin, a small Hsp, and two mitochondrially encoded COX subunits (I and III). Kankare et al. (2010) used a custom microarray with 101 genes known to be involved in diapause, photoperiodism, reproductive behaviour, circadian clock, and stress tolerance to seek out diapause-related genes in a fruit fly (*Drosophila montana* Stone, Griffen, and Patterson, 1942). Rinehart et al. (2010) also searched for diapause responsive genes in *S. crassipalpis* (a cold-hardy species) using SSH, comparing diapause and nondiapause pupae at 20 °C. Seventeen diapause upregulated genes were found including several *hsps* (discussed earlier), ferritin and metallothionein (metal-binding proteins), and Δ9 acyl-CoA desaturase (important for membrane remodeling). Hahn et al. (2009) used massively parallel pyrosequencing to develop a large EST library for *S. crassipalpis* and then created a *S. crassipalpis* microarray (Ragland et al. 2010) to identify diapause-responsive genes and compare the gene responses of flesh fly pupae with those of adult diapause in *D. melanogaster* and larval diapause (dauer) in the nematode *Caenorhabditis elegans* Maupas, 1900. Compared with nondiapause pupae, diapausing *S. crassipalpis* showed enhanced expression of genes associated with glycolysis and gluconeogenesis indicating reduced reliance on aerobic metabolism in the diapause state, features that were also conserved in diapause of the other two species. Enhanced anaerobic capacity in flesh fly pupae fits with the winter biology of the species where pupae are located underground. Transcriptome screening also again revealed enhanced expression of genes encoding HSPs, metalloproteins associated with detoxification, an antimicrobial peptide and multiple components of the insulin-signaling pathway (Ragland et al. 2010). Subsequently, Ragland et al. (2011) analyzed a time course of gene expression changes when cold diapause was broken in *R. pomonella* using a custom cDNA microarray made with ESTs from *R. pomonella*, *S. crassipalpis*, and *D. melanogaster*. In particular, the study showed significant changes in genes in four categories (cell cycle, heat shock, ecdysone-related, circadian rhythm) when comparing late diapause to 24 or 48 h of active development at 24 °C, the data suggesting that the release from cell cycle arrest and metabolic depression occur simultaneously in *R. pomonella*.

Gene responses to extreme desiccation were first analyzed using an expressed sequence tag database for the African sleeping chironomid (*Polypedilum vanderplanki* Hinton, 1951). The analysis showed the importance to survival during anhydrobiosis of antioxidants, late embryogenesis abundant (LEA) proteins, HSPs, genes involved in trehalose (the anhydroprotectant) metabolism, and various transporters (that could redistribute trehalose and other solutes) (Cornette et al. 2010). Significantly, Clark et al. (2009) found similar results when they generated a cDNA microarray for the Arctic springtail (*Megaphorura arctica* (Tullberg, 1876)) to identify genes that contributed to cryoprotective dehydration in this species. Upregulated genes in *M. arctica* included antioxidants (GST), HSPs, trehalose metabolism, and membrane transporters (including aquaporins), as well as genes associated with skeletal reconstruction. Multiple genes co-expressed with trehalose-6-phosphate synthetase, undoubtedly contributing to accomplishing the 100-fold increase in

trehalose concentration needed for desiccation survival. These results emphasize the commonality of responses used by insects to survive extreme dehydration under either hot or cold thermal conditions.

Proteome analysis in insect cold hardiness

Our knowledge of specific proteins that contribute to insect cold hardiness is still relatively limited. Only two novel protein types are recognized that are unique to cold hardiness—AFPs and INPs (Duman 2001). Other proteins featured in cold hardiness are also well known from other systems such as HSPs, antioxidant enzymes, and enzymes of cryoprotectant synthesis and degradation.

