How insects survive the cold: molecular mechanisms - a review

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Abstract

Insects vary considerably in their ability to survive low temperatures. The tractability of these organisms to experimentation has lead to considerable physiology-based work investigating both the variability between species and the actual mechanisms themselves. This has highlighted a range of strategies including freeze tolerance, freeze avoidance, protective dehydration and rapid cold hardening, which are often associated with the production of specific chemicals such as antifreezes and polyol cryoprotectants. But we are still far from identifying the critical elements behind over-wintering success and how some species can regularly survive temperatures below -20°C. Molecular biology is the most recent tool to be added to the insect physiologist's armoury. With the public availability of the genome sequence of model insects such as *Drosophila* and the production of custom-made molecular resources, such as EST libraries and microarrays, we are now in a position to start dissecting the molecular mechanisms behind some of these well-characterised physiological responses. This review aims to provide a state of the art snapshot of the molecular work currently being conducted into insect cold tolerance and the very interesting preliminary results from such studies, which provide great promise for the future.

Keywords

ESTs, Microarray, Proteomics, Stress, Cryoprotection

Introduction

Much of our present knowledge on the manner that insects survive low temperatures stems from studies by R W Salt who conducted physiological experiments in the 1930s using only the basic equipment available to him at that time. Despite this limitation, Salt succeeded in providing the scientific world with descriptions of the fundamental principles of insect cryobiology and a list of working hypotheses (see Ring and Riegert, 1991 for a bibliography, plus Salt 1957; 1959). Since then our understanding of low temperature biology has benefited through technological advances including the application of gas and liquid chromatography, electrophoresis, differential scanning calorimetry, nuclear magnetic resonance spectroscopy and biochemistry (metabolomics). Most recently, gene chips (microarrays) have enabled comparisons between gene expression in control verses treated (cold stressed) animals and upregulated genes can be sequenced and identified by comparison with those stored in a DNA sequence bank. Gene based cold tolerance studies have so far focused mainly on model laboratory organisms, that is to say *Drosophila melanogaster* (see review by Hoffmann et al. 2003) with very little effort being applied to understanding truly cold tolerant animals. The next step is to test emerging hypotheses from this work on other insects.

Early cryobiology research was driven by the need to understand outbreaks of crop pests and more latterly by medical technologists in a bid to protect cells against damage during cryo-preservation. More recently, the recognition of the global implications of anthropogenic climate change has focused attention on understanding biogeography and the control of species distribution patterns and dispersal (Gaston et al, 2008) and the establishment of invasive species (Bale, 2002). This review is

integrates information gained from recent studies, extending our knowledge of seasonal adaptations in insects, by the use of advanced molecular biology techniques.

Genomic resources for studying insect survival at low temperatures

In 2000 the draft genome of *Drosophila melanogaster* was published (Adams et al. 2000). This was the second set of whole genome data to be finished, after the nematode *C. elegans* (The C. elegans Sequencing Consortium, 1998) and has been the reference insect genome ever since. Because of its long history in genetic research, *Drosophila* has always been used as the "model insect". In terms of its utility in environmental studies, particularly with regard to cold tolerance: it is limited as this species is not cold hardy. Chill coma occurs at +7°C with chill injury and developmental arrest occurring only 2°C higher at +9 to 10°C (Hosier et al. 2000; Cohet et al. 1980). Extensive mortality takes place at temperatures below -5°C (Czajka and Lee, 1990). However, the *Drosophila* genome data, along with that from other more recently targeted insects (Table 1) does provide a reference data set with which to compare ESTs (Expressed Sequence Tags) and genes isolated from other non-model insects which are of more utility in cold tolerance work.

This comparative aspect is very important, as there are relatively few characterised proteins in insects. Indeed even with *Drosophila*, which has been sequenced since 2000 and has large numbers of researchers working on it, only one third of predicted protein coding genes have been characterised with some degree of functionality assigned (Table 1). Three insects are currently in the annotation database "ensembl" (www.ensembl.org): *Drosophila melanogaster, Anopheles gambiae and Aedes aegypti* Liverpool LVP strain. This database is intensively curated and regularly

updated, so a brief overview of the status of these three insect genomes reveals the task ahead of those researchers interested in dissecting out the molecular mechanisms of cold tolerance in non-model insects (Table 1).

However with sequencing costs continually decreasing, the number of insect genomes being sequenced either to draft status or the subject of a major EST programme is increasing with 56 currently listed in the GOLD (Genomes On Line Database: www.genomesonline.org) and more within the NCBI dbEST collections (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). Although over 50% of the entries listed in GOLD are represented by *Drosophila* species and the rest are largely crop pests or vectors of human disease, there are also other initiatives, such as Collembase (www.collembase.org) (Timmermans et al. 2007). This targets springtails (*Folsomia candida* and *Orchesella cincta*) as environmental indicators and has recently added data from the EU Sleeping Beauty project, which uses the Arctic springtail (*Onychiurus arcticus*) as a model in desiccation studies (Clark et al. 2007). The sequence data from these latter species, whilst often annotated using the *Drosophila* genome data, will be of greater utility in investigations of insect cold tolerance.

Cold hardening – strategies and adaptations

By the early 1960s it was realised that many factors were involved in insect cold hardening, principally: cryoprotectants, ice nucleators and water content. Insects were originally categorised as being either freeze susceptible or freeze tolerant but these groups were later subdivided when it was realised that some species have low supercooling points (temperature of crystallisation – SCP) but die due to chilling injury before their body fluids freeze. This group has been termed chill susceptible by

Bale (Bale, 1993). Many excellent previous reviews are available, which cover the basic ecophysiological mechanisms that enhance the capacity of insects to survive low temperatures (Bale, 2002; Cannon and Block 1988; Danks 2005; Lee and Denlinger 1991; Michaud and Denlinger 2004; Sømme, 1999).

Insects which are known to survive low winter temperatures may not necessarily be cold tolerant during warm summer periods when they are active and feeding. This is at least partly due to the presence of ice nucleators in their gut. Freeze avoiding insects must enhance their ability to supercool and increase their chill tolerance as winter approaches (seasonal cold hardening). Cold hardening prior to the onset of winter may take several weeks. However, insects that experience unpredictable climate variability or change require a much faster response.

Exposure to a mild cold stress over a period of minutes or a few hours can increase cold tolerance in some insects (Chen et al. 1987; Kelty and Lee, 1999; Worland and Convey, 2001). This phenomenon has been demonstrated in both chill susceptible and chill tolerant insects and termed rapid cold hardening (RCH). Overgaard et al. (2007) used untargeted ^{1}H NMR metabolic profiling to examine the metabolic response in *Drosophila melanogaster* during the 72h period following a low temperature treatment. The most pronounced changes following RCH (gradual cooling to 0°C) were elevated levels of glucose and trehalose, which correlated with improved chill tolerance in terms of survival and reproductive output following a cold shock at -5°C. A similar metabolomic-type study by Michaud and Denlinger (2006) using GC-MS also showed increased levels of glycolytic metabolites associated with RCH in the

flesh fly *Sarcophaga crassipalpis.* However, trehalose levels were reduced, indicating the different biochemical requirements of *Drosophila* and *S. crassipalpis* during RCH.

