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ABSTRACT

Freeze tolerance – the ability to survive internal ice formation – has evolved repeatedly across insects, facilitating survival in environments with low temperatures and/or high risk of freezing. Surviving internal ice formation poses several challenges because freezing can cause cellular dehydration and mechanical damage, and restricts the opportunity to metabolise and respond to environmental challenges. While freeze-tolerant insects accumulate many potentially protective molecules, there is no apparent 'magic bullet' – a molecule or class of molecules that appears to be necessary or sufficient to support this cold-tolerance strategy. In addition, the mechanisms underlying freeze tolerance have been minimally explored. Herein, we frame freeze tolerance as the ability to survive a process: freeze-tolerant insects must withstand the challenges associated with cooling (low temperatures), freezing (internal ice formation), and thawing. To do so, we hypothesise that freeze-tolerant insects control the quality and quantity of ice, prevent or repair

damage to cells and macromolecules, manage biochemical processes while frozen/thawing, and restore physiological processes post-thaw. Many of the molecules that can facilitate freeze tolerance are also accumulated by other cold- and desiccation-tolerant insects. We suggest that, when freezing offered a physiological advantage, freeze tolerance evolved in insects that were already adapted to low temperatures or desiccation, or in insects that could withstand small amounts of internal ice formation. Although freeze tolerance is a complex cold-tolerance strategy that has evolved multiple times, we suggest that a process-focused approach (in combination with appropriate techniques and model organisms) will facilitate hypothesis-driven research to understand better how insects survive internal ice formation.

Key words: freeze tolerance, cold tolerance, ice, overwintering, insects, mechanisms, physiology, evolution, cryopreservation, thermal biology.

CONTENTS

I. INTRODUCTION

Many terrestrial insects encounter temperatures low and sustained enough to freeze their body fluids. Insects have employed a range of strategies to mitigate this risk (Lee, 2010; Sømme, 1999), including behavioural avoidance (*via* migration or burrowing), reducing the likelihood of freezing by promoting supercooling (freeze avoidance), removing freezable water (cryoprotective dehydration; e.g. Elnitsky *et al.*, 2008), and modifying the body fluid composition to prevent ice crystallisation (vitrification; e.g. Sformo *et al.*, 2010). Perhaps the most striking insect cold-tolerance strategy, however, is freeze tolerance, whereby insects tolerate the conversion of as much as 82% of their body water into internal ice (Lee, 2010; Ramløv & Westh, 1993). This strategy was first described by Réaumur (1736) nearly 300 years ago, yet the mechanisms underlying insect freeze tolerance are not fully understood. Here we review our knowledge of freeze tolerance from molecular underpinnings to evolutionary processes, and develop a framework to guide future investigations.

Internal ice formation can cause cellular dehydration and mechanical damage, and restricts the opportunity to maintain homeostasis or respond to environmental challenges (Lee, 2010). Nevertheless, freeze tolerance has clearly evolved multiple times (Dennis *et al.*, 2015; Sinclair & Chown, 2010; Walters *et al.*, 2009*b*) in a diversity of insects (see Table 1 for the freeze-tolerant insect examples discussed herein). However, due to the taxonomically and geographically diverse nature of freeze tolerance, it is unclear whether mechanisms underlying freeze tolerance are consistent across species.

Indeed, ice formation and the conditions under which insects tolerate that ice vary among freeze-tolerant species. The temperature at which ice formation begins (the supercooling point, SCP) can range from –1 °C (e.g. *Chymomyza costata* larvae in contact with ice; Koštál, Zahradníčková & Šimek, 2011) to –54 °C (e.g. *Pytho deplanatus*, an alpine beetle; Ring, 1982). Some insects survive intracellular ice formation (IIF; e.g. larvae of the wasp *Cephus cinctus*; Salt, 1961), while others restrict ice formation to extracellular spaces (e.g. New Zealand alpine weta *Hemideina maori*; Sinclair & Wharton, 1997). Once frozen, freeze-tolerant insects die if cooled to their lower lethal temperature (LLT) or held in the frozen state for their lethal time (Lt). The LLT ranges from moderate (e.g. –11.5 °C, *Pringleophaga marioni*; Klok & Chown, 1997) to extreme (e.g. < –196 °C, *C. costata*; Koštál *et al.*, 2011) low temperatures, and the Lt from several days (e.g. 7 days at –8 °C, *Gryllus veletis*; McKinnon, 2015) to many months (e.g. >205 days at –10 °C, *Cryptocercus punctulatus*; Hamilton, Mullins & Orcutt, 1985).

