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Review

The bugs that came in from the cold: molecular adaptations to low temperatures in insects

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Abstract. The widespread distribution of insects over many ecological niches is a testimony to their evolutionary success. The colonization of environments at high latitudes or altitudes required the evolution of biochemical strategies that reduced the impact of cold or freezing stress. This review focuses on our current interests in some of the genes and proteins involved in low temperature survival in insects. Although the most widespread form of protection is the synthesis of low molecular weight polyol cryoprotectants, proteins with intrinsic protective properties, such as the thermal hysteresis or antifreeze proteins are also important. These have been cloned and characterized in certain moths and beetles. Molecular techniques allowing the isolation of genes differentially regulated by low temperatures have revealed that heat shock proteins, cold stress proteins, membrane protectants, as well as ice nucleating proteins and other less well characterized proteins likely also play a role in cold hardiness.

Keywords. Insect, cold hardening, freeze tolerance, freeze avoidance, molecular adaptation.

Introduction

Temperature is one of the most important abiotic factors in determining the state of activity and geographic distribution of organisms. Outside the lowland tropics and in temperate waters, temperature can decrease to below zero degrees on both a seasonal and occasional basis. Such low temperatures are fundamental determinants of the life history of many ectothermic animals, of which insects form the overwhelmingly majority. To escape or alleviate low temperatures, insects have evolved a battery of physiological and behavioral strategies. For some species, behavior changes play a key role, such as the long distance migratory flights of monarch butterflies [1] that allow them to escape winter altogether. Other insects escape to local shelters, for instance to thermally buffered microclimates that exist under the snow cover or within tree bark crevices. However spectacular, long distance migrations are rare in insects, as is respite in warm local shelters. Thus, many species must still bear some of the brunt of low temperature exposure. A majority of insects that are subjected to seasonal temperatures that approach or exceed the freezing point of water have evolved a set of powerful physiological and molecular adaptations,

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Tabl 1. Cold-related mortality factors and associated cryoprotective strategies in insects. S: sensitive, R: resistant. Adapted from [4].

Cold-related mortality factor			Nedved's (2000) classification	Cold hardiness classification	Typical cryoprotectant	Type of insect	Reference
Cold shock	Freezing	Cumulative chill injury					
S	S	S	"Sneezy"	Chill and freeze-sensitive; opportunistic survival	Cold shock proteins(?), cold responsive genes (?)	*Musca domestica*	[6]
R	S	S	"Snow White"	Chill tolerant, freeze sensitive	Cold shock proteins, cold responsive genes	*Pyrrhocoris apterus*	[67]
R	S	R	"Doc"	Chill tolerant, freeze sensitive	Polyols, antifreeze proteins	*Choristoneura fumiferana, Epiblema scudderiana*	[99, 74]
R	R	S	"Grumpy"	Freeze tolerant	Polyols, ice nucleators (?)	*Eurosta solidaginis*	[184]
R	R	R	"Happy"	Freeze tolerant	Polyols, ice-nucleators (?)	*Pytho deplanatus*	[10]

collectively placed under the umbrella of "cold hardiness", to counter the effects of such stress.

Classification of insect cold hardiness has often been reduced to a dichotomy, pitting species that are "freeze intolerant", those insects that do not tolerate the freezing of body fluids, against "freeze tolerant" insects which survive after a percentage of their extracellular fluids freeze. This simple organization, although prevalent in the literature, is not wholly satisfying since it rarely takes into account the potential additive injury resulting from cold shock. This may occur whether low temperatures are sustained or brief [2]. As a consequence, it has been suggested that insects be classified into five groups from the most to the least freeze hardy [3]. More recently, it has become apparent that insect cold hardiness may be better understood in light of the damage or mortality factors that are cold-induced, as well as those associated with freezing. In this regard, three main types of cold-induced injury have been recognized by Nedved and have been organized into a binary classification scheme that covers all currently known cold survival strategies: cold shock, the freezing of body liquids and cumulative freezing injury constitute, singly or in combination, the three major cold mortality factors affecting insects [4] (Table 1). Eight possible classes of cold hardiness strategies ranging from no adaptation (typical of tropical insects) to adaptation to all three factors (in freeze tolerant species from regions where low temperatures are common) derive from this classification system [4]. These have been designated by whimsical names, a collective known as Snow White and the Seven Dwarfs [4]. In practice, however, only five major cold hardiness classes are known. At one extreme, the most low temperature-vulnerable of the group, which Nedved has dubbed 'Sneezy', comprise the freeze, cold shock and chilling-susceptible species. These are typified by tropical insects such as the housefly, *Musca domestica*, or the fruit fly, *Drosophila melanogaster,* which show extreme sensitivity to temperatures that are below those conducive to normal metabolism [5, 6]. Insects of this group can die after even brief exposure to 0°C, not having evolved specialized cryoprotectants to sustain the integrity and/or function of tissues, cells and macromolecules. However, as recent work in *Drosophila* has demonstrated, even tropical insects are not completely devoid of biochemical adaptive responses against brief episodes of cold [7]. Adaptations to low temperatures in this group of insects are reminiscent of stress adaptations and in fact may have evolved from these basic stress responses [8, 9].

At the other extreme, the most resistant ('Happy') insects are freeze tolerant species such as the arctic beetle, *Pytho deplanatus* [10], and the alpine cockroach, *Celatoblatta quinquemaculata* [11]. Insects from this group have attracted significant attention due to the numerous biochemical layers of protection they have evolved against the three threats of freezing, cold shock and cumulative chill injury. Similarly, insects which show cold shock and freeze injury tolerance but cannot allow their body fluids to freeze (the 'Doc' class), secrete powerful antifreeze proteins (AFPs) to limit ice propagation to microscopic dimensions. Rather than being an exhaustive review of all such adaptations, this paper summarizes our current interests in cold shock responses, the synthesis of cryoprotective proteins and low molecular weight compounds by insects and their integration into the overwintering strategies of a few "model insects".