Considerable recent work has focused on a protein family that had not previously been studied with respect to insect cold hardiness—aquaporins. These water channels render the membrane lipid bilayer permeable to water and some isoforms also transport other nonpolar solutes such as glycerol or urea (Campbell et al. 2008). Hence, they undoubtedly have important roles to play in water movement between intra- and extra-cellular compartments during freeze or thaw of freeze-tolerant species and those that transport glycerol would have key actions in cryoprotectant distribution in both freeze-avoiding and freeze-tolerant insects. Indeed, Izumi et al. (2006) documented the importance of water channels in the freeze tolerance of larvae of *C. suppressalis* by showing that uptake of glycerol and loss of water from fat body cells during freezing was strongly suppressed in the presence of the water-channel inhibitor mercuric chloride. The first analysis of aquaporin proteins in cold-hardy insects used immunoblotting with antibodies to mammalian aquaporins to indicate the presence of homologues for two water channels (AQP2, AQP4) and one glycerol channel (AQP3) in *E. solidaginis* larvae; notably, AQP3 content increased by about 50% in response to desiccation or –20 °C freezing compared with 4 °C controls (Philip et al. 2008). Furthermore, tissues frozen in vitro in the presence of glycerol + mercuric chloride showed substantially lower viability than in the presence of glycerol alone, implicating aquaporin action in the redistribution of both water and glycerol during freeze or thaw. Subsequently, an *E. solidaginis* aquaporin (not a glycerol transporter) was cloned, sequenced, and functionally characterized, showing highest levels in brain tissue and closest sequence similarity to an aquaporin from the desiccation-tolerant *P. vanderplanki* (Philip et al. 2011). Both aquaporin and aquaglyceroporin-like proteins increased in abundance in *E. solidaginis* larvae from July through January and, furthermore, larvae given either acute cold or desiccation exposures in October (the time when natural glycerol accumulation begins) upregulated AQP3-like proteins (Philip and Lee 2010). Cold exposure and diapause also interacted to upregulate aquaporins in *C. suppressalis* (Izumi et al. 2007). All of these data strongly suggest that aquaporins are closely linked to the seasonal acquisition of freeze tolerance. An aquaporin has also been cloned from *B. antarctica*, a species that uses cryoprotective dehydration (Goto et al. 2011). This aquaporin also showed high homology with the homologue from *P. vanderplanki*; however, gene expression was not affected by dehydration or rehydration, suggesting that this may be a constitutive rather than an inducible aquaporin form.

The use of gene screening techniques in recent years has had a major impact on comparative biochemistry and, as noted above, highlighted the putative upregulation of numerous genes whose protein products may make key contributions to insect cold hardiness. However, proteins are the functional players in adaptation and, ultimately, mechanisms are needed to screen insect species to discover prominently expressed proteins associated with cold hardening. This is particularly true for hypometabolic systems because more and more disjoints between gene upregulation and protein response are being identified and linked with post-transcriptional controls on mRNA processing. In hibernating mammals, for example, there is growing evidence that many gene transcripts that are produced when animals enter torpor are not, in fact, translated until arousal; instead, transcripts are stored in nuclear storage bodies or cytoplasmic stress granules until polysomes are reassembled when animals rewarm (Storey and Storey 2010*b*). Hence, a proteome screening approach will add valuable information to the global picture of the changes in cell protein patterns that underlie cold hardiness.