The presence and extent of freeze tolerance varies by life stage and season. For example, only the overwintering prepupae of the goldenrod gall fly (*Eurosta solidaginis*) are freeze tolerant (Storey & Storey, 2013). These seasonal changes may be centrally regulated by the neuroendocrine system (Xu, Neven & Duman, 1990), and often coincide with programmed diapause and seasonal changes in diet. By contrast, other insects, such as the New Zealand alpine cockroach *Celatoblatta quinquemaculata*, maintain year-round freeze tolerance, but nevertheless have a lower LLT during winter (Sinclair, 1997). Some of these changes can be very rapid; for example, the LLT of *Belgica antarctica* decreases after a brief cold exposure (1 h at –5 °C; Teets *et al.*, 2008).

To withstand the challenges associated with freezing, many freeze-tolerant insects accumulate cryo- and cyto-protectants. Cryoprotectants are hypothesised to protect against the

direct effects of low temperatures and ice (Table 2) and cytoprotectants generally to preserve cell structure and integrity [Table 3; see Storey & Storey (2013), Tattersall *et al.* (2012), Zachariassen (1985) and Zachariassen & Kristiansen (2000) for reviews]. For example, overwintering *Chilo suppressalis* accumulate glycerol and ice-nucleating agents (INAs) (Izumi *et al*., 2006; Tsumuki & Konno, 1991), both which are cryoprotectants thought to facilitate freeze tolerance (Table 2). Conversely, *H. maori* does not accumulate glycerol (Ramløv, Bedford & Leader, 1992), and not all populations of *H. maori* produce haemolymph INAs (Sinclair, Worland & Wharton, 1999). Similarly *Dendroides canadensis* accumulate substantial quantities of haemolymph antifreeze proteins (AFPs; Duman, 1980), whereas *C. quinquemaculata* exhibits no evidence of haemolymph AFPs (Wharton *et al.*, 2009). This suggests that no one cryoprotectant or class of cryoprotectants is either necessary or sufficient to allow survival of internal ice formation, and casts doubt on the prospect of a unified set of mechanisms underlying freeze tolerance.

In this review we seek to reframe our understanding of the mechanisms underlying insect freeze tolerance away from identifying specific or general classes of 'freeze tolerance' molecules to focus instead on the inherently dynamic processes of freezing and thawing. Freeze-tolerant insects must withstand the conversion of their body water into ice, maintain cellular integrity while frozen, and re-establish homeostasis and organismal integrity upon thawing. We then place cryo- and cytoprotective molecules within this framework, considering their role in protecting against or mitigating the challenges associated with cooling, freezing and thawing. Finally, we discuss the ecological and evolutionary context of insect freeze tolerance.

II. FREEZING IS A DYNAMIC PROCESS

To be freeze tolerant, an insect must survive ice formation, maintain function (or capacity for recovery) while exposed to cold, and recover function after thawing. Freeze tolerance research has generally focused on the effects of ice itself (reviewed by Lee, 2010; Pegg, 2010; Ramløv, 2000; Storey & Storey, 2013; Zachariassen, 1985). However, low temperatures irrespective of ice formation (reviewed by Koštál, 2010; Lee, 2010; Overgaard & MacMillan, 2017; Ramløv, 2000), and thawing (reviewed in the mammalian cryopreservation context by Pegg, 2010) also pose serious challenges to cellular integrity. Here, we frame cooling, freezing, and thawing as processes, and identify the challenges and mechanisms associated with them.

(1) Cooling

Physiological responses and biophysical changes begin accruing in insects during cooling, prior to ice formation. Most insects supercool: their body fluids remain liquid at temperatures below the melting point of those fluids. Low temperatures impair most biological processes, with well-explored consequences for ion and water balance (Overgaard & MacMillan, 2017). Cold passively impacts the physico-chemical parameters of the internal *milieu*: pH increases by approximately 0.02 pH units per 1 °C decrease in temperature; O_2 solubility increases (but CO_2 solubility is relatively temperature insensitive); and the viscosity of cellular and extracellular fluids increases with cooling (Somero, Lockwood & Tomanek, 2017). Cold can activate cellular signalling pathways (Teets *et al.*, 2008; Teets *et al.*, 2013), and stimulate altered transcription and translation (Štětina *et al.*, 2018; Storey & Storey, 2013), including differential regulation of microRNAs (miRNAs; Courteau, Storey & Morin, 2012). Thus, the cooling process