Cold shock and the chill response in insects

Seasonality imposes on insects the ability to become hardy for long periods of time, while at the same time being able to respond to quick dips in temperatures. There is evidence for both a rapid cold-hardening process as well as a slower hardening response. The slow response can take days, weeks, or even months and is often linked to the entry into a state of quiescence or diapause [12]. In the context of diapause, hardening is likely to be triggered by the same developmental and environmental inducing cues. The response to cold shock, however, is much better studied. A series of metabolic changes, called the rapid cold-hardening process, allows low temperature-susceptible insects to increase their overall cold tolerance. Cold hardening is induced by a brief exposure to moderately low temperatures [13] and results in a reduced susceptibility to the damaging effects of what would otherwise be a lethally low temperature. Thus, it is conceptually similar to rapid heat hardening where a brief exposure to moderately high temperatures induces heat shock proteins which then protect physiological responses against subsequent lethally high temperatures [14, 15]. As little as a 10 min exposure to 0°C allows the flesh fly, *Sarcophaga crassipalpis* to survive a temperature of -10°C, which would be lethal without such prior cold exposure [16].

One of the hallmarks of the cold shock response and the ensuing cold hardening is the synthesis of small organic molecules such as polyhydric alcohols and sugars. Studies with two species of flesh fly including *S. crassipalpis*, as well as the elm leaf beetle, *Xanthogaleruca luteola,* and the milkweed bug, *Oncopeltus fasciatus*, showed that rapid cold hardening is correlated with accumulation of the cryoprotectant glycerol which can shield membranes from temperature-induced phase transitions [13]. In *S. crassipalpis* it also reduces water loss following cold hardening [17]. Glycerol is by far the most common polyhydric alcohol encountered in cold hardy insects, but other low- to moderately- concentrated polyols such as sorbitol, mannitol, erythritol and myoinositol have also been reported in various species [18–21]. A metabolomic study in *S. crassipalpis* showed that sorbitol levels increased during rapid cold hardening [9]. While the lower abundance polyols have not been characterized as well as glycerol and sorbitol, their structural relatedness suggests they likely contribute to low temperature survival in the same manner [22, 23].

Trehalose is a disaccharide of two alpha-linked glucose units which is mainly used as an energy source for insect flight [24] but can additionally protect against cold shock. Indeed, greater chill tolerance was correlated with an increase in glucose and trehalose levels in some species while glycerol concentrations remained unchanged [7]. Trehalose synthesis, along with glycerol synthesis, appears to be an ancient response to low temperatures, and is shared among insects, nematodes and yeasts [25–28]. This fundamental response is probably linked to the capacity of this non-reducing sugar to replace water molecules, and therefore compensate for the loss of water that frequently occurs during cold shock [29].

Sugars and polyols alone do not ensure cold hardening. As cues announce the need to adapt to low temperatures, some organisms produce specific isoforms of certain cellular proteins to deal with cold stress conditions [30, 31]. For instance, *S. crassipalpis* pupae upregulate specific heat shock protein isoforms during diapause, including Hsp70 and Hsp23 [32, 33]. Evidence for the roles of these proteins in low temperature survival comes from RNAi 'knockdown' experiments that reduce the ability of diapausing pupae to survive cold, but do not influence entry into diapause or its duration [34]. Both Hsp23 and Hsp70 are responsive to cold and heat stress in non-diapausing flesh flies, pointing to their protective roles against 'general stress' under normal development. Interestingly, the heat shock cognate 70 (Hsc70) from *S. crassipalpis* is only responsive to cold shock, and not heat shock. Further complicating an explanation for the role of the latter in cold hardiness, it is not upregulated during diapause [33]. In *D. melanogaster*, the heat shock transcription factor 1 (dHSF) can generate three alternative splicing isoforms, dHSFb, dHSFc and dHSFd, with dHSFd responsive to cold stress [35]. However, no research has been done on the importance of this isoform in long-term (longer than 24 h) cold acclimation [36].

A case study of a chill and freeze-sensitive species, *Drosophila melanogaster*

Since the common fruit fly probably remains the insect of choice for genetic and molecular analysis, it is of interest to examine the low temperature susceptibilities of this chill-susceptible species in detail. *D. melanogaster* originally inhabited the tropical regions of the old world, but is now found in almost all temperate regions and on all continents except Antarctica [37]. Humans presumably played an unwitting role in its spread [38]. Temperature and water are two major confining factors, since these flies require moist environments and cannot survive in cold climates [37].

D. melanogaster matures through a complete metamorphosis, with four distinct stages in its life: egg, larva, pupa and adult. At 21°C, the life cycle takes two weeks. Adults are sensitive to low temperatures, and do not survive exposure to –5°C, even though this temperature is much higher than its supercooling point of –20°C. Again, this suggests that cold shock causes lethal injury that is not associated with freezing. For example, no larvae survived 2 h of exposure to –5°C, and exposure of pupae to –8°C was also lethal, but pre-chilling treatments at a higher temperature increased survival of larvae, pupae, and adults at their respective lethal temperatures [5].