Cold acclimation is also known to involve complex restructuring of membranes (cf. Russell, 1997). The most common change is an increase in the proportion of unsaturated fatty acids to saturated fatty acids (Michaud and Denlinger, 2006; Kayukawa et al. 2007). However, this is not always the case as in the firebug *Pyrrhocoris apterus* which increases the relative proportion of 1-palmitoyl-2-linoleyl-*sn*-GPEtn at the expense of 1,2-dilinoleyl-sn-GPChol resulting in a slight decrease in the UFH/SFA ratio during winter acclimation (Tomcala et al. 2006). In addition to changes in membrane composition, longer-term cold exposure has been shown to up-regulate expression of a number of different genes, including antifreeze proteins and heat shock proteins (HSP70s), which will be reviewed more comprehensively under the section on protective mechanisms.

Cryoprotectants

After Chino's publication in Nature (1957) describing the accumulation of sorbitol and glycerol in diapausing eggs of the *Bombyx* silk worm, the importance of cryoprotectants to overwinter survival of insects was soon recognised and further investigated by Wyatt (1963) and Salt (1957, 1959). By the mid 1970s, multi-cryoprotectant systems were recognised consisting of glycerol and other low molecular weight sugars and polyhydric alcohols including sorbitol, mannitol and threitol as well as the blood sugar trehalose (see Sømme, 1982 for a review). The freeze tolerance strategy relies on a variety of molecular adaptations that include the production of high concentrations of polyol cryoprotectants but also the synthesis of

ice nucleators (Zachariassen and Hammel 1976, metabolic rate depression (diapause) and changes in gene expression (Storey, 1997; Pfister and Storey 2006). However, both freeze tolerant and freeze avoiding strategies typically involve the accumulation of low molecular weight polyhydric alcohols, which provide antifreeze protection to liquid compartments (Storey and Storey 1986). The energy investment can be considerable, often in the region of 1-10% of dry mass. Polyols are often derived from the catabolism of glycogen stores accumulated during summer (Worland et al. 1998). The process is initiated by activation of glycogen phosphorylase by a signal transduction cascade system operating at temperatures in the range of 5 to -5°C (Pfister and Storey ,2002; Storey and Storey 1981). Glycogen and other cryoprotectants can be inter-converted in response to changing temperature (Storey and Storey 1986). The regulation of polyol synthesis in insects has been linked to differential cold-responsiveness of glycogen phosphorylase kinase (GPK) and protein phosphatase 1 (PP1) in the control of glycogen phosphorylase (Hayakawa, 1985). Changes in the enzymatic machinery for polyol synthesis are probably also under hormonal direction as part of development or diapause transitions, stimulated by temperature or photoperiod as winter approaches. This is demonstrated in *Ostrinia nubilalis* where only the fifth instar larvae are capable of synthesising glycerol in response to cold stress, indicating that the moult to the overwintering stage is key to completing the synthetic pathway (Nordin et al. 1984). The activity of several other enzymes have been shown to change with season in the goldenrod gall fly, *Eurosta solidaginis,* which has become an ecophysiological model organism for freeze tolerance studies (Storey and Storey, 1991, 1996). These enzymes are involved in fatty acid metabolism, mitochondrial function and antioxidant defense in addition to

polyol synthesis and catabolism. (Joanisse and Storey, 1994a, 1994b; Pfister and Story, 2006).

Desiccation

Most insects avoid water loss by seeking shelter, by for example moving down through the soil profile, however some animals including nematodes (Grewal et al. 2006), tardigrades (Hengherr et al. 2007), an insect, the chironomid *Polypedilium vanderplanki* (Kikawada et al. 2006), some Collembola (Worland et al. 1998) and Diptera (Bennett et al, 2003), are able to withstand significant water loss and even anhydrobiosis. Once water has been removed from their bodies, the threat of freezing is overcome. At the cellular level, the challenges faced through cold or desiccation are very similar, particularly in terms of increases in solute concentration and osmotic stress, reduction in supercooling point and membrane shrinkage (Convey, 2000). Desiccation and cold tolerance are considered to be overlapping adaptations (Ring and Danks, 1994) eliciting similar responses to limit injury during periods of extreme environmental conditions. A common feature of many anhydrobiotic and cold tolerant organisms is the accumulation of trehalose, which stabilises membranes by interacting with the phosphate of phospholipids (Crowe et al. 1992). The accumulation of sugars and polyols seems to be a common trait of both cold and drought tolerant ectotherms. Exposing *D. melanogaster* to desiccating conditions can elicit an increase in desiccation resistance (Hoffmann, 1990) in the same way that cold hardening improves cold tolerance (Hoffmann and Parsons, 1993). Pre-treatment to high and low temperatures can also increase desiccation tolerance a phenomenon termed adaptive cross tolerances (Tammariello et al. 1999) as demonstrated in *D. melanogaster* (Hoffmann and Parsons, 1993). Similarly, acclimation to drought stress can improve

cold tolerance (Forge and MacGuidwin, 1992; Bayley et al. 2001; Holmstrup et al. 2002). The composition of membrane phospholipids fatty acids (PLFA) changes during drought acclimation with an increase in the degree of unsaturation (in the tropical springtail *Folsomia candida)*. (Holmstrup et al. 2002). This is similar to changes that occur in cold stressed ectotherms and is an important physiological adaptation probably involved in maintaining membrane lipid fluidity during drought and cold stress.

Protective mechanisms

As well as physiological adaptation to the cold, there are also a number of well-characterised biochemical and genetic mechanisms which can protect the animal against the effects of lowered environmental temperatures and freezing.

Antifreeze proteins and thermal hysteresis

Antifreeze proteins (AFPs) depress the freezing point of fluids by adsorbing to seeded ice crystals and inhibiting their growth (Raymond and DeVries 1977). AFPs lower the freezing temperature below the melting point, a phenomena known as thermal hysteresis (TH). Typical fish plasma freezes at about 1°C below the freezing point of seawater (-1.9°C). Although this is only a modest degree of undercooling, it is sufficient to protect fish in what is a comparatively stable thermal environment (Duman and DeVries, 1974). Since the discovery of antifreeze glycoproteins proteins in Antarctic fish by DeVries (1971) analogous macromolecular antifreeze compounds have been found in various insects including the darkling beetle, *Meracantha contracta* (Duman, 1977) and the spruce budworm, *Choristoneura fumiferana*

(Tyshenko et al. 2005; Qin et al. 2007). At least 50 insects are now known to produce AFPs (see Duman et al. 2004, for a species list), although many of these have only low levels of thermal hysteresis. This either may be a function of the season the samples were collected or in some cases the way the animals were acclimated. Low levels of AFPs can be beneficial to freeze tolerant species by preventing re-crystallisation while still allowing nucleation and so preventing deep supercooling (Tursman et al. 1994) which would result in rapid ice growth and physical damage to cells. Recently it has become apparent that some insect antifreeze proteins are much more potent than those in fish (Scotter et al. 2006) and are the subject of biotechnological interest. This has come about with the ability to purify the proteins and express them in recombinant systems, as discussed below.

