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REVIEW

Molecular basis of chill resistance adaptations in poikilothermic animals

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ABSTRACT

Chill and freeze represent very different components of low temperature stress. Whilst the principal mechanisms of tissue damage and of acquired protection from freeze-induced effects are reasonably well established, those for chill damage and protection are not. Non-freeze cold exposure (i.e. chill) can lead to serious disruption to normal life processes, including disruption to energy metabolism, loss of membrane perm-selectivity and collapse of ion gradients, as well as loss of neuromuscular coordination. If the primary lesions are not relieved then the progressive functional debilitation can lead to death. Thus, identifying the underpinning molecular lesions can point to the means of building resistance to subsequent chill exposures. Researchers have focused on four specific lesions: (i) failure of neuromuscular coordination, (ii) perturbation of bio-membrane structure and adaptations due to altered lipid composition, (iii) protein unfolding, which might be mitigated by the induced expression of compatible osmolytes acting as 'chemical chaperones', (iv) or the induced expression of protein chaperones along with the suppression of general protein synthesis. Progress in all these potential mechanisms has been ongoing but not substantial, due in part to an over-reliance on straightforward correlative approaches. Also, few studies have intervened by adoption of single gene ablation, which provides much more direct and compelling evidence for the role of specific genes, and thus processes, in adaptive phenotypes. Another difficulty is the existence of multiple mechanisms, which often act together, thus resulting in compensatory responses to gene manipulations, which may potentially mask disruptive effects on the chill tolerance phenotype. Consequently, there is little direct evidence of the underpinning regulatory mechanisms leading to induced resistance to chill injury. Here, we review recent advances mainly in lower vertebrates and in arthropods, but increasingly in genetic model species from a broader range of taxa.

KEY WORDS: Membrane fluidity, Proteostasis, Compatible solutes, Gene ablation

Introduction

Environmental cold poses multiple problems for all living organisms, especially poikilotherms (Cossins and Bowler, 1987). The effects of low temperature on performance are largely determined by the extent to which cooling reduces the rate of biochemical reactions – thus slowing down any rate-dependent processes (Hochachka and Somero, 2002). The impact on survival, however, is determined by the disruptive effects of extreme cold on the molecular processes underpinning normal cellular function, such as protein and membrane integrity, which in turn are closely associated with such fundamental cellular properties such as ion homeostasis and continued activity of excitable cells (Lee, 2010). Thus, at some point during both declining temperatures and diminishing performance, cold becomes stressful, and chilling injuries then accumulate over time, leading ultimately to death. In this context, chilling and cold stress are relative terms, and the temperatures at which they occur can depend on multiple factors, ranging from the species' evolutionary history and geographic origin, to its physiological status and recent thermal history.

Currently, we know very little about the precise molecular changes that characterise the transition from sub-optimal performance to chill-induced stress, and which result in cytotoxicity. Extreme chilling (see Box 1 for terminology) may cause tissue damage either directly, for example, as a result of brief (minutes or hours) exposures to extreme cold (but not freezing) temperatures, or indirectly, over more extended periods at less extreme cold, by interrupting key physiological processes at tissue and organ level (Costanzo and Lee, 2013; Denlinger and Lee, 2010; Knight and Knight, 2012). Chilling injury of insects has been repeatedly linked with a breakdown of diffusion barriers, collapse of ion gradients and loss of ion homeostasis; potassium concentration increases within the haemolymph, with decreases in both sodium and magnesium ions (Kostál et al., 2007; Kostál et al., 2006; Kostál et al., 2004). The progressive disruption to electrochemical gradients of ions eventually causes debilitation at the organ and whole-animal level, leading to loss of neuromuscular coordination and cessation of respiratory movements, as also occurs in fish (Friedlander et al., 1976). In addition, damage induced by chilling may be the result of oxidative stress (Storey and Storey, 2010), including the malfunction of mitochondrial respiration or the production of peroxide (Prasad et al., 1994). Injury can occur at surprisingly high temperatures, well above 0°C, as in tropical plants, insects and fish (Bale, 2002; Denlinger and Lee, 2010; Levitt, 1980), while chill-tolerant species from Palearctic climates may not experience any chilling injury, even after prolonged exposures to temperatures well below 0°C (Sformo et al., 2010). Freezing of body compartments in many species can be even more damaging but presents a quite different scenario, with the extracellular growth of ice crystals causing both mechanical disruption of tissues and a progressive increase in extracellular osmolality. This in turn causes a damaging osmotic dehydration of the intracellular compartment (Costanzo and Lee, 2013; Denlinger and Lee, 2010). In freeze-tolerant species these processes must be tightly controlled (reviewed by Storey and Storey, 2012).

For many organisms, their resistance to chill exposure is an inducible property. Thus, prior exposure to moderate cold, either as a short-term 'shock' or as a more gentle chronic exposure, can substantially decrease the physiological problems caused by either chill or freeze stress. Importantly, fluctuating thermal regimes

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Box 1

Glossary of terms in chill injury

Chill
Cooling sufficient to induce damaging effects or even death in living organisms, in the absence of freezing.

Chill coma
A condition (narcosis) in which cold induces the absence of spontaneous movement (reviewed by Hazell and Bale, 2011).

Cold
Reduction in temperature from some pre-existing condition.

Freeze
Ice crystal formation within a freeze-intolerant organism is lethal, while freeze-tolerant organisms can typically only tolerate extracellular freezing.

Tolerance
Reduction in the negative fitness impacts of an extreme stressor.

Resistance
The ability to withstand the damaging effects of a stressor.

Diapause
A physiological state of dormancy to survive unfavourable conditions.

Hardening
Induced resistance, with specific initiating or inhibiting conditions.

Rapid cold hardening
Fast-acting adaptation of resistance to chill stress, usually assessed by exposure to a challenging chill condition after a brief exposure (i.e. minutes to hours) to a non-lethal chill stress (see Bowler, 2005).

Seasonal cold hardening
Slow-acting adaptation of chill resistance, often as part of a diapause programme.