D. melanogaster does not have antifreeze proteins [39], little is known about cryoprotectant production, and this insect does not spin a cocoon. How do these flies survive when they are exposed to low temperatures? This species appears to use several adaptations to cope with temperature stress, including dispersal to warmer climates. Also, some *D. melanogaster* populations have been reported to overwinter under snow [40] where low temperatures would be moderated. Further diminishing the impact of the cold, they also make changes in their phospholipid profiles that may help maintain membrane fluidity or homeoviscosity [41]. Concurrently, they also increase the production of glycogen, triacylglycerols and proline [42]. Increases in these energy reserves are important in order to fuel cold-hardening mechanisms to cope with the fluctuations of low temperatures causing cold-shock or chilling injury. Cellular membrane structure is concomitantly stabilized by low molecular protectants such as proline, which could serve dual functions in addition to their roles as energy sources. Dipterans show a certain plasticity in their overwintering strategies, as some species can enter a state of pupal diapause [43]. Even a few *D. melanogaster* lines have been reported to show quiescence [44] or reproductive diapause [45]. It is therefore not surprising that the molecular adaptations put in place during cold hardiness are equally varied in this successful insect order.

Like other chill-sensitive insects, *D. melanogaster* can use a rapid cold-hardening process. This increases their overall cold tolerance, presumably induced by changes in temperature that can occur during diurnal thermal cycles. Induction of rapid cold hardening has been shown to be related to the cooling rate: *D. melanogaster* cooled at rates similar to those found under natural conditions (0.05 and 0.1°C/min) showed higher survival after one hour of exposure to –8°C than did flies that were directly transferred to these temperatures in the laboratory, or those flies cooled at a faster rate (0.5 or 1.0°C /min) [46]. As might be expected, limited low temperature protection was also related to the final temperature, with flies cooled to 0°C showing more cold tolerance than those cooled to 11°C.

Multiple genes appear to be important for rapid cold hardening or for recovery from chill coma. When *D. melanogaster* adults were treated for 2 h at 0°C and were allowed to recover only sufficiently so that accumulated transcripts could come off the ribosomes, there was increased abundance of some messages corresponding to a cluster of stress protein genes, including some heat shock proteins [47] (Fig. 1). These chaperones facilitate refolding, presumably of proteins that are denatured by cold or oxygen stress. Remarkably, some of the transcripts that were identified in this study have also been implicated in mammalian hibernation, including a locus involved in the sleep response. More than one-third encoded membrane proteins. Also, the Frost (*Fst*) gene product is likely a mucin [47], which may protect membranes. Previously, elevated levels of *Fst* transcripts were seen after low temperature exposure and recovery [48]. Supporting these findings, reduced levels of certain transcripts suggested that cold hardening flies were exposed to some oxidative stress even in the brief period at 0°C [47], and that this stress is likely associated with chilling injury [49–51]. Further, there was evidence of apoptosis in cold shock flies that was ameliorated by eliciting a rapid cold hardening response [52].

In other experiments, a subset of the transcripts associated with the acquisition of cold hardening was also reported after a brief recovery from moderate cold stress [53]. Quantitative trait loci mapping has assigned a portion of the chill recovery phenotype to the second chromosome [54], and selection of strains resistant to low temperature stress showed altered transcript abundance in a number of different genes in one study (unpublished observations), but little difference in another [55]. Clearly, even though *D. melanogaster*, as a chill sensitive insect, shows high mortality to low temperatures, the understanding of its modest adaptations are complex and not fully understood despite the ready availability of molecular tools.

Cold shock tolerance and freeze sensitivity

The freezing point of water, the equilibrium temperature at which ice and water co-exist under one atmosphere pressure, is 0°C [56]. However, water usually freezes at lower temperatures, especially in small volumes [57–59] or when containing dissolved solutes or other organic molecules. Whether supercooled or not, once ice crystallization is initiated, the growth of ice crystals can lead to significant damage to

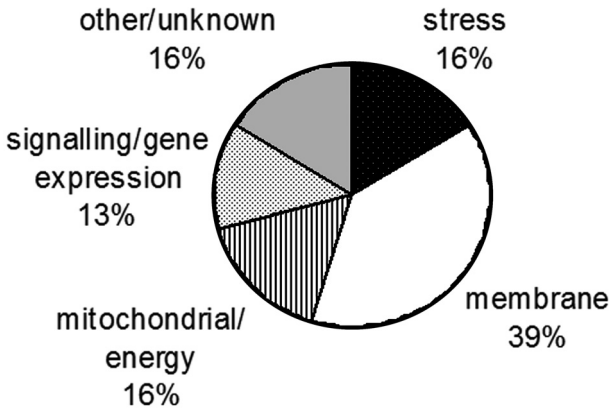

Figure 1. *D. melanogaster* genes showing increased transcript abundance after 2 h at 0°C (see text), as determined by microarray analysis [47]. The 'stress' group includes Hsp83, Hsp26, Hsp23, ubiquitin-63E and Frost. The 'membrane' group includes many putative membrane proteins, a G-protein, ABC transporters, a glutamate receptor, a GTP-binding membrane protein, a Na^+/H^+ transport protein, and a cuticle membrane. The 'mitochondrial and energy' group includes a mitochondrial transporter, an electron transporter (*mtacp1*), an enoyl CoA hydratase, a glycoside hydrolase and a β-galactosidase involved in cell death. The 'signaling and expression' group includes a nervous system splicing factor (*sl6*), ATP binding (*Madm*), a transcription coactivator (nervous system; *mbf1*) and a protein binding expression protein. The 'other/unknown' group includes ATP/GTP binding proteins, and an odorant protein involved in behavioral responses. The analysis was updated to include new biological processes or functions available on FlyBase (http://FlyBase.net) and BioGrid (http://www.thebiogrid.org).