158 can both perturb homeostasis and be a signal for physiological changes to protect against ice
159 formation.
160
161 **(2) Ice nucleation and propagation**
162 In some freeze-tolerant animals, the process of freezing appears to involve active
163 responses by the animal. For example, the wood frog *Lithobates sylvaticus* (formerly *Rana*
164 *sylvatica*) increases metabolic rate when ice formation begins (Sinclair *et al.*, 2013*b*). In
165 addition, both the wood frog (Storey & Storey, 1984) and enchytraeid worms (Pedersen &
166 Holmstrup, 2003) mobilise glucose stores upon freezing. However, the freeze-tolerant insect *P.*
167 *marioni* does not appear to increase metabolic rate during freezing (Sinclair, Klok & Chown,
168 2004), suggesting that the process of freezing in insects may be passive, with changes during
169 freezing and thawing (e.g. water and osmolyte redistribution) driven by simple physical and
170 chemical principles. We discuss these passive processes here.
171 Ice formation is nucleated at the SCP when sufficient water molecules are arranged into
172 an ice-like structure to form an ice crystal (Fig. 1; Lee, 2010). The probability of homogeneous
173 (spontaneous) nucleation in insects is low at temperatures above about –20 °C (Zachariassen *et*
174 *al.*, 2004*b*). Ice crystals are excellent nucleators and are often responsible for nucleation,
175 especially from the environment (Fig. 2). For example, contact with external ice increases the
176 SCP of *C. costata* from –20 °C to –1 °C (Shimada & Riihimaa, 1988) and is necessary for freeze
177 tolerance in this species. Many biological molecules are INAs, some of which can be extremely
178 efficient (Fig. 2). Endogenous INAs are produced by the insect, and may include proteins
179 (Duman & Horwath, 1983; Wilson & Ramløv, 1995), other organic macromolecules such as
180 lipoproteins (Duman *et al.*, 1985), or inorganic crystals (e.g. $CaPO_4$ in *E. solidaginis* Malpighian

tubules; Mugnano, Lee & Taylor, 1996). Exogenous nucleators – apart from ice – can include bacteria (Worland & Block, 1999) and fungi (Tsumuki *et al.*, 1992), and plant material, such as algae (Worland & Lukešová, 2000), which may be either external or in the gut.

Following ice nucleation, ice propagates throughout the insect (Sinclair *et al.*, 2009). The bulk of ice formation occurs at the SCP, generating an exotherm due to heat released by ice formation (Fig. 1; Sinclair, Alvarado & Ferguson, 2015). The exotherm duration may range from a few seconds in the approximately 1 mg larvae of the drosophilid *Chymomyza amoena* (Sinclair *et al.*, 2009) to an hour or more in a 7 g *H. maori* weta (Ramløv *et al.*, 1992). Ice formation (and heat production) continues beyond the exotherm, until the insect reaches equilibrium ice content. For example, *E. solidaginis* takes approximately 48 h to reach equilibrium ice content at –23 °C, despite a relatively brief (< 5 min) observable exotherm (Lee & Lewis, 1985). Equilibrium ice content increases with decreasing temperature: even after completion of freezing at one temperature, fluctuations in temperature will lead to changes in ice content (Lundheim, 2002). Equilibrium ice content also depends on the availability of 'freezable water' – less ice will form as the osmolality of a solution increases (Storey & Storey, 1988; Tattersall *et al.*, 2012; Zachariassen, Hammel & Schmidek, 1979*a*).

The extracellular freezing model (Fig. 3) is the predominant model of ice formation in freeze-tolerant insects (Scholander *et al.*, 1953; Zachariassen, 1985). In this model, ice forms extracellularly. Solutes are excluded from the growing ice, and the osmotic pressure of the unfrozen fraction of the haemolymph consequently increases, dehydrating cells *via* osmosis (Asahina, Aoki & Shinozaki, 1954; Izumi *et al.*, 2006). This osmotic dehydration of cells continues until the cytoplasm is at equilibrium with the unfrozen fraction of the haemolymph (Sinclair & Wharton, 1997). Once in equilibrium, the cytoplasm is theoretically unfreezable by

virtue of its osmotic pressure, and IIF is avoided. However, the final distribution of ice varies considerably among freeze-tolerant insects: survivable IIF has been documented in cells from many freeze-tolerant insects (Sinclair & Renault, 2010), including fat body cells of *C. cinctus* (Salt, 1961) and *E. solidaginis* (Lee *et al.*, 1993), and *C. quinquemaculata* midgut cells (Worland, Wharton & Byars, 2004). The mechanisms allowing IIF survival are not understood, but in the Antarctic nematode *Panagrolaimus davidi*, ice is restricted to the cytoplasm and osmotically dehydrates organelles in a process analogous to the extracellular freezing model (Wharton *et al.*, 2005).

We expect several factors to impact ice propagation. Whether ice is restricted to the haemolymph or propagates into cells will depend on the rate of ice formation: external ice nucleation at high subzero temperatures and slow cooling prevent IIF in *C. cinctus* and *C. quinquemaculata* cells, while nucleation at lower temperatures results in faster ice formation and propagation into cells (Salt, 1961; Worland *et al.*, 2004). Dehydration may also limit ice propagation: at the SCP, ice propagates through the abdomen (71% water content) of *Exechia nugatoria*, while the head and thorax (47% water content) remain unfrozen (Sformo *et al.*, 2009). If ice is nucleated externally (e.g. *via* contact with external ice, or ice nucleators in the gut), it must propagate across epithelia, i.e. the cuticle and epidermal epithelia or gut epithelia (Sinclair & Renault, 2010). One potential route for paracellular ice movement across gut epithelia is the rectal paracellular channels, which are wide enough to accommodate ice crystals (e.g. 10 nm in *Gryllus pennsylvanicus*; Des Marteaux, Stinziano & Sinclair, 2018). For a review of biophysical factors that affect ice growth and movement, see Mazur (2010). Ice can also propagate between cells (Berger & Uhrik, 1996), which may be facilitated by (but does not require) gap junctions (Acker, Elliott & McGann, 2001).