Several studies have begun to take this approach. Li and Denlinger (2008) used two-dimensional electrophoresis followed by mass spectrometry to identify 14 high abundance proteins that responded to rapid cold hardening (a 2 h exposure to 0 °C) in pharate adults of *S. crassipalpis*. Proteins that respond to rapid cold hardening may also be those that contribute to seasonal hardening. Increased expression in response to cold exposure was indicated for ATP synthase alpha subunit, Hsp23, and tropomyosin-1 isoforms 33/34. Other proteins were suppressed by cold exposure including another tropomyosin-1 isoform which argues that cytoskeletal remodeling may be a key response to cold shock. Colinet et al. (2007) used the same methodology to identify proteins that responded to constant cold or a fluctuating cold and warm regime in a parasitic wasp (*Aphidius colemani* Viereck, 1912). Viability was greater under the fluctuating regime than during constant cold exposure presumably because of the ability to rapidly synthesize protective proteins during brief warm pulses. Proteins that were upregulated in response to fluctuating exposure included several associated with energy metabolism (glycolysis, TCA cycle, F0F1-type ATP synthase alpha subunit), protein chaperones (Hsp70 or Hsp90), and protein degradation (proteasome). The identification of the mitochondrial ATP synthase alpha subunit during cold exposure in two species argues for the necessity to adjust subunit composition of this key transmembrane protein to ensure continued ATP production at low temperature. Proteins that contribute to cryoprotective dehydration were analyzed in *M. arctica*, comparing +5 °C controls, animals acclimated to –2 °C (a temperature than induces trehalose synthesis as the anhydroprotectant), and –6 °C (temperature at which trehalose plateaus) (Thorne et al. 2011). Three prominent categories of upregulated proteins were identified, as well as one membrane transporter, the V-ATPase. Five proteins associated with carbohydrate metabolism were upregulated, most notably UDP-glucose pyrophosphorylase, a key enzyme in the synthesis of trehalose. Several chaperones including protein disulfide isomerase were also expressed. Finally, three proteins involved in cytoskeletal organization (actin, tropomyosin, and a LIM protein) were also elevated in –6 °C animals; these may indicate a need for muscle re-

structuring when severe dehydration collapses cell volume. Interestingly, a gene encoding a LIM protein was also identified as cold responsive in *E. scudderiana* (Bilgen et al. 2001). Transcript and protein levels rose 3- to 4-fold over midwinter and protein peaked at 8.5-fold higher in April compared with November. LIM proteins are involved in myogenesis during both embryogenesis and metamorphosis and play roles in the development and maintenance of cytoarchitecture (Stronach et al. 1996). Hence, their functions in the winter might include (*i*) a role in temperature-dependent restructuring of muscle cytoskeleton or (*ii*) a role in diapause development during the preparatory phases preceding the extensive myogenesis that will occur in the spring.

At present, proteomic screening studies typically highlight only high abundance proteins, but nonetheless they suggest multiple new targets for follow-up to fully evaluate their contributions to cold hardiness. Much remains to be explored.

Conclusions and future directions

The field of insect cold hardiness is vast and we have attempted here to focus mainly on topics of metabolic regulation, cell preservation, and gene and protein expression, primarily as they relate to known cold-hardy insect species. Going forward from here, there are many intriguing biochemical mechanisms to explore to continue to improve our knowledge of how insects adapt and endure at very low environmental temperatures. Many of the "hot topics" in mammalian biochemistry will have application to insect cold tolerance. We have discussed aspects of reversible phosphorylation control of metabolism, but many other forms of covalent modification of proteins exist that can alter enzyme or protein structure and function in response to signals and stresses; these include acetylation, methylation, ubiquitinylation, SUMOylation, GlcNAcylation, and others. We are only beginning to appreciate the complexity of controls that are possible from these. For example, protein modification by small-ubiquitin-like modifier (SUMOylation) is proving to be of major importance for the reversible suppression and storage of transcription factors when hibernating mammals enter torpor (Lee et al. 2007); the role of this mechanism in other forms of hypometabolism is wide open for discovery. Similarly, stress-responsive control of the genome by DNA methylation and (or) chromatin control by histone modification is a burgeoning field with potentially important roles for epigenetic modifications on phenotypic plasticity and adaptation to environmental stress among insect species, particularly in our era of rapidly changing climate (Morin and Storey 2009; Glastad et al. 2011). Furthermore, mechanisms of post-transcriptional regulation of gene expression by microRNA and long noncoding RNA species are now proving to be very powerful factors in biochemical adaptation including hibernation, anaerobiosis, and freezing survival (Biggar and Storey 2011) and will undoubtedly also have key roles to play in insect cold hardiness. Much also remains to be learned about the web of signal transduction cascades that trigger and coordinate both metabolic and gene responses to low temperatures and that regulate both rapid and seasonal cold hardening. All of these regulatory mechanisms and more will be fertile areas for study over the next years as researchers continue to map out the principles of insect cold hardiness.

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