cells and tissues. This is partially due to the exclusion of solutes into the unfrozen fraction as ice crystals grow, changing the osmotic environment. For most organisms, freezing of water occurs first on the outside of cells [60], resulting in an osmotic gradient between the unfrozen extracellular fraction and the interior of the cell. The osmotic disequilibrium across the membrane then forces the water to leave the cell, dehydrating it and increasing the concentration of cytoplasmic components such as small (*e.g.* ions, sugars) and large (*e.g.* proteins) molecules. The increase in concentration of these solutes, in turn, increases the viscosity of cellular fluids, affects enzyme function and changes the cellular pH [61, 62], all of which can be highly damaging to cells and tissues. Also, loss of water makes cells shrink, resulting in additional stress on the membranes [63], and reducing cell volumes until they are unrecoverable, dramatically affecting survival [64, 65]. This damage is in addition to any mechanical damage to cell membranes caused by ice crystal growth, again resulting in ionic leaking or other imbalance.

Membrane and osmotic stress must be avoided in order for freeze susceptible, but cold shock tolerant insects to survive, and thus it is important that freezing does not take place. Indeed, this appears to be the most common cold resistance strategy of arthropods [66]. A portion of the species in this group that are also susceptible to chronic chill injury, dubbed 'Snow White', not only makes up a huge class but is also climatically widespread [4] (Table 1). These insects are typified by the red firebug, *Pyrrhocoris apterus* [67], which survives overwintering temperatures of −13°C. Coincident with autumn and diapause, these insects increase their supercooling capacity and accumulate low levels of polyols such as ribitol, sorbitol, arabinitol and mannitol, but at levels that are insufficient to function as typical cryoprotectants [68]. Actually, the chronic chill injury that characterizes these insects may be due to problems in maintaining ion gradients, since prolonged exposure at 0°C leads to K^+ imbalance [18]. In the field, periodic warm shifts likely restore membrane gradients, and polyols may help to protect electrochemical gradients under these conditions [18]. Proteomic analysis in other species in this class has identified several proteins that increase in abundance after similar temperature shifts [69]. In addition, various genes from a number of insects have been implicated in resistance to chilling stress [70–72], but their roles are not yet established.

Freeze intolerant insects also include a sister class ('Doc') that survives chronic chilling [4] (Table 1). This class of insects is typified by the goldenrod gall moth, *Epiblema scudderiana,* which might be more cold hardy than those grouped in the previously described class due to higher glycerol production. Sorbitol is also present and it likely acts both as a cryoprotectant and to maintain diapause [73]. Temperature-regulated enzymes are crucial for the synthesis of glycerol levels that approach 2 M, and these enzymes utilize large glycogen stores that are accumulated prior to overwintering [74]. Maximum rates of glycerol synthesis occur at relatively high subzero temperatures (0°C to -5°C) and the triggering event can be traced to the activation of a cAMP-dependent protein kinase A (PKA), following a rise in intracellular cAMP [75]. In a cascade fashion, the catalytic subunit of PKA (PKAc) regulates two enzymes having opposite roles in the phosphorylation of glycogen phosphorylase, the enzyme that converts glycogen into glucose. PKA thus regulates a phosphorylase kinase as well as phosphorylase phosphatase (also called protein phosphatase 1) [76]. At the same time, PKA activity may also inhibit pyruvate kinase, funneling carbon flow away from glycolysis and into glycerol production [75]. The activity of antioxidative enzymes in *Epiblema* is also increased during overwintering; enzymes such as superoxide dismutase, catalase, and gluthathione reductase amongst others, maintain reactive oxygen species at low levels during overwintering [77]. This suggests

that damaging free radicals can be produced even though general metabolism has been minimized.

Choristoneura fumiferana (Cf), another insect in the same 'Doc' class, remains unfrozen even at temperatures which can dip to −30°C or lower in the boreal forest [78]. The first instar larvae spin cocoons, molt to second instars in the early fall and then enter a diapause stage for overwintering. They survive partially aided by the accumulation of glucose, trehalose, and glycerol [79]. Dehydration also indirectly helps increase the concentration of cryoprotectants. However, even such high concentrations of cryoprotectants do not sufficiently depress the freezing point if ice nucleators are present [80, 81]. In consequence, *C. fumiferana* second instars eliminate materials from their midguts that could act as ice nucleators, resulting in a lowering of the supercooling point [79]. Thus these insects can avoid freezing by lowering the freezing point of the body fluids in order to supercool. They further protect themselves by avoiding the initiation of ice growth by sealing themselves in hibernacula, and through the synthesis of AFPs.

The fire colored beetle, *Dendroides canadensis*, is also in the same freeze intolerant class. It produces polyols (principally glycerol) and AFPs [82, 83]. Curiously, polyols have not been reported in *Tenebrio molitor,* a related beetle, which overwinters in a larval quiescent state and not a true diapause. The ability of this latter beetle to supercool to low temperatures without glycerol [84] may be due to a combination of its ability to desiccate, perhaps aided by specific proteins such as the desiccation stress protein (dsp28) [85], as well as its highly active AFP.

Both these classes of freeze intolerant insects, in contrast to more low temperature-susceptible insects, are classified as resistant to cold shock (Fig. 1). Low temperatures induce stress proteins including the chaperone Hsp70 for protection [61, 77, 86–88]. The silk moth, *Bombyx mori,* produces *Samuri* (Japanese for cold) transcripts in response to cold shock, and the corresponding protein may also be involved in the long-term chilling response during diapause [71].