Although the vast majority of insect AFP work has concentrated on detecting and measuring thermal hysteresis in haemolymph (c.f Duman et al. 2004), a number of insect AFPs have now been purified and sequenced from the mealworm (*Tenebrio molitor*) (Graham et al. 1997), the pyrochoroid beetle (*Dendroides canadensis*) (DAFP) (Duman et al. 1998; Andorfer and Duman, 2000), the snow flea (*Hypogastrura harveyi*) (Graham and Davies, 2005) and the spruce budworm (*Choristoneura fumiferana*) (cfAFP) (Tyshenko et al. 2005). Comparison of sequence data from these four insects reveals very little sequence similarity between them, indicating that similar to the situation of antifreeze evolution in teleost fish (Chen et al, 1997), insect AFPs have evolved independently from different progenitors (Graham and Davies, 2005). Again, similarly to fish, the AFPs studied in detail consist of multi-gene families with at least 17 isoforms of cfAFP (Doucet et al. 2002; Tyshenko et al. 2005) and 13 isoforms of DAFP (Andorfer and Duman, 2000)

characterised to date. The existence of multiple copies of slightly different isoforms of the same gene would appear to have adaptive significance, as seasonal analysis of two DAFPs (*dafp-1* and *dafp-7*) reveals differential transcription and the 6 isoforms of cfAFP show developmental-specific expression patterns (Qin et al. 2007). Hence, it has been hypothesised that these proteins may serve additional functions to that of antifreezes depending on the environmental signals (Andorfer and Duman, 2000), if indeed they are under environmental control (Qin et al. 2007).

Cloning, sequencing and subsequent manipulation of these insect AFP genes has produced a greater understanding of their function. Some have been expressed and studied in *E.coli* transgenic systems (Graham et al. 1997; Graham and Davies, 2005), whilst others have been expressed either in insect transgenic lines (Tyshenko and Walker, 2004; Nicodemus et al. 2006; Qin et al. 2007) or transformed into a plant (*Arabidopsis thaliana*) (Huang et al. 2002). Such techniques have revealed that some insect AFPs are more active than those so far described in fish by over 2 orders of magnitude. For example the antifreeze protein isolated from the beetle *Tenebrio molitor* has a specific activity 100 times that of a typical fish AFP (Graham et al. 1997) and has been termed "hyperactive". Similarly high activity levels have been observed in *Hypogastrura harveyi* and *Choristoneura fumiferana* (Graham and Davies, 2005, Qin et al. 2007).

So how are these antifreezes activated and how do they protect cells from cold damage? Whilst expression of a DAFP in *Arabidopsis thaliana* produced transgenic plant lines with measurable thermal hysteresis activity in apoplast fluid, the plants did not show an improved ability to survive freezing when compared with wild-type,

although the transgenic lines with AFPs in the apoplast fluid froze at significantly lower temperatures than wild-type (Huang et al. 2002). This lack of improved ability to survive freezing was unexpected, but may have been due to problems in the intrinsic structural properties of the AFPs such as post translational modifications, or more likely, expression in a heterologous non-insect system. More recent studies have either expressed insect AFPs in insect cell lines or produced transgenic flies, which has been a more successful approach. Luciferase reporter assays of genomic clones from (CfAFP2.26 and CfAFP2.7) in a *C. fumiferana* cell line has lead to the identification of two 5' promotor regions (Qin et al. 2007). This is a first step towards understanding the complex regulatory patterns of AFP expression in this species, in which 17 isoforms display markedly different developmental patterns. Expression studies indicate that these genes are potentially under neuroendocrine control, as they appear relatively insensitive to environmental triggers. Earlier studies introduced a DAFP-1 gene (from *D. canadensis*) and cfAFP337 (from *C. fumiferana*) into *Drosophila* stocks (Tyshenko and Walker, 2004; Nicodemus et al. 2006, respectively). Whilst DAFP-1 transgenic flies were able to live longer at 0°C and 4°C than controls, the effects were small. In the case of the cfAFP337 transgenic line, high levels of thermal hysteresis were measured, but the transgenic lines did not display any cold resistance when compared to wild type.

These experiments demonstrate that transgenic AFPs can be correctly processed and are demonstratively active in transgenic systems, but they do not absolutely emulate the thermal hysteresis properties of AFPs in the native animals. There are several potential reasons for this:

- AFPs are generally multiple gene families and thermal hysteresis measurements of haemolymph cannot distinguish between the different isoforms, which frequently exist as a mix and it may be this combination of forms, which confers the cold tolerance (Nicodemus et al. 2006).
- Stabilisation of cell membranes is thought to play a large part in cold resistance and the transgenic AFPs may not be present in high enough concentrations to contribute to membrane stability, even if the proteins are correctly post-translationally modified and integrated into membranes (Tyshenko and Walker, 2004; Nicodemus et al. 2006).
- Additional synergistic components (such as glycerol) may be required to stabilise cellular membranes at low temperatures (Tyshenko and Walker, 2004; Nicodemus et al. 2006). Certainly it has bee shown that *D. canadensis* increases the THP activity of AFPs by accumulating glycerol and enhancer proteins (Wu and Duman, 1991; Duman 2001).

It is only now, with the production of transgenic constructs in insect vector systems, that we are beginning to understand the complexity of AFP action in insects. It certainly is not a simple case of producing AFPs and achieving cold tolerance.

Recrystallization inhibition

Freeze tolerant species may also produce antifreeze proteins along with ice nucleating proteins. One explanation by Knight and Duman (1986) for this seemingly anomalous situation is that these proteins prevent a size increase of individual ice crystals (recrystallization), which would otherwise cause physical damage to cells.