(FTRs), in which periods of chilling are broken by brief warmer spells, can significantly reduce chilling injury (Colinet et al., 2006; Rinehart et al., 2011). Together, these phenomena constitute both resistance adaptations (see Box1) and an increase in tolerance, in the sense of endowing fitness benefits. They suggest that the damage that builds up during chill exposure can be either prevented or repaired by molecular processes that are either activated before the imposition of chill stress or re-activated during warming periods. For example, ion gradients are re-established (Kostál et al., 2007), heat shock proteins (HSPs) or other cytoskeletal components (Michaud and Denlinger, 2004) are upregulated, depleted energy reserves (Cheng-Ping and Denlinger, 1992) are recharged, and accumulated toxic metabolites are removed (Colinet et al., 2007).

Induced resistance to freeze stress has a substantial research literature mainly focused on the induced production of compatible solutes displaying colligative properties or of ‘antifreeze’ or ice nucleation proteins (Costanzo and Lee, 2013). These solutes are often induced prior to the onset of freeze, during the time that chill injury might well occur, and it is entirely possible that these solutes have protective effects other than via colligative properties (Ødegård and Holmstrup, 2008). However, our understanding of the mechanisms endowing chill resistance are much less well described than for freeze injury, and have been largely focused on compositional modifications of the cell and sub-cellular membranes rather than on compatible solutes or on the production of chaperones. The relationship between the mechanisms for chill and freeze damage is not clear, but given the different underlying lesions it is likely that the protective mechanisms are also very different.

Here, we discuss our current understanding of the molecular mechanisms accounting for both chill injury and chill resistance adaptations in poikilothermic animals, focusing particularly on evidence implicating cellular membranes, specific metabolites and protein homeostasis. Danks has previously pointed out the need for studies of chill hardiness to integrate from a much wider range of tools and approaches (Danks, 1996), a point that has more weight given the advent of molecular genetic and system-wide, post-genomic screening technologies. Indeed, in the past 10 years, bias-free screening of transcriptomes has revealed rich patterns of chill-induced responses in fish and insects (Gasch and Werner-Washburne, 2002; Gracey et al., 2004; Zhang et al., 2011). However, converting this into a precise molecular understanding of the underpinning mechanisms responding to chill damage and mediating resistance is not straightforward, not least because changes in gene or protein expression and metabolite accumulation might be secondary effects rather than direct or adaptive responses. An effect of single gene ablation upon inducible chill resistance phenotypes provides the most direct and compelling evidence of the associated mechanisms, but this approach has rarely been utilised in studies of chill resistance mechanisms to date.

Ion gradients, induced permeability, synaptic malfunction and the central nervous control of resistance thermo-adaptation

The most obvious symptom of chill injury in animals is the progressive failure of neuromuscular coordination as indicated by the development of spasmodic movements, difficulty with locomotion, and the loss of the righting response when equilibrium is disturbed. Chill exposure in insects leads to progressive loss of coordinated movements and defects in crawling. This is referred to as the critical thermal minimum (CT_{min}) and is followed by the eventual loss of all neurophysiological activity (Hazell and Bale, 2011), immobility (chill coma) and ultimately death. A convenient and ecologically relevant measure of thermal damage is the ‘knockdown’ temperature, which is the temperature during progressive cooling or heating at which insects lose the ability to cling to an inclined or vertical surface. At some point during chill exposure, re-warming can lead to a full recovery, though beyond this point the damage becomes irreversible to the point of death. Chill coma recovery (CCR) is another common metric of cold resistance, and often reflects biogeographic or evolutionary variation in cold exposure, with species from cooler climates demonstrating faster CCR times (MacMillan et al., 2012). CCR may be energetically very costly if it encompasses, for example, not just the recovery of muscle potentials and movement, but also the complete re-establishment of normal trans-membrane ion gradients (MacMillan et al., 2012).

The underpinning molecular lesions of chill injury have been linked to the properties of cellular membranes. In insects, chill exposure has been linked with a slow but progressive reduction in ion gradients for Na, K and Mg in muscle cells (Kostál et al., 2006) consistent with the time course of injury and death. The same effects were noted in locusts (Findsen et al., 2013) and in a field cricket (MacMillan and Sinclair, 2011), which also displayed large-scale movements of water from the haemolymph to gut lumen. In these respects, chill injury shows similar effects to heat-induced injury in arthropods (Gladwell et al., 1976) and thermal inactivation of membrane-bound proteins at temperatures causing heat death of the whole animal (Cossins and Bowler, 1976). To our knowledge, the basis of neurophysiological deficits and coma following either warm or chill exposure has not yet been resolved at a more fundamental level.

In fish, the early work of Prosser and colleagues (Friedlander et al., 1976) on goldfish established that the sequence of increasingly severe behavioural and locomotory deficits that were induced by

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progressive cooling could be replicated simply by direct cooling of the brain cerebellum using a stainless steel thermode. They showed that the electrical activity of Purkinje neurons was affected over the same thermal range, with inhibitory synapses being more affected by prior thermal acclimation than excitatory synapses. Other somatic tissues were largely unaffected, suggesting that the brain was the most thermo-sensitive and thermo-adaptive site in the body. The threshold temperatures for these different symptoms were found to be modified by prior thermal conditioning of the warm-acclimated fish to reduced temperatures over a period of days to weeks (Cossins et al., 1977), along with changes in the biophysical properties and lipid saturation of synaptic membranes (Cossins, 1977). More recent work using the common carp (Vernon, 2007) has shown that differences in the chill resistance of resistant and susceptible individuals within a population was a robust and reproducible trait, but that successive chill shocks to the point of coma, separated by a month of re-conditioning, showed accumulating resistance. In other words, the degree of chill resistance was influenced not simply by chronic thermal conditioning (acclimation) but also prior history of brief chill shocks.