Antifreeze proteins

AFPs have been identified in numerous terrestrial arthropods including spiders [89–91], mites [92, 93], centipedes [94, 95], the arctic lepidopteran, *Embryonopsis halticella* [96], various beetles [91] including the longhorn beetle, *Rhagium inquisitor* [97], moths [96, 98, 99], the pine needle gall midge, *Thecodiplosis japonensis* [100], the milkweed bug, *Oncopeltus fasciatus* [101], and more than 50 other species of insects [92, 102, 103]. These proteins are of particular interest because of their unique properties. They depress the freezing point of the hemolymph in the presence of ice or ice nucleators by adsorbing to ice and thereby inhibiting further ice growth [104]. AFPs lower the non-equilibrium freezing point while not significantly affecting the melting point, a phenomenon termed thermal hysteresis [105]. As a result, AFPs are also called thermal hysteresis proteins (THPs). Ice crystal surface recognition and van der Waals interactions [106, 107], as well as hydrophobic groups, complementarity of fit and optimal orientation [108–111] seem to be features of the association of different AFPs to different ice crystal planes. Some AFPs even appear to change their conformation when adsorbed to ice [112, 113].

One of the first reports of what we now know as thermal hysteresis (TH) or AFP activity in any organism came from observations in *T. molitor* (Tm) in the mid and late 1960s [114, 115]. TmAFP cDNAs encode a group of small proteins (8.4–13 kDa), possessing very high TH activity, with some isoforms having up to 100 times the specific activity of fish AFPs [116, 117]. TmAFPs are rich in Cys and Thr residues, which together represent ~40 % of the amino acids [118]. They are composed of varying numbers of tandem 12-residue repeats: (Thr-Cys-Thr-X-Ser-X-X-Cys-X-X-Ala-X, where X can be any amino acid), a unique AFP sequence save for *D. canadensis.* A model structure was obtained using X-ray crystallographic analysis [119], showing that the protein folds as a right-handed β-helix, with each repeat representing a single turn of the helix, stabilized by intra-turn disulfide bonds (Fig. 2). The first three residues of each repeat, Thr-Cys-Thr, together form a flat surface with the 'Thr buttons' precisely aligned and acting as the ice-binding site [119, 120]. The different isoforms, some of which are glycosylated, are encoded by a moderately large gene family with 30–50 unique AFP loci [117]. Approximately half of these have been at least partially sequenced [117, 122].

The related beetle, *D. canadensis* (Dc), has an AFP that is sufficiently similar to TmAFP that a common evolutionary origin may be inferred [122]. TH in this insect is amongst the highest known with enhancer proteins and glycerol perhaps helping to explain this extraordinary activity [102]. DcAFP shares 40–66 % amino acid identity with TmAFP and the 83–84 residues are divided into 12 to 13 amino acid repeats [123]. Although the structure of DcAFP has not been reported, the repeats contain the Thr-Cys-Thr motif, and the two Cys residues within each repeat form a disulfide bond with each other [124]. Thus, it is very likely that DcAFP and TmAFP share a similar three-dimensional structure. DcAFPs are also encoded by a multigene family and similarly, many cDNAs have

been isolated and sequenced [125]. Analysis of the coding regions suggests that there is a bias in codon usage in both beetle AFPs; Tm and DcAFPs show a low GC content at the third wobble position [122]. It is possible that this bias is an adaptation to facilitate transcription or translation at low temperatures. In this regard, it is of interest to note that *C. fumiferana* AFP, too, shows a lower GC content than other coding regions in its genome. It is not known if codon bias is a hallmark of transcripts utilized at low temperatures, but one can hope that other researchers will examine this question in the future.

The *C. fumiferana* AFP (CfAFP) has an obvious independent evolutionary origin from the beetle AFPs, but shares a remarkable number of similarities with TmAFP and DcAFP, so much so that these genes are a good example of convergent evolution [118]. Hints of the structure of CfAFP were first obtained by sequence comparisons [99]. Alignment of 'conserved' amino acids found in the translated sequence revealed occasionally imperfect 'Thr-X-Thr' motifs spaced approximately 15 residues apart. Substitution mutagenesis of Thr residues to Leu in individual Thr-X-Thr motifs resulted in up to 80–90 % loss of TH activity [126]. It is thought that these 'Thr buttons' may play a central role in ice-adsorption [99], similar to Thr residues in certain fish AFPs [107, 127, 128]. This hypothesis was strengthened when a model of the CfAFP structure was obtained using NMR spectroscopy and subsequently by X-ray crystallography; the protein is folded into a left-handed β-helix, with a triangular cross section (Fig. 2) and ~15 residues to each loop [126,110] with a flat ice adsorption face.

The Thr-X-Thr motifs and the disulfide bonds are critical to ice interaction and the overall structure [126], with a longer isoform having increased TH activity [110]. *C. fumiferana* AFPs probably appeared prior to the divergence of the *Choristoneura* sister species approximately 4 million years ago, coincident with the Pleistocene ice age [129] and at a time of changes in the coniferous forest cover [130]. The longer forms with the extra 30-residue repeat forming two more loops likely originated from a duplication and addition event prior to their divergence [129]. Selective pressure for more active isoforms would have retained the insertion and also probably resulted in gene duplication events to increase gene copy number. A similar correlation between increased activity and the number of loops in the isoform has also been observed in beetle AFPs [120].