Ice Nucleators

The temperature at which ice forms in insect body fluids is regulated by ice nucleators which seed the formation of ice crystals (Salt, 1961, 1966). Initial studies on bacterial nucleators indicated that protein, carbohydrate and lipid components of the outer membrane are essential for nucleation activity. Ice nucleators have also been identified in the extracellular fluids of freeze tolerant invertebrates (Neven et al. 1989). These restrict ice formation to the extracellular fluids

Removing such nucleators can result in an increase in the animal's ability to supercool (Sømme and Block, 1982) a process which is further enhanced by the colligative action of low molecular weight cryoprotective compounds (Zachariassen, 1991). Particles of food and bacteria in the guts of insects are thought to be potential ice nucleators (Lee et al. 1991; Worland and Lukešová, 2000) but can be removed by cessation of feeding at low temperatures, although there is no evidence for deliberate ejection of gut contents in response to low temperatures. Recently, the effect of moulting on supercooling ability has been studied in Collembola (Worland, 2005; Worland et al. 2006). Moulting might be expected to depress the SCP, because in Collembola the mid-gut and its entire contents are shed during this process (Thibaud, 1968) resulting in the expulsion of potential ice nucleators. Although this is still the subject of debate, recent microarray studies similarly indicate a correlation between low SCPs and moulting (Purać et al. submitted). Genes coding for cuticle proteins are up-regulated in low SCP animals, interpreted as indicating that a significant proportion of the low SCP population were undergoing moulting (Purać et al. submitted).

Heat shock proteins

Heat shock proteins are expressed in most organisms in response to a wide range of stressful environmental conditions (reviewed in Feder and Hofmann, 1999) and are generally viewed as a protective cellular mechanism. The role of HSPs in protecting insects against heat and desiccation stress is well understood (Goto and Kimura 1998; Goto et al. 1998). These genes are a family of highly conserved proteins, which act as chaperones to stabilise and refold denatured proteins, preventing the formation of cytotoxic aggregates (Parsell and Lindquist, 1993; Hartl, 1996; Fink, 1999). Numerous families of heat shock proteins have been identified, the naming of which is related to their molecular weight in kiloDaltons. For example, the most studied of the family members are the 70kD heat shock proteins (HSP70s), comprising both constitutive (HSC70: heat shock cognate 70) and stress inducible (HSP70s: heat shock protein 70) isoforms. Their popularity in molecular studies is largely because these genes are highly conserved at the molecular level and therefore relatively easy to clone and evaluate in different species, even where there is limited or no genome data available for that species.

The classical activation of the inducible HSP70 genes is in response to elevated environmental temperatures and expression is tightly regulated by the heat shock transcription factor (HSF1) (reviewed in Morimoto, 1998). However, heat is not the only activator, with anoxia, starvation and desiccation also identified as inducers (Salvucci et al. 2000; Parsell and Lindquist, 1993). The induction and expression of the HSP70's is highly plastic, with cellular levels influenced by factors such as thermal history and seasonal temperature cycling (reviewed in Hofmann, 2005).

Indeed, it has been suggested that the temperatures for onset (T_{on}) and maximal induction (T_{max}) of gene expression of HSPs are useful indicators of thermal tolerance (Tomanek and Somero, 1999) and can be used to help define geographical distribution limits, as has been demonstrated in the case of two *Liriomyza* species (Huang and Kang, 2007).

The temperature, at which heat shock proteins are produced, varies according to the species and habitat; for example they are induced above 43°C in *S. crassipalpis* (Joplin and Denlinger, 1990) and 45°C in the desert locust *Locusta miggratoria* (Whyard et al. 1986). But high temperature stress (although the classical activator of HSPs) is not the subject of this review *per se* and this section will concentrate on their activation by cold stress. Transcribing, translating and folding proteins at high temperatures is well known, but cold denaturation of proteins is also documented (Privalov, 1990). For example, the constitutive production of inducible HSP70 is a known feature of polar marine species (Place et al. 2004, Clark et al. 2008) as a presumed adaptation to life in the cold and the consequential problems of folding proteins at low temperatures.

HSPs are induced in response to cold shock in most insects studied to date, although the number of examples is limited (Nunamaker et al. 1996; Rinehart and Denlinger, 2000; Yocum, 2001; Chen et al. 2005, Rinehart et al. 2006a; 2007). The exception to this general rule would appear to be *Drosophila* where studies of HSP70 and its associated regulator gene, the heat shock transcription factor (HSF-1) show negligible change of expression levels in either cold stress resistance or rapid cold hardening (RCH) (Kelty and Lee, 2001; Nielsen et al. 2005). However, as stated earlier,

Drosophila is not an ideal model when it comes to the investigation of insect cold tolerance and although HSP70 is not up-regulated in response to cold shock, this does not mean that other HSP genes, particularly the smaller HSPs are not. No doubt this will be investigated in the future. Interestingly HIF-1α was substantially increased in the goldenrod gall fly larva (*Eurosta solidaginis*) with cold exposure (Morin et al, 2005).

An interesting twist to the use of HSPs as a protective cellular mechanism in cold shock is provided by the work of Rinehart et al. (2006b). Their studies on the Antarctic flightless midge *Belgica antarctica* show continuous up-regulation of HSPs (Hsp70, Hsp90 and smHsp) in larvae, but not the adult stages, with the latter displaying the classical activation patterns in response to both high and low temperatures. The reason behind these stage–specific differences in gene expression has been suggested as being linked to niche thermal stability. The larval stages last up to two years with the animals surviving under rocks and in soil and vegetation, experiencing a temperature range of no more than 4°C. In contrast, the adults live only a couple of weeks in the austral summer and can be exposed to highly fluctuating temperatures between 0°C and +22°C. Therefore, the adults need protection against high, as well as low temperatures and the inducible mechanism is clearly the most energetically efficient way to achieve this. Conversely, the larvae, with their restricted stable temperature regime are in a situation akin to the Antarctic marine organisms and in this case the constitutive expression can be seen as a common adaptation to the Antarctic environment, with a strong requirement for facilitating proper folding of proteins at low temperatures (Rinehart et al. 2006b; Place et al. 2004; Clark et al. 2008).

HSPs can be induced by cold shock and a range of other stressors and clearly the regulatory mechanisms governing these genes are complex. They are also associated with diapause, and also recovery from cold shock. In each case, the nature and extent of the shock is different. Cold shock is an unpredictable acute stress, whereas diapause is a programmed elongated chronic stress and HSPs have the necessary regulatory capacity to deal with all situations (Rinehart et al. 2007).