A central role for membranes and membrane-bound proteins

Invoking cellular membranes as a key site of thermal damage and adaptation is common in the research literature, an idea that is supported by compensatory changes in membrane properties with thermal pre-conditioning of different kinds. A particularly well-known and consistent response to cold conditioning of plants, microbes and animals is an increase in the proportion of unsaturated fatty acids in the phospholipids that make up cellular membranes (Cossins, 1983). This has long been thought to represent a homeostatic response at the cellular level, in that a change in membrane physical properties caused by increasing unsaturation compensates for the cooling-induced ordering of the hydrocarbon interior of cellular membranes (Johnston and Roots, 1964). This effect is illustrated in Fig. 1 in a comparison of synaptic membranes from vertebrate animals with diverse habitat or thermo-regulated body temperatures, and with very different chill sensitivities (Logue et al., 2000). Fig. 1A indicates a clear, orderly relationship of percentage lipid unsaturation of the major synaptosomal phospholipid classes with adaptation temperature across the full range of species. Fig. 1B shows that this is correlated with changes in membrane hydrocarbon static ordering as indicated by the fluorescence anisotropy of the membrane probe 1,6-diphenyl-hexatriene (DPH).

Any functions and phenotypic traits linked to these membrane physical attributes would also be altered in a compensatory manner, perhaps including susceptibility to thermal stress and acclimation. For fish, this view was supported by a series of papers in which the static ordering of the membrane hydrocarbon interior of cold-acclimated animals was shown to be more ‘fluid’ or more disordered than corresponding membranes of warm-acclimated animals (Cossins, 1983), thereby offsetting the direct effects of cooling on hydrocarbon molecular order. This response was subsequently linked to conserved phase properties of mosaic membranes (Hazel, 1995; Hazel et al., 1998), and the conservation of the balance between lamellar and hexagonal phase lipids (Zehmer and Hazel, 2004). Most published work relates to lipid adaptations due to pre-conditioning treatment for several days and weeks, though rapid cold hardening (RCH; see Box 1) has been linked with biophysical adaptations of membranes using ^{31}P-nuclear magnetic resonance (NMR) spectroscopy (Lee et al., 2006), mass spectrometry (Tomcala et al., 2006) and gas chromatography-mass spectrometry (GC-MS) (Overgaard et al., 2005). This last study identified an increase in linoleic acid (18:2 n-6) during RCH, while oleic acid (18:1) decreased during RCH and also in response to cold tolerance selection.

These thermally related effects also occurred when comparing different species from diverse thermal environments from polar to tropical, notably using synaptic membrane preparations from fish and other vertebrates (Fig. 1) (Logue et al., 2000). Moreover, the compositional differences between species was largely resolved to the substitution of mono-unsaturates for saturates in the *sn*-1 of the ethanolamine phosphoglyceride fraction (Farkas et al., 2001; Logue et al., 2000). Cloning of acyl Δ9-desaturase genes from carp allowed

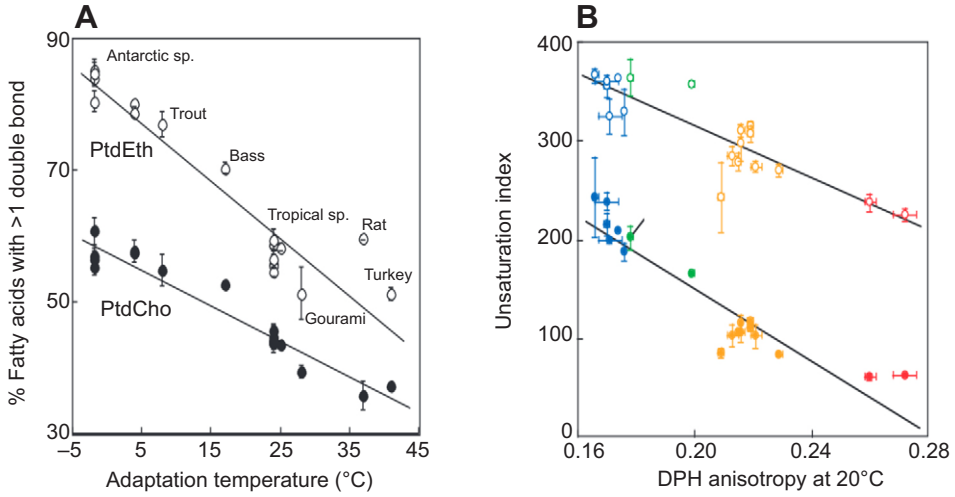

Fig. 1. A comparative analysis of brain synaptic membrane lipid composition and physical structure in relation to habitat or thermo-regulated body temperature. (A) A linear, orderly relationship for five fish species, one bird and one mammal in the percentage of fatty acids that were unsaturated in phosphatidylethanolamine (PtdEth) and phosphatidylcholine (PtdCho). (B) The unsaturation index of membrane phospholipids, which is a measure of the number of unsaturated bonds in fatty acids of membrane phosphoglycerides, is related to a measure of membrane static physical structure, namely the fluorescence anisotropy of the membrane probe 1,6-diphenyl-hexatriene (DPH). Lower values of anisotropy indicate reduced constraint on intra-membrane molecular mobility and *vice versa*. High values of the unsaturation index were correlated with reduced membrane rigidity or high membrane ‘fluidity’. Species were divided into four groups: Antarctic (blue), temperate (green), tropical (orange) and homeothermic (red). For graphs in both A and B, the correlation coefficients are all highly significant (P<0.0001) (adapted from Logue et al., 2000).

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exploration of the molecular mechanisms leading to increased unsaturation in the cold, which were shown to include activation of latent enzyme with moderate cooling, and transcriptional induction with more extreme cooling (Tiku et al., 1996). Together, the two processes provide for graded responses depending on the magnitude of the cold experience. Subsequent work showed that the two desaturase isoforms in the common carp resulting from a presumed genome duplication led to divergence of one gene as cold responsive and the other as diet inducible (Polley et al., 2003).

However, despite the plausibility and widespread acceptance of these ideas, the precise status of the membrane hypothesis remains technically unproven. The problem, at least in animal studies, lies in the purely correlative nature of the evidence presented in most published work. While this research clearly links lipid unsaturation, induced desaturases and altered membrane physical structure with changes in the chill tolerance phenotype, the possibility that this is not causal but a correlated effect has rarely been addressed. By contrast, in plants and microorganisms, the same hypothesis has been greatly strengthened by the use of single gene ablation techniques, which link the abolition of an adaptive response (i.e. conditioning-induced chill tolerance) to inhibited or ablated expression of a specific gene(s) (i.e. desaturase) either through interference RNA (RNAi) or by mutational knockout (Nishida and Murata, 1996). This conclusion was then supported by experiments in which the cold-induced upregulation of desaturase expression was mimicked by the catalytic hydrogenation of membrane lipids independently of changes in temperature (Vigh et al., 1993).