The full repertoire of AFPs encoded in the spruce budworm genome was estimated to be around 17 genes, using Southern blotting [131]. Over the last decade, genomic and cDNA cloning efforts [131, 132] as well as peptide sequencing from purified antifreeze proteins [99] have allowed the characterization of roughly half that number at the sequence level. In order to further explore AFP isoform diversity, a recently generated expressed sequence tag (EST) library constructed from second instar spruce budworm RNAs was mined (unpublished). Out of 5000 sequenced ESTs, three were deemed homologous (Table 2). One EST (CFDP_037_C23) was identical to the translated sequence of a previously characterized short isoform. The two other ESTs, however, encoded novel isoforms. One of these (CFDP_037_P04) was closely related to a genomic sequence encoding a short isoform, but another, a long isoform (CFDP_0312_A17), had only 65 % amino acid identity to the closest known sequence. When conceptually translated, all three ESTs showed 'Thr buttons' (Table 3), but divergence from the consensus was observed in a few motifs. This included Val-X-Thr, Thr-X-Ala and Thr-X-Lys. Whether these isoforms would show TH activity if expressed *in vitro* remains to be determined.

In the snow flea, *Hypogastrura harveyi*, an arthropod, two related glycine-rich AFP isoforms of 6.5 and 15.7 kDa have been reported [133]. Similar to the insect AFPs, the longer isoform is more active, with TH values similar to those reported for DcAFP. A model [134] of the short isoform shows Gly-gly-X motifs (in which about half of the second residues are Gly).

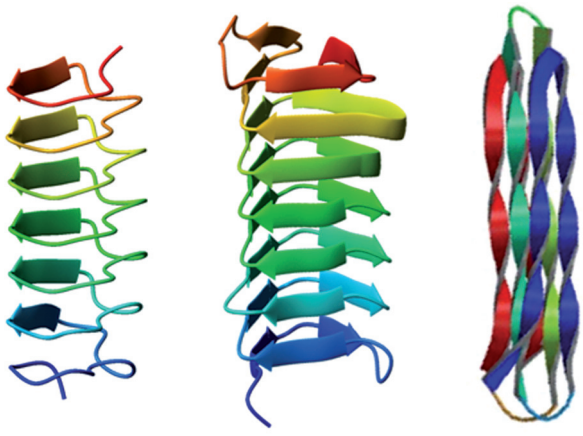

Figure 2. Models of the crystal structures of antifreeze proteins. On the left, the 12–amino acid repeats containing the consensus Thr-Cys-Thr motifs of *T. molitor* AFP are folded into a bread loaf shape formed by a right-handed β-helix, stabilized by intra-loop disulfide bonds [119]. In the middle, the *C. fumiferana* AFP, with 15-amino acid repeats containing consensus Thr-X-Thr motifs (where X can be one of many amino acids), is folded into a left-handed β-helix with a triangular cross section, stabilized by inter-loop disulfide bonds and side chain stacking [110]. On the right, the *H. harveyi* AFP has a repetitive Gly-gly-X motif (where about half the second residues are Gly) that fold into six alternating parallel and antiparallel helical strands that are stabilized by disulfide and hydrogen bonds [135]. All three models generate a rather flat surface on one side, which is believed to be involved in ice adsorption.

F

Table 2. Novel CfAFP sequences obtained from an EST database of diapausing *C. fumiferana* larvae

EST ID	Length (bp)	ORF (start-end)	No. of amino acids	Note
CFDP_037_C23	580	92–253	107	Identical to CfAFP339
CFDP_037_P04	804	59–385	108	70% identical to CfAFP2.7a
CFDP_0312_A17	765	70–480	136	65% identical to CfAFP10

Table 3. Repeat composition of CfAFPs. Two novel sequences obtained from the EST library are bolded.

CfAFP isoform	Repeat position							
	1	2	3	4	5	6	7	
AFP-Lu1	TNT	TLT			TCT	TST	IIT	
CfAFP333	TNT	TLT			TCT	TST	IIT	
CfAFP337	TNT	TLT			TCT	TST	IIT	
CfAFP339	RNT	VPT			TCT	TTT	TIT	**"Short"**
CFDP_037_P04	**VST**	**TLT**			**TCT**	**TTT**	**TIT**	
AFP-2.7a	TKT	TLT			TCT	TTT	TIT	
CFDP_0312_A17	**VST**	**TAN**	**TCA**	**STT**	**TCA**	**TIK**	**TIT**	
CfAFP501	VNT	TAT	ICT	VTT	TCT	TTT	TIT	
AFP-Iu1	VNT	TLT	TCT	RTT	TCT	TTT	TIT	**"Long"**
CfAFP10	VNT	TLT	TCT	RTT	TCT	TTT	TIT	
CfAFP104	RNT	TLT	TCT	RTT	TCT	TTT	TIT	

These repetitive motifs make helical turns that are regularly disrupted by the presence of four prolines (and one serine), so that the protein is folded into alternating parallel and antiparallel helical strands with the Gly residues facing the interior of the folded AFP. Hydrogen and disulfide bonds stabilize this structure which has been likened to a polyproline type II helix [134]. The attractiveness of the model is that the even numbered helical turns together form a rather flat hydrophobic surface, thought to be the ice-adsorption face, but there is as yet, no experimental evidence for this. Recently, the crystal structure for this AFP was solved by another group, substantiating the model [135] (Fig. 2).

AFP transcripts were present in snow fleas found in late spring in melting snow [133]. In *T. molitor, D. canadensis* and *C. fumiferana*, the presence of AFPs is correlated with the developmental stage, but there does not appear to be a consistent mode of regulation in these insects or for each isoform within a species. In *T. molitor*, weeks of low temperatures and desiccating conditions increased larval AFP transcript levels, but developmental timing was crucial [136]. Short photoperiods were also reported to increase levels of hemolymph AFPs [84, 136, 137], but others have not

seen evidence of elevated TmAFP mRNAs correlating with photoperiod length [136]. Despite extensive sequence searches and gene transfer experiments, no hormonal regulatory motifs were found adjoining TmAFP genomic DNA [138]. Generally, *D. canadensis* AFP expression appears to be regulated by overall seasonal temperature changes with peak transcript levels in winter, although some isoforms are present in warmer months [125].