Desiccation pathways

Can desiccation protect against cold shock? Are the biochemical pathways involved in each of these two types of stresses the same? There are few reports in the literature on molecular level studies to investigate the response of insects to desiccation. The heat shock proteins (Hsp23 and Hsp70) were upregulated in desiccating flesh fly pupae (*S. crassipalpis*) (Tammariello et al. 1999). This was substantiated in the adults, whilst Hsp90 and Hsc70 were shown to be unresponsive (Hayward et al. 2004). However, expression levels of these genes did depend on the diapause status of the insect. Hsp 23 and Hsp70 are highly expressed during diapause, but not responsive to additional desiccation stresses, whilst in this state. Similarly, expression levels of smHsp, Hsp70 and Hsp90 are permanently upregulated in the larvae of *Belgica antarctica* and expression levels cannot be further enhanced under any type of desiccation stress (Hayward et al. 2007). So there is potentially a trade-off in the cellular energetics for Hsp requirements. As regards non-hsp genes, so far only a novel desiccation protein (dsp28) has been identified in the beetle *Tenebrio molitor* (Graham et al. 1996), smp-30 in *Drosophila* (Sinclair et al. 2007), although this gene was originally a candidate

for cold hardening and an LEA (late embryogeneis abundant) protein in the chironomid *Polypedilum vanderplanki* (Kikawada et al. 2006). No doubt, with more high throughput technologies, such as microarrays and proteomics becoming integrated within insect physiology, this paucity of information will change, enabling us to draw comparisons between biochemical pathways activated by different stresses i.e. is cold stress different to desiccation stress at the cellular level?

Diapause (developmental-stage-specific cold survival)

Winter dormancy is common across a range organisms. Diapause is an endocrine mediated dormancy that occurs at a specific developmental stage, requiring specific cues for its initiation and is also widespread among insects. Facultative diapause is programmed by environmental factors, (normally temperature and daylength) that are perceived before diapause begins allowing insects to predict the onset of harsh seasonal conditions (see Denlinger 2002 for a review of diapause regulation). The insect thus anticipates the adverse season and prepares for the dormant period by sequestering additional nutrient reserves and seeking a suitable hibernaculum. Although we know that developmental arrest is due to an alteration in hormone titers, we do not know how the brain interprets environmental information and how it is relayed via the endocrine system. Diapause and cold hardiness are potential elements of winter survival for many insects in temperate zones but few studies have examined how the two are related. It should be noted that diapause is thought to be rare in Polar invertebrates as the environments are extreme enough to make false cueing likely (Convey, 1996).

Molecular information on diapause is limited to a few species. These include *D. melanogaster*, which has an adult diapause stage. However, it is not considered to be the most appropriate model organism for diapause studies (Denlinger, 2002). Studies so far, have concentrated on cloning and analysis of candidate genes, of which, the heat shock proteins predominate. However a distinction has to be drawn between those genes, which are involved in diapause regulation and those, which have closely associated regulatory mechanisms or function alongside diapause programming. Of the latter, there is not an all-encompassing pattern for a named gene as species-specific differences have been identified (Table 2) such as the differential up- and down-regulation of Hsp90 in *S. crassipalpis* and *D. antiqua.*

The situation, certainly with regard to the HSPs is more complex. Although there are documented associations of HSP gene expression with diapause (Table 2), these genes are also still responsive to additional external environmental stimuli, even when the insects are in a dormant state (e.g. Rinehart and Denlinger, 2000; Yocum, 2001; Chen et al. 2005) and their expression, even during diapause can be dependant on the thermal history of the animal under study (Yocum et al. 2005). Hence it has been proposed that the HSP association with diapause is a continuation of their known function of stress adaptation and that this enables insects to survive dormant stages such as diapause, which are effectively bridging stages in environmental adversity (Hayward et al. 2005). This is supported by RNAi experiments using Hsp23 and Hsp70 in the flesh fly. Suppression of Hsp23 and HSP70 expression did not alter the decision to enter diapause or its duration, but had a profound effect on the pupa's ability to survive low temperatures and so is considered to be a major factor contributing to cold hardiness of overwintering insects (Rinehart et al. 2007).

Certainly, at least in the case of the flesh fly, diapause is accompanied by the accumulation of cryoprotectants (glycerol), which again, although the glycerol pathway genes do not control diapause, the resultant product affects the ability of the flesh fly to survive this environmentally stressful period (Hayward et al. 2005). This gene-based study was supported by metabolomics data in which the metabolites involved in glycolysis were elevated by diapause treatments (Michaud and Denlinger, 2007) and it is particularly rewarding when different approaches to the same question produce supporting evidence. In the metabolomics study, the amino acids alanine and leucine were also increased and high alanine levels have been shown to have a synergistic colligative effect with other solutes, emphasizing again the complex interactions involved in such adaptations and these may not necessarily be identified solely at the transcription level.

As regards the identification of genes, which act as triggers for diapause, so far only two candidates have been identified. The action of one of these, the aptly named "diapause hormone" (DH) is discordant in the two species examined so far. It has been shown to induce embryonic diapause in the silk moth (*B. mori*) (Denlinger, 2002), yet conversely injections of DH prompt the termination of pupal diapause in both *H. virescens* and *H. armigera* (Xu and Denlinger, 2003). The picture with p38 MAPK is more consistent, with a role emerging in both the initiation and termination of diapause in the three species examined to date (*Atrachya menetriesi, Bombyx mori* and *Sarcophaga crassipalpis*), an involvement which has been monitored by both transcription and protein phosphylation studies (Kidokoro et al. 2006; Fujiwara et al. 2006; Fujiwara and Denlinger 2006).

As stated earlier, so far the studies on diapause have involved the investigation of single genes. However, with microarrays and proteomics, there are now technologies for examining hundreds of genes/proteins at the same time. Indeed the continuing interest on diapause has expanded via a proteomics study comparing brain extracts of diapausing and nondiapusing flesh fly pupa (Li et al. 2007). Thirty-seven diapause-unique or upregulated proteins were identified, of which, not surprisingly given previous studies, the HSPs predominated. This nicely complements the transcriptomics study of Rinehart et al. (2007) and adds confirmatory evidence that mRNAs upregulated during diapause are indeed translated into functional proteins (which does not always necessarily follow). Forty-three proteins were downregulated, some of which could be identified and broaden our picture of diapause-related cellular events. These included phosphoenolpyruvate synthase, fatty acid binding protein and endonuclease, which are related to metabolic processes and consequently less active during diapause (Li et al. 2007). One point highlighted during this study was the inability to identify some of the abundant protein spots. This will always be a problem with non-model species, but hopefully the possibilities of identifying genes and proteins by comparative database approaches will improve as the number of insect sequences in the databases increase.