(3) Changes while in the frozen state

The conversion of the bulk of body water into ice precludes haemolymph circulation, and presumably therefore excretion and endocrine communication. However, even at equilibrium ice content, the frozen state is not necessarily static: insects die when kept frozen at an otherwise-survivable temperature for a critical time (Lt), suggesting that changes occur after completion of ice formation. Ice structure changes over time: recrystallisation, the accretion of water molecules onto large ice crystals from smaller crystals, occurs readily at temperatures above about –8 °C (Knight & Duman, 1986; Knight, Hallett & DeVries, 1988; Ramløv, Wharton & Wilson, 1996). Cellular activity may continue in frozen insects, as indicated by detectable CO_2 production (Irwin & Lee, 2002; Sinclair *et al.*, 2004), ATP consumption (Storey & Storey, 1985), changes in miRNA expression (Courteau *et al.*, 2012), and accumulation of metabolites such as glycerol (Michaud *et al.*, 2008; Walters *et al.*, 2009*b*), alanine (Michaud *et al.*, 2008) and lactate (Storey & Storey, 1985). In addition, frozen insects continue to lose ion balance, probably due to equilibration of ion gradients through leak channels, or slow leak of calcium ions from storage (Boardman, Terblanche & Sinclair, 2011; Kristiansen & Zachariassen, 2001; Štětina *et al.*, 2018). Thus, frozen insects, at least at ecologically relevant temperatures, are not ‘cryopreserved’ in a static state.

(4) Thawing and recovery

A frozen insect will begin to thaw when the environmental temperature increases above its melting point (Fig. 1). In some cases, the insect is active immediately post-thaw (Sinclair *et al.*, 2004), implying that freeze-tolerant insects restore biological processes during thawing.

However, many frozen insects (e.g. *C. costata*; Koštál *et al.*, 2011) remain inactive post-thaw for minutes to days, during which the insect is presumably recovering physiological function, and/or repairing freeze injury. Some insects that are active post-thaw (e.g. *C. cinctus* frozen to –15 °C) may still die within the next few days (Salt, 1961), suggesting that recovery of movement (the typical method for determining 'survival') does not imply complete recovery from freezing. In addition, insects may appear to recover from freezing, but experience sub-lethal effects that prevent development to the next life-history stage (Štětina *et al.*, 2018).

Despite their probable importance, our understanding of thawing and recovery are limited. Thawing appears to be passive: there is no apparent change in adenylate charge (ATP availability) during thawing in *E. solidaginis* (Storey & Storey, 1985), nor is metabolic rate elevated during thawing in *P. marioni* (Sinclair *et al.*, 2004). Thawing may thus be a simple reversal of the processes associated with cooling and freezing: ice content decreases and cells rehydrate (Zachariassen, 1985). However, because recrystallisation is energetically favoured with increasing temperature, ice crystals can grow during thawing, even though overall ice content is decreasing (Mazur, 2010). In addition, thawing is unlikely to be spatially or temporally uniform. We expect that peripheral body regions will warm more quickly and should thaw earlier and at lower ambient temperatures than the abdomen and thorax. Thus, any challenges associated with thawing (e.g. osmotic stress) may occur at different times and intensities across the insect. During recovery, freeze-tolerant insects presumably expend energy to restore homeostasis (e.g. ion gradients), and recognise/repair any damage that occurred during freezing and thawing (see Section IV.5).

III. CHALLENGES ASSOCIATED WITH COOLING, FREEZING AND THAWING

Freeze-tolerant insects must tolerate the physiological and biophysical impacts of low temperatures, freezing, and thawing. Here we draw from theory on insect cold tolerance, mammalian cryopreservation, and the physical chemistry of ice to speculate on the nature of these challenges. We summarise the effects of low temperatures and ice, and how they challenge survival in Fig. 4.