Evidence of such multifarious regulation is also apparent in *C. fumiferana.* Transcripts for the majority of isoforms were most abundant in the second instar overwintering stage, but some were also detected in late summer first instars and even in mid-summer egg stages [121]. Significantly, these transcripts were localized to the fore and midguts, and immunological assays showed AFPs in the gut lumens, but not in the hemolymph. Since certain freeze susceptible insects clear their midguts of ingested plant material, it would not be surprising if these summer isoforms served to reduce the efficacy of potential ice nucleators in case of an early frost [139]. As expected, however, at the overwintering second instar stage, transcripts corresponding to the majority of isoforms were most abundant and AFP mRNAs were detected by *in situ* hybridization in almost all tissues [140]. However, even at this stage, there was heterogeneity, with the ubiquitous distribution of one AFP mRNA and a more limited appearance of another transcript. These studies underscore the temporal and spatial complexity of AFP gene regulation even in an insect with a relatively small AFP gene family [140] and make a strong case for the need for continuing study in this area.

Freeze tolerance

In freeze-tolerant insects, extracellular fluids freeze in a controlled manner to maintain the cellular contents in an unfrozen state. Metabolic pathways for production of cryoprotectants such as sugars, polyhydroxy alcohols, amino acids as well as possibly antifreeze proteins may be upregulated [30, 39, 87]. For example, when acclimatized to low temperatures, the freeze tolerant, cold shock-resistant gall fly *Eurosta solidaginis* larvae dramatically alter their metabolism to produce glycerol and sorbitol [141]. Although the absolute levels may be lower than those of freeze-susceptible ('Doc') insects, the concentrations dramatically rise in the non-frozen fraction as ice forms. The gall fly is freeze hardy but it appears susceptible to cumulative chill injury, and thus this class is distinguished from insects that are freeze, cold shock and chill tolerant [4] (Table 1). In practical terms, however, there is of yet insufficient data on chilling injury in freeze-tolerant insects, and these two classes are discussed together.

The freeze-tolerant Arctic insect *Gynaephora groenlandica* enhances its freeze-tolerance by producing glycerol [142] presumably to slow ice formation [143], but the selection of suitable microhabitats for overwintering is also reported to be crucial [144]. Mitochondria degrade in *G. groenlandica*, possibly reducing damaging free radical generation [145, 146]. Some freeze-tolerant insects also produce trehalose, which can reportedly facilitate the formation of a glassy frozen state, inhibiting solute crystallization [147]. Freeze-tolerant insects freeze at high subzero temperatures, raising their supercooling point by producing ice nucleators or accumulating other macromolecules [39, 141, 148].

As previously indicated, many insects which produce AFPs are freeze-avoiding, but AFPs can also be found in certain species known to be freeze-tolerant at temperatures as low as -40°C to –70 °C [91]. Why do some insects freeze but produce AFPs? The ability of these proteins to inhibit ice recrystallization [149] and thus prevent the formation of large ice crystals at temperatures close to melting would reduce tissue damage [150]. This is likely more important than TH activity to these insects at times of freeze-thaw or at temperatures close to melting, and is probably particularly significant for polar insects, since spring on the tundra is often characterized by multiple freeze-thaw cycles [151]. Since high temperatures are known to inactivate bacterial ice nucleating proteins [152, 153, unpublished], it is of interest that it has been further argued that the presence of AFPs may be important to protect insects in the spring, when in the absence of nucleators it might be advantageous to supercool [154]. In addition, AFPs may afford some protection from nucleators themselves (see below).

Ice nucleators

As noted, small volumes of pure water have the capacity to supercool and do not freeze at temperatures close to their melting points. It is only the presence of ice nucleators that ensures that water crystallizes near 0°C. As a consequence, unless ice nucleators or ice crystals are present, the small size of insects dictates that they are unlikely to spontaneously freeze until their body fluids drop to 25°C below that of the equilibrium freezing point [155]. Although some have argued that crystallization in insects is due to homogeneous ice nucleation [156], the weight of evidence suggests that heterogeneous nucleators are important in biological systems [155, 157, 158].

Membranes, unspecified large biological molecules, salts, proteins, lipoproteins, bacteria and fungi [103, 159, 160] have all been cited as nucleators in insects. *E. solidaginis* uses calcium phosphate crystals to reduce supercooling points, and other crystals such as calcium carbonate, potassium phosphate, uric acid and several periodic table group one and two urates have also been reported in insects [161].

Inoculative freezing by external ice is also possible in those species that are unprotected, but even then the cuticle likely offers some resistance to ice crystal growth. In this regard, it may be significant that cocoons can offer some protection, but some silks can also act as ice nucleators [162]. Indeed, several insects, including some larval dipteran and coleopteran species [155], depend on inoculative freezing to prevent supercooling. Thus, no internal ice nucleators with higher activity would be present in these freeze-tolerant insects.

The most active ice nucleators, apart from ice itself, are ice nucleating bacteria. These were initially reported as plant epiphytes, those bacteria that colonize the surface of leaves and stems [163–165], and thus have been historically linked with plant pathogens such as *Pseudomonas syringae.* Given sufficient numbers, these bacteria typically allow ice to form at temperatures as high as -2°C. Therefore, the proteins that mediate this effect, likely by providing a repetitive scaffold for ice growth, have been called type 1 or the most active of the ice nucleator proteins (INPs). INPs and ice lipoproteins have also been reported in freeze-tolerant insects, but with weaker nucleating activities at -6 to -10°C [166–169]. Large ice crystals are formed in the presence of highly active type 1 INPs, and thus it is a formal possibility that insect INPs are less active in order to generate smaller, potentially less damaging initial ice crystals upon freezing.