Consequently, we sought a more tractable animal model of adapted chill tolerance in which the role of single gene expression could be systematically explored (Hayward et al., 2007; Murray et al., 2007). We showed (i) that the nematode worm *Caenorhabditis elegans* displayed induced chill tolerance following cold conditioning, (ii) that the chill-resistant phenotype was reversible in that it was lost within 24 h of warming the culture to 25°C, (iii) a cold-induced increase in lipid unsaturation of the magnitude widely observed in poikilotherms and (iv) that the worm possesses three desaturases, one of which, *fat-7*, displayed a strong 20-fold cold-induced increase in transcript expression. All of these features were consistent with the membrane hypothesis of chill adaptation. We then used mutational ablation combined with RNAi to suppress the activity of the *fat-5*, *-6* and *-7* genes to generate substantial changes in lipid saturation independently of any change in culture temperature. Importantly, this had surprisingly little effect upon chill tolerance of adult worms, determined as just 16% of the change in chill tolerance achieved by pre-conditioning of worms at lower growth temperatures (Fig. 2) (Murray et al., 2007). To date, this experiment is the only direct and quantitative test in a metazoan animal of the role of membrane saturation in mediating the transition from a chill-susceptible to a chill-resistance phenotype. It raises questions about the true significance of cold-induced changes in fatty acid saturation.

It is of course possible that additional changes in membrane lipid composition may occur beyond those controlled by just the Δ9-desaturase (*fat*) genes. For example, a rather neglected group of polar lipids in animal cold tolerance research are the lysophospholipids (LPLs), produced in conjunction with free fatty acids (FFAs) when phospholipases (PLAs) hydrolyse phospholipids. LPLs have been identified as an important part of the cold response in plants (Welti et al., 2002), but have only recently been studied in insects (Koštál et al., 2013). Interestingly, LPLs activate transient receptor potential (*TRP*) ion channels in mammals, which function as neuronal sensors of temperature change (Andersson et al., 2007),

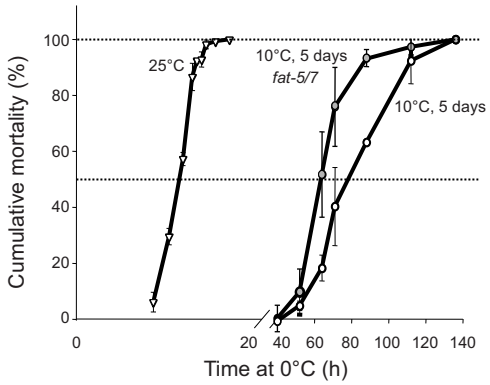

Fig. 2. Comparison of cumulative mortality curves for cultures of *Caenorhabditis elegans* after exposure to 0°C. We compared worms cultured at 25°C and transferred directly to 0°C with those grown at 10°C for 5 days prior to transfer to 0°C, which showed much extended survival. Manipulation of the desaturase activity of the worm using mutation and RNA interference (RNAi) of two Δ-9 desaturase genes, *fat-5* and *fat-7*, resulted in a substantial increase in membrane phospholipid saturation but this failed to shift the mortality curves much to the left. This manipulation thus shifted the chill resistance phenotype induced only by ~16%. Data from Murray et al. (Murray et al., 2007).

and so could represent an early step in the cold response cascade. Other critically important components of the membrane architecture that have also received little attention in studies of cold resistance include both sterols and tocopherols (Koštál et al., 2013).

Metabolite contributions to acquired chill resistance responses

Metabolites, such as glucose, glycerol, trehalose, sorbitol and ribose, have long featured in studies of cold and freeze responses and resistance adaptations. Much of the early focus, particularly in insects, was on metabolites present in freeze-intolerant species at sufficiently high concentrations (molar) to depress the supercooling point (SCP) through colligative effects. However, often the concentration changes observed in cold-responsive metabolites were too low to explain the sometimes dramatic seasonal or acclimation-induced changes in SCP (e.g. Sformo et al., 2010). Thus, a heteropteran insect, *Pyrrhocoris apterus*, experiencing cold exposure under different regimes showed an increase of ~100 mmol l^{-1} of four different polyols combined (Kostál et al., 2001). This was linked to an increase in cold resistance but with no change in SCP. Moreover, direct injection of these compounds into the haemolymph improved chill survival but again failed to shift the SCP. This suggests a protective role through a non-colligative mechanism, perhaps through changes to the structure of bound water and protein-folding state (Cacela and Hincha, 2006; Leslie et al., 1995) or, perhaps, via an as yet unappreciated mechanism. New methods of global metabolite screening now offer scope for genuinely system-wide and bias-free screening of metabolite responses as a result of changed physiological state, which might identify novel metabolites contributing to chill resistance adaptation.

Metabolomic analysis seeks to identify and quantify all, or more realistically a substantial fraction of, low molecular weight metabolites in a given organism, organ, tissue or cell type, which can then be used to characterise stress response phenotypes (Patti et al., 2012). While gene and protein expression represent the potential of organisms to respond to adverse conditions, metabolites constitute the integration of these two aspects, as well as both upstream regulatory events and environmental influences. Furthermore,

metabolomics data and its interpretation are possible without prior knowledge of an organism's genome, providing accessibility to 'non-model', and often more ecologically relevent, species. However, the metabolome encompasses a huge chemical diversity and dynamic range, and multiple preparative and analytical techniques are needed comprehensively to characterise any metabolic changes in response to cold (Weckwerth, 2007), notably gas or liquid chromatography-mass spectrometry (GC- or LC-MS) and NMR (Arbona et al., 2013; Kostál et al., 2011; Malmendal et al., 2013; Teets et al., 2012). As a result, most studies focus on particular classes of metabolites (i.e. lipids or sugars) rather than on all classes.