Membrane lipids

The transition of cell membrane lipids from a liquid crystalline phase to a gel phase is one of the important causes of cold injuries under non-freezing conditions (Drobnis et al. 1993). Increasing the concentration of unsaturated fatty acids is considered to have a role in maintaining the liquid crystalline phase of cell membranes at low temperatures. Hence it is not surprising that changes are detected in the composition

of membrane phospholipids associated with diapause and increased cold tolerance in insects (Cossins, 1994). This remodelling of cell membrane lipids in response to changes in temperature is termed homeoviscous adaptation (HVA). HVA at low temperatures has been studied in several insects: *Pyrrhocoris apterus* (Slachta et al. 2002); *Drosophila* species (Ohtsu et al 1998), *Eurosta solidaginis* (Bennett et al. 1997), *Delia antiqua* (Kayukawa et al. 2007), *Chymomyza costata* (Koštál et al. 2003) and *Sarcophaga crassipalpis* (Michaud and Denlinger, 2007). In the *C. costata* example the transition to diapause was accompanied by an increase in the molar proportion of molecular species containing palmitic/linoleic (16/18.2) fatty acids esterified to *sn*-1/*sn*-2 positions of glycerol. In *Delia antiqua*, palmitoleic acid (C16:1) in phosphatidylethanolamine (PE) and oleic acid (C18:1) in phosphatidylcholine (PC) were significantly increased in pupae with enhanced cold hardiness (Kayukawa et al. 2007) and in *S. crassipalpis* oleic acid contributed to 58% of the total fatty acid pool on entering diapause from a basal level of 30%, which represents a considerable re-modelling of both fatty acid metabolism, but also membrane structure (Michaud and Denlinger, 2007).

The enzyme Δ9-acyl-CoA desaturase plays an essential role in HVA by increasing the ratio of unsaturated to saturated fatty acids in cell membranes and is expressed at low temperatures in fish (Tiku et al. 1996), bacteria (Sakamoto and Bryant, 1997), plants (Vega et al. 2004), and insects (Kayukawa et al. 2007). Insect Δ9-acyl-CoA desaturase sequences have been characterised in *D. melanogaster* (Dallerac et al. 2000) the cabbage looper moth, *Trichoplusia ni* (Liu et al. 1999), the silkworm *Bombyx mori* (Yoshiga et al. 2000) and the house fly *Musca domestica* (Eigenheer et al. 2002). Based on their broad tissue distribution, it has been proposed that they are

important for homeostasis and development in addition to being involved in sex-specific pheromone production (Eigenheer et al. 2002). However, Kayukawa et al. (2007) also demonstrated their role in cold hardiness, showing an increase in expression of the D*elia antiqua* Δ9-acyl-CoA desaturase with cold hardiness treatments, which were accompanied by a concomitant increase in the production of palmitoleic and oleic acids (the presumed products of Δ9-acyl-CoA desaturase).

Mitochondrial degradation

Studies have linked polyol production to diapause, metamorphosis, hypoxia, cold shock, and heat shock (Lee et al. 1987). These metabolic states apparently involve oxidative metabolism, possibly linked to changes in mitochondria (Kukal 1991). Investigations of the lymantriid moth *Gynaephora groenlandica* show that prolonged cold acclimation results in glycerol synthesis together with a large reduction in the number of mitochondria in fat body and brain cells (Kukal et al. 1989). A more recent study used molecular techniques (densitometry of dots blots and Northern blots) to analyse mitochondrial copy number and gene transcription in larvae of *E. solidaginis* and *G. groenlandica* to assess whether mitochondrial degradation is an adaptive physiological response to cold (Levin et al. 2003). This study concluded that although mitochondrial (mt) DNA content was reduced in cold adapted larvae, stable mitochondrial-specific mRNAs were preserved over winter in fat body cells of both *E. solidaginis* and *G. groenlandica.* This enabled mtDNA content (and consequently metabolic activity) to recover rapidly to original levels when larvae were warmed to 10 or 15°C (Levin et al. 2003).

Unique gene and proteins associated with cold shock

As HSP genes are relatively easily cloned from a wide range of organisms, their characterisation in association with cold shocks and cold acclimation predominate in the available molecular data. Genetic analyses have also concentrated on antifreeze proteins and enzymes involved in the production of cryoprotectants such as glycerol, as these are known chemicals that accumulate during cold acclimation. As these genes have largely been discussed previously, the aim of this section is to concentrate on other types of gene (summarised in Table 3).

Subtractive hybridization and selection techniques have identified candidate genes, such as Dca and EsMlp (Goto, 2000; 2001; Bilgen et al. 2001). In the first study a gene (Dca: Drosophila cold acclimation gene) was identified in *Drosophila*, which showed homology to the mammalian senescence marker protein-30 (SMP-30) (Goto, 2000). This gene was suggested to have a role in the maintenance of cytosolic Ca^{2+} levels, which may become raised as part of the stress response. Transcripts for a gene with 94% identity at the amino acid level to *D. melanogaster* LIM protein (Stronach et al. 1996) were found in the goldenrod gall moth *E. scudderiana* after exposure to low temperatures (Bilgen at al 2001). Muscle LIM proteins are essential for myogenesis and promote differentiation of striated muscle (Arber et al. 1994). As EsMlp was also upregulated in field populations overwinter it was proposed that this protein may have some role to play in either muscle restructuring at sub-zero temperatures or as a preparative function to facilitate rapid development and metamorphosis in the spring. In an alternative approach Fujiwara and Denlinger (2007) studied the phosphorylation of the p38 MAPK (mitogen activated protein kinase) in *S. crassipalpis* in response to RCH. They were able to show an increase in phospho-p38 MAPK activity within 10 minutes of cold hardening treatments. This is

an interesting study in that it targets a signalling molecule (and by inference a molecule at the upper end of the RCH gene cascade) rather than the gene end products, which is more usually the case in transcriptomics studies.

Although to a large extent, the subtractive and candidate gene approach has been replaced by microarray type techniques, there is still an interest in particular types of gene, which based on their function in other organisms will, almost certainly play a role in cold acclimation. This category includes the aquaporins. These genes were discovered as recently as 1992 by Agre et al. (1993) and are integral membrane proteins. They are characterised by six transmembrane helices forming pores that selectively allow water or other small-uncharged molecules to pass along an osmotic gradient (Kruse et al. 2006). Aquaporins have been identified and cloned in *D. melanogaster*, two species of mosquito: *Aedes aegypti* (Pietrantonio et al. 2000) and *Anopheles gambiae* and most recently identified in an Artic springtail (*O. arcticus*) EST project (Clark et al. 2007). There are no publications so far which show a direct involvement of aquaporins in cold hardening in insects. However, their role in controlling the flow of water and glycerol (in the case of the glycerol-permeable homologs- aquaglyceroporins) and additional functions in osmoregulation and metabolite transport, suggest that further studies of aquaporin gene expression may show this to be the case.

Other approaches include the use of microarrays (Qin et al. 2005), exploitation of genome data (Sinclair et al. 2007) and proteomics (Colinet et al. 2007). Only the latter investigated a non-model organism: the parasitic wasp (*Aphidus colemani*), whilst the others concentrated on *Drosophila.* Hybridization and microarray analysis of 12,000

Drosophila clones only identified 37 transcripts that were up-regulated in response to cold hardening in *Drosophila.* Not surprisingly, given the previous discussions, the majority of these transcripts coded for either heat shock proteins or membrane proteins. However, it was also possible, using functionality of proteins derived from sequence similarity data to identify a potential shift from carbohydrate to fatty acid oxidative pathways during this process. A similar shift appears during hibernation and it is plausible that both cold hardening and hibernation may share similar mechanisms (Qin et al. 2005).