(1) Low temperatures impair cellular function

During cooling and freezing, low temperatures likely inhibit macromolecular function, impairing membrane- and protein-dependent cellular processes, and causing damage (Fig. 4). Enzymes are less flexible in the cold, which decreases binding affinity (or prevents enzyme–substrate binding altogether) thus impairing function (Somero *et al.*, 2017). This reduced enzyme function will decrease metabolic capacity, reducing ATP availability, potentially increasing anaerobic metabolism, and facilitating the accumulation of harmful metabolic intermediates and by-products that damage macromolecules (Storey & Storey, 1988; Watson & Morris, 1987). Reduced antioxidant enzyme function, as well as inhibited function of the mitochondrial electron transport system, can lead to reactive oxygen species (ROS) accumulation and oxidative damage to macromolecules (Gulevsky, Relina & Grishchenkova, 2006; Lalouette *et al.*, 2011; Rojas & Leopold, 1996; Somero *et al.*, 2017). Reduced protein function will truncate cellular responses to stressors (Ramløv, 2000), for example by slowing transcriptional and translational machinery (Farewell & Neidhardt, 1998; Miguel *et al.*, 2013), or dissociating multi-subunit proteins (e.g. cytoskeletal polymers) necessary for structural integrity or intracellular transport (Des Marteaux *et al.*, 2018). The structural stabilisation of proteins by the hydrophobic effect declines at low

temperatures, potentially causing irreversible denaturation (Dias *et al.*, 2010; Marqués, 2006), leading to both functional failure and cytotoxic aggregates of denatured proteins (Korsloot, van Gestel & Van Straalen, 2004).

Low temperatures reduce membrane fluidity (Somero *et al.*, 2017), likely impairing the function of membrane-bound proteins, and processes such as endo- and exocytosis that depend on membrane fluidity. Membrane-associated enzymes, such as ATP-dependent ion pumps, have reduced activity in the cold – which is associated with loss of ion homeostasis (Koštál *et al.*, 2007) and accumulation of chilling injury in chill-susceptible insects (MacMillan, Baatrup & Overgaard, 2015). Very low temperatures cause membrane phase transitions from a fluid to a crystalline (gel) state, potentially disrupting cells and causing death. This loss of fluidity occurs at the gel-transition temperature, which is determined by membrane composition itself (Hazel, 1995). Thus, low temperatures alone cause stress, accounting for the majority of insects which are killed by cold-induced injuries unrelated to ice formation (Sinclair *et al.*, 2015).

(2) Mechanical damage during freezing and thawing

Internal ice formation kills most insects (Sinclair *et al.*, 2015), and probably damages cells/tissues even in freeze-tolerant insects (Collins, Allenspach & Lee, 1997; Izumi *et al.*, 2005; Marshall & Sinclair, 2011; Worland *et al.*, 2004; Yi & Lee, 2003). Two properties of ice can cause mechanical stress (Fig. 4): ice is less dense than water (i.e. when water crystallises, it expands), and ice is of sufficient hardness to pierce/tear biological tissue (i.e. ice crystal growth may cause shear stress). Internal ice formation can cause whole-body distension (e.g. *C. amoena* dorsal area increases by up to 5.5% with freezing; Sinclair *et al.*, 2009) and damage – the latter presumably dependent on ice location and quality (crystal size and shape). Ice formation may

rupture cells, for example by expansion of intracellular ice, or compromise tissue integrity if ice forms between cells (Pegg, 2010; Sinclair & Renault, 2010; Storey & Storey, 1988). Extracellular ice may have minimal impact on organismal or cell viability in freeze-tolerant insects (Asahina *et al.*, 1954); although if extracellular ice recrystallises it may damage cells, as seen for thawing mammalian cells (Pegg, 2010). Freeze-tolerant insects therefore likely control both the location and quality (e.g. size or shape) of ice crystals.

(3) Damage caused by freeze-induced cellular dehydration

Frozen insects lose very little water to the environment (Lundheim & Zachariassen, 1993; Sinclair *et al.*, 2013*a*). However, internal ice formation reduces available liquid water inside the animal (i.e. decreases water activity; Bradley, 2009), resulting in increased haemolymph and cytoplasmic solute concentrations (increased osmotic stress) and low cellular water content (dehydration stress) (Fig. 4; Lee, 2010; Zachariassen, 1985). When ice melts, the decreased osmotic pressure could also cause damage if cell rehydration is too rapid ('osmotic shock', as reviewed by Elliott, Wang & Fuller, 2017). Thus, both dehydration and/or osmotic stress could damage cells in frozen and thawed insects (Pegg, 2010; Worland *et al.*, 2004; Yi & Lee, 2003).

Increased osmotic pressure associated with freezing (i.e. freeze concentration) might destabilise proteins and damage cell membranes, causing cell death (Lee, 2010). The reduced water availability will increase the concentrations of individual solutes (Lee, 2010), including cations (Zachariassen, Kristiansen & Pedersen, 2004*a*), which can have specific consequences. Increased $[H^+]$ will decrease pH, which can alter protein structure and stability (Harrison, 2001). Cations such as Ca^{2+}, alter signalling (Teets *et al.*, 2013), often in a concentration-dependent manner, and can activate processes such as apoptosis (Orrenius, Zhivotovsky & Nicotera, 2003),

while others, such as Fe^{2+} will facilitate ROS formation (e.g. *via* the Fenton reaction; Storey & Storey, 2013), causing oxidative damage. High concentrations of some trace metal ions (Cu^{2+}, Mg^{2+}) may have toxic effects (Zachariassen *et al.*, 2004*a*). Hyperkalemia (high extracellular $[K^+]$) disrupts muscle function and causes injury in chilled locusts (MacMillan *et al.*, 2014), but freeze-tolerant *C. costata* appear to restore ion balance rapidly after freezing (Štětina *et al.*, 2018), and the consequences of high cation concentrations in frozen insects warrant further exploration. Concentration of other solutes may also cause damage: trehalose can crystallise (and potentially cause mechanical damage) under freezing conditions (Wen *et al.*, 2016), although this has not yet been demonstrated *in vivo*.