The metabolomic characterisation of animal responses to cold has again tended to focus on insects. These include studies on mild chilling and developmental acclimation (Colinet et al., 2012b; Foray et al., 2013), stress-selected lines (Malmendal et al., 2013), RCH (Michaud and Denlinger, 2007; Teets et al., 2012), as well as freeze resistance (Hawes et al., 2008; Kostál et al., 2011; Michaud and Denlinger, 2007). Overwintering diapause has also been studied in some detail (Colinet et al., 2012b; Michaud and Denlinger, 2007; Zhang et al., 2012), and, while not necessarily representing a direct response to low temperature, often displays a similar metabolic 'fingerprint' to the cold stress response. Distinguishing unique metabolic characteristics of freezing versus chilling versus diapause is not yet possible, as the very few studies conducted to date fail to disentangle stress-specific from species-specific responses. Where different types of cold response have been investigated in the same species, for example RCH versus seasonal preparations for chronic cold exposure as part of the diapause programme in *Sarcophaga crassipalpis* (Michaud and Denlinger, 2007), very few differences were noted. RCH uniquely perturbed the urea cycle and upregulated glutamine, cystathionine, sorbitol and urea, while only leucine was upregulated as part of diapause. As with other 'omic levels, separating causative responses from secondary effects is not easy, but they can constrain and direct what is an open and bias-free search for a wider range of possibilities than might occur through conventional hypothesis-driven approaches.

Patterns of metabolic downregulation and modulation of energy production in response to both cold and diapause have a number of common themes across species, most notably the reduction of tricarboxylic acid (TCA) cycle intermediates and a larger dependence on glycolytic and gluconeogenic pathways (Colinet et al., 2012b; Michaud and Denlinger, 2007; Ragland et al., 2010; Xu et al., 2012). Metabolites that are upregulated in response to cold and diapause are dominated by those thought to enhance cold stress tolerance, including: sugars, e.g. trehalose, glucose, fructose and sucrose (Colinet et al., 2012b; Michaud and Denlinger, 2007); sugar alcohols, e.g. glycerol, sorbitol and myo-inositol (Colinet et al., 2012a; Colinet et al., 2012b; Khodayari et al., 2013; Michaud and Denlinger, 2007; Vesala et al., 2012); and free amino acids (FAAs), e.g. proline and alanine (Kostál et al., 2011; Michaud and Denlinger, 2007; Teets et al., 2012). Glycerol is by far the most common polyol identified in overwintering insects or in insect responses to cold conditioning (reviewed by Storey and Storey, 2005). Surprisingly, glycerol is also accumulated up to 500 mmol l^{-1} in arctic fish species, such as the rainbow smelt (Clow et al., 2008; Hall et al., 2012; Raymond, 1995), where it is believed to provide part of the necessary freeze resistance along with antifreeze proteins.

However, in most instances it remains uncertain whether glycerol itself, or metabolites derived from glycerol, e.g. glycerol-3-phosphate (G-3-P), account for the enhanced stress tolerance. This is partly because the assays used in pre-metabolomic studies often did not distinguish between glycerol and G-3-P, or because only 'snap-shot' samples were taken. This highlights the importance of conducting intensive time series metabolomic investigations of the stress response, and the equally important recovery period when the chill-sensitive phenotype is restored (see Clark and Worland, 2008), because metabolite variations can occur rapidly (Nicholson et al., 2002). Yet, few studies have undertaken temporal sample collections (but see Colinet et al., 2012b; Teets et al., 2012). Even with detailed time series data, interpreting metabolite flux remains a significant challenge, and to date no insect temperature stress studies have employed isotopic tracers, e.g. ^{13}C, although this so-called 'fluxomic' technique has been employed in plants (Iyer et al., 2008). A further challenge will be to explore tissue-specific responses to cold. These can differ dramatically, for example brain versus liver responses to hypoxia in turtles (Storey, 2007), but will have been masked in whole-body extractions typical of insect studies.

Metabolomic profiles, and earlier assays of individual metabolites, again only provide correlative evidence linking the increased abundance of certain metabolites with induction and loss of cold-adaptive status. Very few studies have provided unambiguous evidence that specific metabolite changes directly contribute to or control enhanced chill resistance, although one (Kostál et al., 2011) has demonstrated that supplementation with proline significantly enhanced freezing tolerance in the Drosophilid fly *Chymomyza costata*. However, no insect studies to date have undertaken genetic manipulations of metabolite synthesis pathways, followed by assessment of effects on cold tolerance, as determined, for example, with lipid unsaturation in *C. elegans* (Murray et al., 2007) or HSP synthesis in *S. crassipalpis* (Rinehart et al., 2007). Furthermore, it is not known whether the accumulation of specific metabolites during cold acclimation or recovery is a direct protective response or an indirect consequence of, for example, the temperature-dependent functioning of the TCA cycle. Answers to this question will only come from more detailed investigations to characterise downstream (effector) and upstream (regulatory) mechanisms. This has begun to some extent with investigations of diapause induction, initiation and termination in the cotton bollworm, *Helicoverpa armigera*, and a key role for metabolic change has been identified in regulating the diapause programme (Xu et al., 2012; Zhang et al., 2012). Thus, while it is apparent that compatible solutes such as sugars, free fatty acids and polyols can protect cells both osmotically (Kostál et al., 2001) and by stabilising membranes and macromolecules even at low physiological concentrations, we still lack a detailed understanding of the precise functional role(s) that most cold-responsive metabolites play, or the stress response regulatory cascades that induce them.

In metabolomic studies of cold stress in insects, whole-body extractions dominate the literature. This clearly masks any subtleties arising from tissue-specific responses and frustrates further any understanding of regulatory mechanisms controlling metabolite responses to cold. In this respect, investigations of diapause are again at the vanguard, and tissue-specific metabolomic studies have identified clear differences in fat body, brain and haemolymph profiles (Zhang et al., 2012). A more interesting issue is the extent to which metabolite responses are initiated centrally but implemented in peripheral tissues. In the rainbow smelt, isolated hepatocytes display cold-induced glycerol production (Clow et al., 2008) but, given the recent demonstration in *C. elegans* of central nervous control over HSP production (Prahlad et al., 2008), it might be that in whole smelts a central control mechanism also dominates. Progress will likely depend on detecting and perhaps controlling organ or tissue responses in relation to cell-specific responses.