Some ice nucleation activities have been seen in hemolymph, but other ice nucleators have been reported in whole insects. The composition of some of the insect ice nucleators has been reviewed [170, 159], including a 74 kDa protein that was reported in queens of the hornet, *Vespula maculata* [171]. However, the most detailed studies on an insect nucleator are from the ~800 kDa lipoprotein from the cranefly, *Tipula trivittata* [39]. These insects overwinter as frozen larvae. The lipoprotein showed some cross reactivity with antibodies raised against bacterial ice nucleation proteins as well as a synthetic peptide containing a bacterial INP consensus 8-mer repeat. In addition, the crane fly protein similarly appeared to depend on phosphatidylinositol, likely for membrane anchoring [103, 167]. A 70 kDa INP purified from the hemolymph of *D. canadensis* has been partially characterized, with a reported primary function of enhancing DcAFP activity [172]. It is not known if the INP activity of this protein is fortuitous.

Since many overwintering insects feed on plant material, or come in contact with precipitation or soils which also contain ice nucleating bacteria [173, unpublished], care must be taken to ensure that potential insect INPs do not originate from microorganisms. Indeed, one might argue that there would be little need for certain insect groups to evolve such endogenous INPs if they feed upon or are associated with such ice nucleating microorganisms. In fact, ice nucleating bacteria and fungi, many of which only have nucleation activity when kept at 'seasonal' temperatures [152], have been described in insect guts (reviews: [155, 174]).

Whether or not insect ice nucleators contribute to overwintering survival is a difficult question. It has been argued that because freeze tolerant insects survive freezing, there would be little to be gained by eliminating either physiologically or by selection, 'incidental nucleators', those molecules that can accidentally act as nucleators [159]. However, there would be strong pressure to remove 'incidental nucleators' from the freeze-susceptible classes and hence the presence of nucleators would be fortuitously correlated with freeze tolerance. Support for this thesis comes from studies of high latitude species which show no significant differences in nucleation temperatures, irrespective of acclimation temperatures [159, 175]. Analogously, it is possible that the production of AFPs by some freeze-susceptible insects serves to decrease the activity of 'incidental' and hard to eliminate ice nucleators, since the presence of antifreeze glycoproteins, and likely also AFPs, reduces the activity of INPs [176]. Thus it is a most interesting observation that DcAFPs have been reported to bind to 'enhancer proteins' with ice nucleator activity [172]. Yet another possible manner by which 'incidental' or insect-encoded ice nucleators could increase survival is that the large ice crystals formed in the presence of nucleators do not appear to grow even larger when retained at temperatures close to melting (unpublished observations). This suggests that ice nucleators may inhibit ice recrystallization, and thus themselves be useful to reduce freeze-thaw stress.

Conclusions

It has become clear in the last 30 years that insects encode a great diversity of proteins to alleviate cryoinjury, and frequently supplement this protection with a battery of low molecular weight polyols and

sugars. On the other hand, cold shock and cold hardening responses seemingly involve changes in macromolecules linked to cell membrane fluidity and metabolic reserves (*e.g.* glycerophospholipids and glucose [7, 177]). How these different components are regulated to protect the insect optimally, either under short-term exposure or over whole seasons including diapause states, is a question which will still see intense investigation for years to come. Microarrays and proteomic technologies will facilitate an understanding of those cold adaptation mechanisms which depend on *de novo* gene expression, as has been done previously to assess the impact of cold shock in *Drosophila* [47]. A recent proteomics study on diapausing flesh flies [178] has highlighted the importance of several HSPs during this period of cold hardiness, and is the harbinger of more such work in this area. However, genome- and proteome-wide studies should also be initiated on much more cold-hardy insects of the 'Doc' and 'Happy' classes, such as the spruce budworm moth and the arctic beetle, *P. deplanatus*, respectively. Apart from AFPs and INPs, the suite of genes responsible for helping to sustain life during periods of extreme cold are not known.

The model proposed by Nedved also underscores our scant knowledge on protective mechanisms against cumulative cryoinjury, in contrast to short-term protective responses [4]. Very few studies have been done to identify the proteins or small molecules involved, but the role of polyols and sugars for such long-term protection can be logically suspected [179]. Indeed, many freeze-intolerant insects accumulate polyols to levels which would be insufficient to significantly reduce their supercooling points [21, 180]. Polyol protection against cumulative cryoinjury might be linked to their known osmoprotective properties, or by acting directly and indirectly as chaperones [181, 182]. It remains to be explored whether freeze-tolerant and freeze-intolerant insects control long-term cryoinjury in the same manner. Finally, the evolutionary origin of insect AFPs and the nucleators found in insects remains a mystery. The widely different AFP sequences isolated to date from Lepidoptera and Coleoptera orders parallels the situation of teleost fishes from polar seas, which express a striking diversity of AFPs [183]. The striking but divergent sequences of CfAFPs and TmAFPs, as well as that of the snow flea, consisting of repeats of short ice-binding units, make the identification of a non-AFP precursor in insect genomes a technical challenge. Thus, questions remain on the evolutionary origin of insect AFPs, on the mechanism of action of low molecular weight cryoprotectants, as well as on the pleiotropic roles these molecules play in cold hardiness strategies. However, a classification scheme centered on all three sources of injuries related to low temperature (cold shock, freezing and cumulative chill injury) is a useful guide for future investigations, in that it provides the canvas on which these fascinating molecular adaptations have evolved.

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