Freezing may damage cells by dehydrating them below a critical 'minimum cell volume' (MCV) threshold that precludes cellular recovery (Lee, 2010). Cellular dehydration will cause molecular crowding (Ramløv, 2000), increasing the probability of unfavourable intracellular interactions that can damage macromolecules (e.g. between proteins, and between proteins and ions or ROS). Dehydration can also reduce structural stability of macromolecules (e.g. by removing the hydration shell), as demonstrated *in vitro* for globular proteins (Morisaku, Arai & Yui, 2014; Prestrelski *et al.*, 1993), and membranes (Crowe, Crowe & Chapman, 1984). Extreme cytosolic volume loss can lead to potential shear stress on the cytoskeleton and cell–cell adhesions, or cause cell membrane fusions that result in cell death (Li *et al.*, 2009). The effect of freezing on macromolecule stability and its consequences (e.g. protein aggregation), have not been examined in insects.

(4) Damage due to metabolic limitations

Gases do not diffuse well through ice, and freezing may therefore impose hypoxia or anoxia (Fig. 4; Scholander *et al.*, 1953). Frozen insects accumulate anaerobic end products such as lactate, succinate, and alanine (Michaud *et al.*, 2008; Storey & Storey, 1985; Storey, Baust & Storey, 1981), suggesting a shift to anaerobic metabolism. However, the larger tracheae of frozen *C. amoena* do not collapse (Sinclair *et al.*, 2009), and frozen *E. solidaginis* (Irwin & Lee, 2002) and *P. marioni* (Sinclair *et al.*, 2004) appear to exchange CO_2 with their environment. Thus, it is unclear whether frozen insects are only partially hypoxic (we hypothesise that this could vary among tissues), or if being frozen is accompanied by a facultative shift to anaerobiosis. Lack of aerobic metabolism is associated with adenylate charge reduction in frozen *E. solidaginis* (Storey & Storey, 1985), which may impede any energy-requiring processes during recovery.

If oxygen supply is restricted in frozen insects, then thawing – especially given the efficient tracheal system – likely rapidly increases oxygen availability to tissues. This influx of oxygen has been considered analogous to ischaemia-reperfusion injury (Storey & Storey, 2013), and could therefore be accompanied by a large increase in the formation of potentially damaging ROS. Repeatedly frozen *E. solidaginis* accumulate more oxidative damage than *E. solidaginis* frozen and thawed once, potentially reflecting reperfusion injury from multiple thaw events (Doelling, Griffis & Williams, 2014).

(5) Limits of freeze tolerance: life and death while frozen

Most freeze-tolerant insects cannot survive being frozen indefinitely: they die after a critical period of time (Lt) or below a threshold temperature (their LLT). This implies that the

challenges of surviving freezing can be exacerbated while the insect is frozen. We suggest parameters that may determine these lethal limits in Table 4.

One explanation for these lethal thresholds is that some molecules, organelles, or tissues may be more susceptible to the challenges of the frozen state than others (e.g. differential damage of tissues observed by Marshall & Sinclair, 2011; Yi & Lee, 2003). By this logic, mortality of these 'weak links' accrues in a time- or temperature-dependent manner. Lethal freezing in *E. solidaginis* is associated with damage to nuclei (in the brain), mitochondria (in Malpighian tubules), and myofilaments (in the muscle; Collins *et al.*, 1997) – suggesting these organelles (and tissues) are most sensitive to freezing stress, but the mechanisms of this damage are unclear. Alternately, the LLT could be simply associated with direct effects of temperature on macromolecules (i.e. unrelated to ice; Table 4, Hypothesis 1). For example, cell death at the LLT in *H. maori* (Sinclair & Wharton, 1997) suggests that membranes are damaged, which could be caused by cold-induced membrane phase transitions, or dissociation/denaturation of the cytoskeleton (e.g. Des Marteaux *et al.*, 2018).