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We have recently undertaken several preliminary screens of *C. elegans* including screens of transcriptomic and metabolomics responses to cold conditioning, and of gene knockdowns that elicit changes in chill tolerance (S.A.H., B.M. and A.R.C., unpublished). These have revealed that cooling causes large-scale and distinctive responses of gene expression that form into three clusters linked to depression of central metabolism, induction of immediate early genes and the sustained induction of chromatin-handling genes. The first two clusters were reversed on warming the culture whilst the third was not. This indicates a high level of complexity in responses to cold conditioning and a large number of potential contributory mechanisms of acquired chill tolerance. Metabolite analyses of worms transferred to 5°C indicated the reduced levels of almost all intermediary metabolites, including glycerol, a well-established cryoprotectant. But a few core metabolites showed significantly increased concentrations in the worms and these have guided us in seeking new candidate genes involved in generating the inducible chill-resistant phenotype.

Protein aggregation phenomena and responses

Given their intrinsically weak intra-molecular bonding systems (Hochachka and Somero, 2002), proteins can adopt a range of different folding configurations, many of which are non-functional. Cold stress, like other stressors, can cause proteins to lose their compact folded structure, and hence functional competence (Lopez et al., 2008). Thermodynamic arguments have linked this to changes in the interaction of water with non-polar domains of proteins, causing a weakening of the hydrophobic interactions that are necessary for correct folding (Lopez et al., 2008). Some of these unfolded states show a propensity for aggregation, as in the case of amyloid protein, which unless corrected accumulates within cells causing progressive cytotoxicity (Aballay, 2013). Consequently, the cellular maintenance of a correctly folded and functional suite of proteins, termed proteostasis, is now regarded as central to cellular and organismic functioning (Balch et al., 2008; Jovaisaite et al., 2014). When proteostasis is disrupted by environmental stresses, a series of unfolded protein response (UPR) pathways are activated principally via induction of HSPs and other elements of what is a highly coordinated and powerful cellular response (Fig. 3). Disruption to the UPR leads to the accumulation of damaged proteins and typically results in cell death (Aballay, 2013).

The induction of protection from extreme thermal stress by mild or brief thermal pre-exposure of organisms and isolated cells applies famously to the induction of HSPs. Whilst a number of studies have found that HSPs are transcriptionally induced by cold shocks or by FTRs (Kostál and Tollarová-Borovanská, 2009; Kostál et al., 2001; Rinehart et al., 2006; Rinehart et al., 2007), only a few have firmly linked this to induction of the encoded protein or, more importantly, have provided direct evidence of a role in the chill-resistance phenotype. First, Rinehart and colleagues (Rinehart et al., 2007) demonstrated an active role for *hsp23* and *hsp70* genes in chill resistance, by means of the direct injection of the respective double-stranded RNAs into larvae. Second, Kostál and Tollarová-Borovanská (Kostál and Tollarová-Borovanská, 2009) showed in adult, non-diapausing *P. apterus* that the inducible form of *hsp70* was transcriptionally upregulated in fat bodies by both heat and cold stress, and also that mild heat induction led to an increase in chill resistance. In this study the chill exposure was severe at −5°C and *hsp70* induction only occurred on the warming after cold experience. Transcript abundance increased by over 1000-fold, but HSP70 protein levels only increased 1.6-fold. However, the important observation was that ablation of *hsp70* using RNAi substantially suppressed recovery from the injury caused by chill exposure, as well from heat shock. Interestingly, chill resistance was also increased when the insects were first exposed to a mild heat shock, indicating the thermal versatility of the HSP induction. Both studies directly invoke *hsp* genes as being central to acquired protection to chill injury.

While much attention is focused on the HSP70 family of HSPs, small HSPs (sHSPs) may also play an important role in responses to cold. sHSPs have molecular masses of 16–40 kDa and typically possess a conserved α-crystallin domain (Basha et al., 2012). They are known to undertake conventional chaperone functions under stress, usually by forming oligomers with extensive β-pleated sheets.

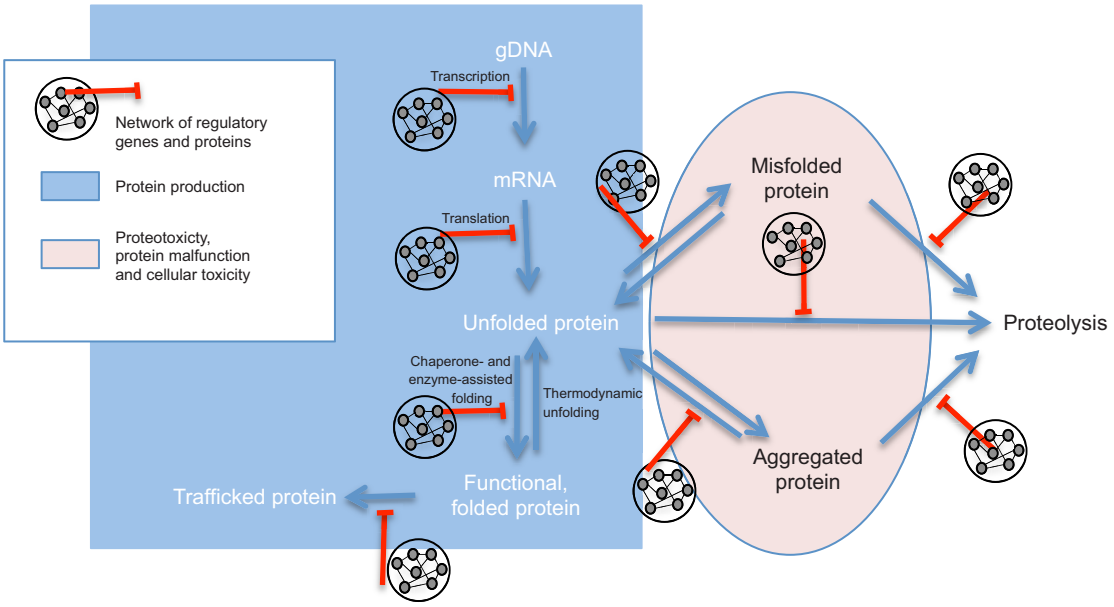

Fig. 3. Schematic diagram illustrating proteostasis as the balance between the continual production of protein and its folding into a functional entity, and the loss of the folded state or misfolding. An imbalance can lead to cellular malfunction, disease and toxicity. The coordinated regulation of the indicated events is the means by which balance is achieved, ameliorating environmentally induced protein folding (modified from Balch et al., 2008).