Sinclair et al. (2007) took a slightly different approach, by exploiting the *Drosophila* genome data and analysing the transcription of 5 candidate genes (Frost, Smp-30, Hsp23, Hsp70a and desaturase 2) during cold stress in *Drosophila*. Some of these genes had been previously implicated in cold stress (Goto, 2000; 2001), but real time PCR enabled far more accurate time course experiments to be performed. Cold stress did not up-regulate the expression of any of these genes, but Hsp70 and Frost were implicated as being important during the recovery phase. This study emphasized the importance of being able to accurately differentiate between the exposure and recovery phases and this aspect was mirrored in the proteomics study (Colinet et al. 2007) of *A. colemani,* which studied both fluctuating temperature regimes (FTR) and constant cold temperature (CLT) treatments. Most proteins were associated with recovery phases, whilst only 4 were associated with CLT; a chitin binding protein, a cuticular protein, an ATPase and a leucine rich repeat protein of unknown function. The lack of significant amounts of up-regulated protein during exposure to cold temperatures may be due to a reduction in protein synthesis in the cold, with the production of cuticle proteins continued under this regime as development continues

even under low temperature conditions (Colinet et al. 2007). So, although these most recent approaches are providing valuable information on cold tolerance (even for non-model organisms), they are intriguingly, posing further questions about the nature and complexity of cold adaptation biochemistry.

Injury and Recovery from cold shock

Although the causes of injury responsible for death in chill susceptible insects are not full understood (Koštál et al. 2004, see Chown and Terblanche (2007) for a review of chill injury), insights have been gained from thermal cycling experiments where the cumulative effects of exposure to low temperatures are repaired during short recovery periods at a higher temperature (Chen and Denlinger 1992; Koštál et al. 2007). Koštál et al. (2007) found increased concentrations of potassium ions in extracellular fluids of *Pyrrhocoris apterus* exposed to constant low temperature (-5°C) suggesting a failure in the function of an active metabolic component (ion pump), which can result in dissipation of trans-membrane ion balance (Overgaard et al. 2007). Fluctuating temperature regimes (FTRs) can also trigger the initiation of a metabolic response involving the synthesis of free amino acids (FAAs). Levels of essential amino acids (Lys, Iso, Leu, Phe and Trp) are known to be significantly higher in the tropical beetle, *A. diaperinus* treated at 0°C compared to those at 20°C (Lalouette et al. 2007). Some FAAs have been shown to have cryo-protective properties.

The molecular mechanisms behind recovery from cold shock are alluded to in the previous section and it certainly seems from the limited evidence so far that more genes/proteins are activated in the recovery phases when compared to the actual period of the cold shock itself. Of the studies to date, 10 genes were identified using

subtractive techniques (Ellers et al. 2008) and 16 proteins in *A. colemani* after a fluctuating temperature regime (Colinet et al. 2007) (Table 4). The microarray analysis of Qin et al. (2005), chose experimental conditions that avoided the possibility of accidentally detecting genes involved in recovery. Targeted gene analysis of Hsp70 and Frost showed association with recovery from cold stress (Goto, 2001; Rinehart et al, 2006a; Sinclair et al. 2007). Interestingly, desaturase 2, a gene implicated in the alteration of membrane lipid content in response to cold (Kayukawa et al. 2007), was unaltered in expression levels, even during recovery (Sinclair et al. 2007), when, given its presumed function, one would have expected a down regulation as cellular membrane composition was changed back to "normal".

Of the genes involved in recovery, there are Hsps, membrane associated proteins and altered energy and metabolic pathways (Rinehart et al, 2006a; Colinet et al. 2007; Sinclair et al. 2007; Ellers et al. 2008). The results are consistent with a ramping up of energy production during favourable environmental periods to produce energy for essential physiological processes. So, for example genes such as Aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, all components of the glycolytic pathway were found to be up-regulated in the wasp, along with an ATP-synthase subunit, involved in buffering ATP levels and a receptor of protein kinase C (RACK), involved in protein synthesis, cell division and signal transduction (Colinet et al. 2007). This shift in energy metabolism and requirements was also identified in *Orchesella cincta* (Ellers et al. 2008). In addition cytoskeletal components were up-regulated and these have previously been implicated as being important in cold survival (Bilgen et al. 2001; Kim et al. 2006; Colinet et al. 2007). So, even now with limited evidence, there are several pathways identified which play

significant roles in recovery from cold shock and certain components, such as the cytoskeleton and lipid membranes, the manipulation of which are essential for cold survival.

Conclusions: the future

With the application of mass screening techniques (microarrays and 2-D gel electrophoresis, as described here) and the evaluation of both lab-based and environmental data, insect physiologists are gradually unlocking the clues to the biochemical mechanisms behind insect cold tolerance. *Drosophila*, despite its limitations as a model organism in cold tolerance studies, will remain an experimental favourite. It also has the advantage of a fully sequenced genome, well-characterised breeding lines, mutants and cell lines. Therefore it is possible to use this organism to conduct more traditional genetic studies to identify areas of the genome with associations to thermotolerance (Rako et al. 2007) and chill coma recovery (Norry et al. 2007). This will facilitate the identification of candidate genes for these traits, which if characterised can be identified and functionally evaluated in other insect species. However, it is still possible to identify areas of research, which with minimal effort should be promoted to advance research in non-model insect species and also areas where ecologically relevant insect species have significant contributions to make, over and above those provided by model species:

With the number of insect genomes completed to draft standard being increased, alongside numerous EST projects, genomic resources for the insect community are growing. This effectively means that it will become increasingly easy to identify genes and proteins isolated by these various techniques and there will be fewer

restrictions imposed on those working on non-model genomes. However, such studies should be community driven with at least pilot studies in a number of relatively closely related ecologically relevant species (i.e. not necessarily *Drosophila* or *Anopheles*). This will enable us to determine not only how closely related some of the insects are, but also scope future co-operations for the sharing of resources, such as microarrays and gene primers/data for targeted gene studies and Q-PCR. Such studies are ultimately synergistic and provide added value in a field where resources can be limited.

Within such studies, it should also be remembered in our technology driven era that genomics cannot provide all the answers. It is essential to maintain such investigations within multidisciplinary teams, particularly with ecologists and physiologists who can provide the context in which to place the molecular results. That is not to say that emerging techniques such as metabolomics (the "new" biochemistry) and proteomics should not be exploited, as ultimately the applied aim is to identify changes associated with cold tolerance and proteins and biochemical compounds are the last stage in expression analyses (Michaud and Denlinger, 2007). As regards triggers of response to low temperature, the answers will not lie in the gene products themselves, but in transcription factors and signalling molecules (such as p38-MAPK) (Fujiwara et al. 2006; Kidokoro et al. 2006; Fujiwara and Denlinger, 2007), an area which is largely unexplored in non-model insects to date.