The quantity, quality, and distribution of ice change with both time and temperature (Lee, 2010; Ramløv, 2000). In particular, the increase in ice content with decreasing temperature will exacerbate osmotic and dehydration stress, and could contribute to mechanical damage/distortion (Table 4, Hypothesis 2). This hypothesis of a critical ice content at the LLT is supported in *E. solidaginis* (*ca*. 66%; Lee & Lewis, 1985), but ice content does not differ between non-lethal and lethal freezing temperatures in *H. maori* (*ca*. 82%; Ramløv & Westh, 1993) or *Heleomyza borealis* (*ca*. 80%; Worland, Block & Grubor-Lajsic, 2000). While recrystallisation over time (especially at high subzero temperatures; Mazur, 2010) could mechanically damage cells or tissues, there is no evidence for or against a role for recrystallisation in the Lt of freeze-tolerant

insects (Table 4, Hypothesis 3). Finally, the location of ice may change with temperature or time, and ice propagation into particularly weak cells or tissues could define the lethal limits (Table 4, Hypothesis 4).

Alternately, the Lt could be associated with a threshold accumulation of toxic products (Table 4, Hypothesis 5) [e.g. ROS (Joanisse & Storey, 1996) and lactate (Storey & Storey, 1985)] or unrepaired macromolecular damage. Depletion of adenylate charge (energy reserves) while frozen (Storey & Storey, 1985) could also limit recovery (Table 4, Hypothesis 6). For example, prolonged freezing may result in loss of ion homeostasis (Boardman *et al.*, 2011; Kristiansen & Zachariassen, 2001), and considerable energy stores may be required to restore neuromuscular function post-thaw. If these energy stores are compromised prior to recovery, then the lethal limits may be a consequence of disrupted thawing/recovery processes. Thus, metabolic processes could drive the Lt either by producing too many by-products, or through depletion of energy resources required for recovery; either way we might predict that the Lt should be positively correlated with the strength of metabolic suppression.

IV. MECHANISMS CONFERRING FREEZE TOLERANCE

Freeze-tolerant insects prevent or repair damage caused by low temperatures and ice, and preserve (or recover post-thaw) the cellular and organismal processes important for survival. Most of the putative cryo- and cytoprotectants that may contribute to these strategies (Tables 2 and 3) have been identified by correlating an accumulation of those molecules with the acquisition of freeze tolerance, usually across seasons (e.g. Baust & Lee, 1981; Marshall *et al.*, 2014; Philip & Lee, 2010). Glycerol and other polyols are the best-known low molecular weight cryoprotectants associated with freeze tolerance (Miller & Smith, 1975; Salt, 1957; Walters *et*

al., 2009*b*). However, the free amino acid proline (Koštál *et al.*, 2011; Leader & Bedford, 1978; Ramløv, 1999) and lipid-related cryoprotectants (Marshall *et al.*, 2014; Sinclair & Marshall, 2018; Walters *et al.*, 2009*a*) were identified more recently, and because they were not included in earlier screens of potential cryoprotectants, their prevalence among freeze-tolerant species is unclear. Furthermore, our understanding of the proteins involved in freeze tolerance is incomplete. Ice-binding proteins [INAs (Knight & Duman, 1986); recrystallisation inhibitors (Wilson & Ramløv, 1995)], heat shock proteins (HSPs; Lee *et al.*, 1995; Lu *et al.*, 2014; Rinehart *et al.*, 2006; Zhang, Storey & Storey, 2011), and aquaporins (AQPs; Goto *et al.*, 2011; Izumi *et al.*, 2006; Philip *et al.*, 2008; Yi *et al.*, 2011) are all associated with freeze tolerance, but most were identified *via* targeted exploration rather than an untargeted ('-omics'-style) approach that might identify unexpected molecules. Thus, the identification of cryoprotectants in freeze-tolerant species (Tables 2 and 3) has been correlative in nature, and biased towards *a priori* expectations, which makes it challenging to ascribe functional roles to them.

The most informative cryoprotectant studies examine the effects of loss- or gain-of-function. For example, we infer that AQPs facilitate freeze tolerance because their inhibition by mercuric chloride (loss-of-function) reduces survival of frozen cells from *B. antarctica* (Yi *et al.*, 2011), *C. suppressalis* (Izumi, Sonoda & Tsumuki, 2007) and *E. solidaginis* (Philip *et al.*, 2008). Proline is clearly cryoprotective, given that elevated concentrations (gain-of-function) confer freeze tolerance on *Drosophila melanogaster* (Koštál *et al.*, 2016). Here we eschew a focus on (groups of) cryo- and cytoprotectants, but rather approach their function within a framework of hypothesised strategies and mechanisms underlying insect freeze tolerance – these mechanisms are summarised in Fig. 4. We hope that this strategy/mechanisms paradigm will facilitate a

hypothesis-driven approach to unravelling the role of cryoprotectants and freeze tolerance in general.

(1) Controlling ice formation and propagation reduces mechanical damage

We hypothesise that freeze-tolerant insects control ice crystal location and size to minimise mechanical damage (Fig. 4). Ice location could be controlled by modifying the site of ice nucleation (e.g. *via* INAs), by physical barriers such as cell–cell tight junctions, and by the redistribution of water during freezing (e.g. *via* AQPs), while ice-binding proteins (e.g. AFPs) could alter ice quality.