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HSP16.11 has recently been implicated in mediating protection from heat shock-induced necrosis in both *C. elegans* and cultured human cell lines (Kourtis et al., 2012). Work from our group also indicates that both Hsp16.11 and Hsp17 transcripts demonstrate a strong response to chilling in *C. elegans* (Fig. 4A). In some plants, sHSPs have also been implicated in inducible resistance to chill stress (Soto et al., 1999), while in animals sHSPs have been shown to underpin the cold resistance phenotype associated with diapause in *S. crassipalpis* (Rinehart et al., 2007), and play a critical role in chill coma recovery in *D. melanogaster* (Colinet et al., 2010b). However, of particular relevance in the present context is work on the cyanobacterium *Synechocycstis* indicating effects of sHSPs on the physical properties of cellular membranes (Nakamoto and Vígh, 2007). HSP17 was shown to bind to unfolded proteins as expected, and increased HSP17 expression led to heightened resistance to high temperature. However, the Vigh laboratory has offered some evidence that these proteins also associate with phospholipid headgroups of biomembranes with effects in the hydrophobic core. They suggest that this conserves the balance between inverted hexagonal phase and lamellar phase structures of membranes in the face of thermotropic distrubance (Tsvetkova et al., 2002). These phase structures and transitions between them have profound functional implications, and the *shsps* are thought to stabilise thermally induced instability. In support of this theory, there is evidence that transcription of the *hsp17* gene is strongly regulated by small changes in membrane physical order, which suggests a feedback of some kind between the two, with HSP17 acting as a signalling element (Horváth et al., 1998). The general idea of a feedback system linked to a 'fluidity' sensor was proposed long ago (Maresca and Cossins, 1993), and more recently a compelling candidate was identified in *Synechocystis* as the Hik33 histidine kinase (Suzuki et al., 2000; Suzuki et al., 2001), which activates the transcription of many downstream genes.

To date no such sensor has been identified in animals, but there is emerging evidence supporting the idea that membrane composition plays a direct role in regulating the HSP response. As described earlier, our work on *C. elegans* combining RNAi for *fat-7* with a genetic knockout (mutant strain) of the associated *fat-5* substantially increases the saturated fatty acid content of worms – thus mimicking the membrane response to warmer temperatures. Accordingly, these manipulated worms had a distinctly lower abundance of *Hsp70* transcripts when placed under heat stress, compared with wild-type animals (Fig. 4B). Other circumstantial evidence exists, with data from a variety of organisms indicating that temperature acclimation, which influences membrane lipid composition, also changes HSP expression thresholds (Barua and Heckathorn, 2004).

Despite these developments it seems that at the whole-animal level other HSP control systems operate. Indeed, in addition to the central control of thermo-acclimatory responses in fish described earlier, a controlling role for central neurons in the induction of the HSP was discovered by Morimoto and colleagues in *C. elegans* (Prahlad et al., 2008). A pair of brain neurons, the AFDs, was previously known to sense environmental temperature and initiate thermoregulatory searching behaviour via the associated AIY neurons. Introducing a loss-of-function mutation into either of these paired cell types caused loss of heat-inducible HSP70 expression in somatic cells, and this ablated protection from heat injury. Mutational knockout of other sensory neurons had no such effect. Thus, the widely held view that HSP induction was a cell-autonomous function, based on HSP70 induction in cultured cells, was likely not to be true *in vivo*. Because the AFD neurons do not directly innervate any of the HSP70-producing cells, the authors invoke a neuroendocrine signalling mechanism. This implies that responses to chill or heat shock observed in isolated, cultured cells are overruled by central control processes operating through the action of neuroendocrine systems to peripheral tissues, and that *in situ* rather than *in vitro* assessment of HSP mechanisms is necessary to understand the adaptive and ecological significance of thermally inducible expression.

The idea that compatible solutes, such as glycerol, act independently as agents for the reduction of protein stress (Hu et al., 2009; Rösgen et al., 2005) has been challenged in recent work on volume adaptive responses to hypertonic stress by the model nematode *C. elegans* (Burkewitz et al., 2012). Hypertonic treatment leads to the production of large amounts of body glycerol acting as the balancing osmolyte to maintain cell volume and bodily hydration (Lamitina et al., 2005). But hypertonicity has also been linked *in vivo* to an equally rapid and irreversible aggregation and misfolding of a constitutively expressed, transgenic, fluorescence-labelled protein (Burkewitz et al., 2011). Glycerol has thus been proposed as a 'chemical chaperone' that mitigates protein folding and aggregation damage. However, deletion mutants expressing reduced