Finally although, the mechanisms of cold tolerance are interesting their own right *per se*, there is definitely future value in combining such studies with those on genome plasticity and evolution. How insects will potentially react and adapt in the face of

climate change is a major challenge in future predictions of biodiversity. Examples of insect alien invasion have already been documented and are a major concern for the future, particularly in areas such as the poles (Sinclair and Stevens, 2006; Frenot et al. 2005) where the numbers of endemics are limited and could easily be replaced by aggressive invasive species.

So, by applying a number of different approaches and using a range of species with a comparative approach, the answers as to how insects survive the cold will become apparent. This is certainly an area of research with great promise for the future with relevance in many different fields of science.

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	Drosophila melanogaster	**Anopheles gambiae**	**Aedes aegypti**
Genome paper	Adams et al, 2000	Holt et al, 2002	Nene et al, 2007
Genome coverage	Complete	10x	8x
Assembly	BDGP 4.3 July 2005	AgamP3 Feb 2006	AaegL1 Oct 2005
GeneBuild	FlyBase Mar 2006	VectorBase Jun 2007	VectorBase Jun 2006
Known protein coding genes	4,751	1,316	757
Novel protein coding genes	9,288	11,141	14,662
Gene transcripts	19,841	13,133	18,061
% protein coding genes characterised	33.8	10.5	4.9

Table 1: Summary data on the three major insect genome projects curated in ensembl (at Feb 2008).

Species	Common name	Up-regulated	Down-regulated	Unchanged	References
Coleoptera					
Anthonomus grandis	Boll weevil	AgSP-1			Lewis et al (2002)
Atrachya menetriesi	False melon beetle	P38 MAPK			Kidokoro et al (2006)
Leptinotarsa decemlineata	Colorado beetle	LdHSP70A		LdHSP70B	Yocum (2001)
Diptera					
Culex pipiens	House mosquito	Actin-1 Actin-2			Kim et al (2006)
Delia antiqua	Onion maggot	Hsp90 DaTCP-1			Chen et al (2005) Kayukawa et al (2005)
Drosophila triauraria	Fruit fly	Drosmycin-like Drosmycin		Hsp23 Hsp26 Hsp83 (Hsp90) Hsrw Hsp70 Defensin Drosocin	Goto et al (1998) Daibo et al (2001) Goto et al (2004)
Lucilia sericata	Blow fly			Hsp23 Hsp70 Hsp90	Tachibana et al (2005)
Sarcophaga crassipalpis	Flesh Fly	Hsp70A Hsp70B Hsp60 Hsp25 Hsp23 Hsp23 pseudogene SmHsp	Hsp90 pnca	Hsc70	Yocum et al (1998) Rinehart and Denlinger (2000) Rinehart et al (2000) Hayward et al (2004) Hayward et al (2005)
Lepidoptera					
Bombyx mori	Silk moth	DH P38 MAPK			Yamashita (1996) Fujiwara et al (2006)
Chilo suppressalis	Rice stem borer	Hsp90		CsSP1 Hsc70	Sonoda et al (2006a) Sonoda et al (2006b)
Galleria mellonella	Greater wax moth	Lhp76 Lhp82			Godlewski et al (2001)
Heliothis virescens	Tobacco budworm		DH-PBAN PTTH		Xu and Denlinger (2003)

Table 2: Examples of genes that have had expression levels monitored in conjunction with diapause states in different insect species.

Organism	Gene	Function
Diptera		
Delia antiqua	Δ9-acyl-CoA desaturase	Phospholipid biochemistry
Drosophila melanogaster	Hsp83	Stress protein
	Hsp26	Stress protein
	Hsp23	Stress protein
	HIF-1α	Hypoxia/stress control
	CG10912	Membrane protein
	CG9568	Membrane protein
	CG13510	Membrane protein
	Enoyl-CoA hydratase	Fatty acid oxidation
	Smp-30	Maintenance of cytosolic Ca^{2+}
Lepitoptera		
Culicoides variipennis sonorensis	Hsp23	Stress protein
	Hsp40	Stress protein
	Hsp43	Stress protein
	Hsp48	Stress protein
	Hsp60	Stress protein
	Hsp70	Stress protein
	Hsp92	Stress protein
Hymenoptera		
Aphidius colemani	Homolog to pupalcuticular protein	Component of the rigid cuticle
	Gasp precursor	Chitin metabolic process
	Bellwether ATP synthase	Energy production and conversion

Table 3: Genes/proteins involved in cold hardening. Data taken from Qin et al.(2005) (data only included from microarray that was verified by Q-PCR); Goto (2000); Kayukawa et al. (2007); Bilgen et al. (2001); Nunamaker et al. (1996), Morin et al. (2005) and Colinet et al. (2007). Only annotated genes are noted.

Organism	**Gene**	**Function**
Drosophila melanogaster	Frost	Secreted protein
	Hsp70	Stress protein
Aphidius colemani	Hsp70/Hsp90	Stress protein
	ATP synthase	Energy production and conversion
	Fumarase	Energy production: TCA cycle
	Phosphoglycerate kinase	Energy production: glycolytic cycle
	Aldolase	Energy production: glycolytic cycle
	Arginine kinase	Energy production and conversion
	Guanine nucleotide-binding protein	Regulation of signal transduction
	Mitochondrial malate dehydrogenase	Energy production: TCA cycle
	Proteasome	Proteolytic complex
	Cofilin/actin-depolymerising factor	Actin regulatory protein
	Hsp70	Stress protein
	Glyceraldehyde-3-phosphate	Energy production: glycolytic cycle
	Aconitase	Energy production: TCA cycle
Orchesella cincta	Pantothenate kinase	Energy metabolism
	Carbonic anhydrase precursor	Control of pH and ion transport
	Histone H4	Transcription
	Ankyrin-like protein	Cell growth and maintenance
	Dynamin related protein 1	Cell growth and maintenance
	Vitelline membrane protein	Reproduction
	Cytochrome b	Electron transport
	28s rRNA	Protein synthesis
	16s rRNA	Protein synthesis
Culex pipiens	HSP70	Stress protein

Table 4: Genes/proteins involved in recovery from cold stress in *Drosophila*, the parasitic wasp (*A. colemani*), the springtail (*O. cincta*) and the northern house mosquito (*Culex pipiens*). Data taken from Goto (2001); Rinehart et al. (2006); Sinclair et al. (2007); Colinet et al. (2007) and Ellers et al. (2008). Only annotated genes are noted.