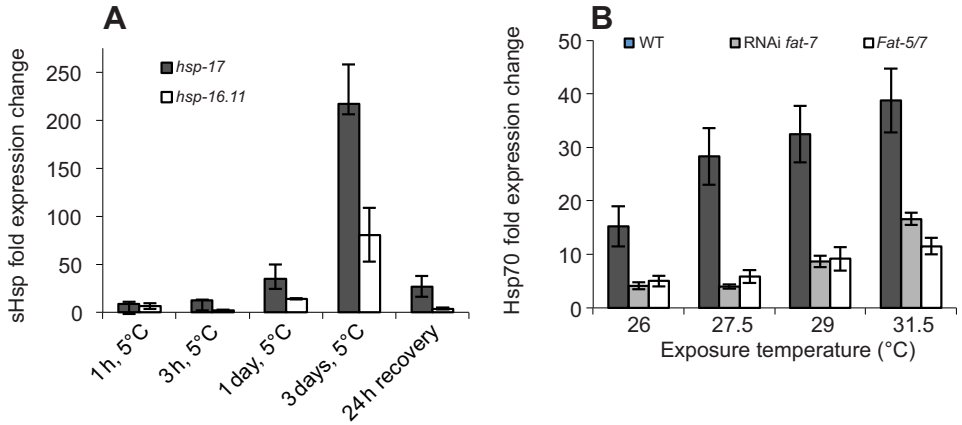

Fig. 4. Transcriptional heat shock protein (*Hsp*) responses in *C. elegans* in relation to cold conditioning (A) and how heat induction is affected by manipulations of lipid composition (B). (A) A progressive and very substantial induction by cold conditioning of two small *hsp* (*shsp*) genes (*hsp-17* and *hsp-16.11*) followed by a substantial reduction after rewarming for 24 h (unpublished data of A. Ong and S. A. Hayward). (B) Comparison of the heat induction responses for the *hsp70* transcript (Wormbase gene C12C8.1) of *C. elegans* whose whole-body phospholipid saturation had been manipulated by either RNAi for just fat-7, or of this treatment applied in combination with a mutationally ablated genetic background of *fat-5*. WT, wild-type. Manipulation substantially reduced the heat inducibility of this gene (adapted from Hayward et al., 2007).

glycerol responses or point mutations causing the constitutive expression of very high glycerol levels failed to affect the *in vivo* dynamics of either ageing-induced or hypertonically induced protein aggregation (Burkewitz et al., 2012). This work clearly indicates the value of gene manipulation approaches in dissecting the components of a complex system as the primary mechanism limiting protein damage following hypertonic stress was shown to be increased chaperone activity only.

Apparently unrelated to proteostasis, the *Frost* (*FsT*) gene of *Drosophila* has also been implicated in chill resistance. First, it is transcriptionally upregulated ~40-fold during the recovery period following chill exposure (Colinet et al., 2010a). Second, the silencing of this gene using a GAL4-UAS system significantly reduced the ability of *Drosophila* adults to recover from chill coma (Colinet et al., 2010a); thus, control flies recovered within 60 min whilst the knockdown females either failed to recover or recovered at approximately half the rate. This gene evidently plays a significant role in recovery from chill damage, but at present its function is not clearly understood.

Conclusions

Chill-induced injury is complex, with primary molecular lesions leading to a range of secondary impacts at cellular and higher levels of organisation. There is strong evidence from across different taxa that the normal functions of excitable cells, particularly in central nervous systems, are most seriously impacted by extreme damaging cold, and that excitable cells are the focal tissues mediating chill resistance. There is also some evidence in both vertebrate and invertebrate species that, at least under some circumstances, the control of adaptive responses mediated by peripheral tissues lies in the central nervous systems rather than in peripheral cells only.

We have focused on three focal molecular mechanisms that mediate a cold-induced chill resistance response. But in at least two, the evidence pointing to underpinning mechanisms is less than definitive. Thus, despite the intense focus over several decades on the increased unsaturation of membrane lipids in the cold, the vast majority of evidence in animals, at least, is correlative and underpinned by plausibility, linking cold with altered composition and in some cases with partially compensated membrane biophysical structure. But without manipulating lipid composition independently of temperature or other potentially confounding factors, the relationship is not formally tested, except in one instance where only a limited effect was evident. Thus, at this time the status of lipid adaptations in chill responses has not been definitively confirmed, at least in respect of the chill-resistance phenotype. Membrane adaptations might have other phenotypic outcomes

Regarding the inducible production of metabolites as a result of cold exposure, the use of improved assays and screens allows ideas of 'chemical chaperones' to be advanced for an increasing range of compounds expressed at increasingly lower concentrations. Effects at these lower concentrations likely denote more specific interactions with protein domains, but which to date are not clearly defined or demonstrated. Specific evidence linking these compounds with chill protection is lacking, again due to a dearth of studies where candidate solutes have been manipulated independently of temperature. The network structure of metabolite maps makes this difficult but perhaps not impossible to achieve. However, single gene ablation of proteostatic functions, particularly HSPs and associated proteins, has proved much more successful in blunting the cold-induced resistance phenotype either partially or completely. This is largely due to the intense focus on these systems in a wide range of organisms and stressors, the high degree of HSP conservation across these species, and the adoption of genetically tractable models from which definitive evidence of stress resistance could be adduced. *Caenorhabditis elegans*, in particular, emerges as one of the most useful animal models, providing direct evidence of mechanisms contributing to chill resistance, and highlighting the close interaction between metabolite synthesis, membrane compositional change and HSP response mechanisms.

To date, single gene effects undoubtedly provide the most compelling evidence of the causal mechanism(s) underpinning environmentally adaptive phenotypes, although the approach is not without its problems (Robbins, 2011). However, many physiological phenotypes are influenced by multiple 'substrate dependence' level pathways (Huang and Sternberg, 2006), each with a series of sequential steps that produce the final outcome, and each of which may be controlled by distinctive pathways of 'switchable' regulatory genes, which can be turned 'on' or 'off' (Huang and Sternberg, 2006). As a consequence, the separation of different contributory mechanisms and their regulatory pathways in quantitative terms is much more difficult, particularly when one pathway can compensate for fluctuations in the effectiveness of another. A classic example of this functional redundancy was the lack of a molecular phenotype of myoglobin ablation in mice, in which the expected exercise physiology phenotypes were masked by compensatory adjustments to tissue vascularisation, increased haematocrit and so on (Gödecke et al., 1999; Meeson et al., 2001). Recent experiments using *C. elegans* suggest that multiple mechanisms may contribute to the expression of inducible chill resistance, but, where the contributory components have separable and non-overlapping roles, ablation of individual mechanisms at either substrate or switch genes allows us to quantify each of their respective phenotypic contributions. They also provide the first compelling evidence of the dominance of central over peripheral, and neuroendocrine over cellular level systems in the regulatory control of the effector mechanisms. Our attempts to understand the mechanisms of phenotypic environmental adaptation will benefit from an expansion of these technical approaches.

Competing interests

The authors declare no competing financial interests.

Author contributions

All authors contributed equally to this review paper.

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