Ice-nucleating agents initiate ice formation, and differential distribution of INAs will thus localise ice formation, perhaps to compartments that are robust to ice-induced mechanical damage. A core hypothesis is that INAs confine ice to extracellular spaces (Fig. 3), preventing IIF (and the associated mechanical damage) by facilitating osmotic dehydration of cells (Lee, 2010; Zachariassen, 1985). In addition to controlling the location of ice, INAs elevate the temperature at which ice formation begins (Sømme & Zachariassen, 1981; Zachariassen & Kristiansen, 2000). This promotes slow formation of large ice crystals (Salt, 1961), which can promote osmotic dehydration by extending the time available for water and cryoprotectants to redistribute during freezing (Storey & Storey, 1988). High SCPs can substantially improve survival of internal ice, for example *C. costata* is freeze tolerant if ice formation is nucleated above –2 °C, but not at –20 °C (Shimada & Riihimaa, 1988). However, a high SCP is not sufficient for freeze tolerance: for example, *Eleodes blanchardi* beetles held at +20 °C retain their high SCP (–6.5 °C), but lose their freeze tolerance (Zachariassen & Hammel, 1976).

This model of osmotic cellular dehydration to avoid IIF depends on transmembrane water movement during ice formation. The bulk of water movement across cell membranes is through AQPs, which may also transport glycerol (aquaglyceroporins, GLPs; or entomoglyceroporins, EGLPs) and other small polar molecules (Finn *et al.*, 2015; Hub & De Groot, 2008), and likely facilitate intracellular accumulation of low molecular weight cryoprotectants (Izumi *et al.*, 2007). If AQPs are inhibited (e.g. by mercuric chloride), cells from *C. suppressalis* and *E. solidaginis* do not survive freezing (Izumi *et al.*, 2007; Philip *et al.*, 2008). Water moves very quickly through AQPs (Izumi *et al.*, 2006), which will be particularly important for osmotic dehydration if ice formation is rapid (e.g. if nucleated at low temperatures; Duman & Horwath, 1983). While AQPs may be important for preventing IIF, these transporters may facilitate other dynamic processes associated with freeze tolerance (e.g. glycerol distribution, recovery during thawing), and are necessary for general cellular homeostasis. We also note that avoiding IIF is not necessary for freeze tolerance, but the prevalence of, and mechanisms underlying, survival of IIF are unknown (Sinclair & Renault, 2010).

Ice-binding proteins can regulate ice crystal shape and size (Fig. 4). The insect AFPs examined to date inhibit recrystallisation, at least *in vitro* (Table 2; Horwath *et al.*, 1996; Knight & Duman, 1986; Walters *et al.*, 2009*a*; Wharton *et al.*, 2009). This recrystallisation inhibition may reduce damage due to ice crystal growth at high subzero temperatures (Mazur, 2010). Thus, while AFPs prevent growth of nascent ice crystals in many freeze-avoidant species *via* thermal hysteresis (TH) activity (i.e. non-colligative depression of freezing point), AFPs in freeze-tolerant insects are hypothesised to modify the behaviour of existing ice crystals (Duman, 2001; Walters *et al.*, 2011). We note that recrystallisation inhibitors (RIs) do not always have TH activity (e.g. in the nematode *P. davidi*; Wharton *et al.*, 2005), and that some freeze-tolerant

insects do not exhibit RI activity e.g. *Tipula trivittata* (Knight & Duman, 1986) and *H. maori* (Ramløv *et al.*, 1996). Thus, it is not clear if recrystallisation is unimportant for some freeze-tolerant species, or if we cannot comprehensively identify the mechanisms that regulate it, and we suggest characterizing RI activity in more freeze-tolerant species (e.g. *via* the high-throughput gold nanoparticle aggregation assay; Mitchell *et al.*, 2015). We are not aware of *in vivo* observations of the quality of ice or the recrystallisation process in freeze-tolerant insects.

(2) Controlling ice content reduces damage due to dehydration

The proportion of water that is converted into ice depends on solute concentration, temperature, and time. Because insects cannot control temperature or time spent frozen, we assume that frozen insects are at their equilibrium ice content in nature. Freeze-tolerant insects may reduce equilibrium ice content by accumulating low molecular weight cryoprotectants (Fig. 4, Table 2; Rozsypal *et al.*, 2018; Storey & Storey, 1988; Tattersall *et al.*, 2012; Zachariassen *et al.*, 1979*a*), which act as compatible osmolytes (Somero *et al.*, 2017). We assume that the concentration of these low molecular weight cryoprotectants equilibrates between intracellular and extracellular spaces, and that high haemolymph osmolality will therefore be associated with low ice content (Fig. 5). Minimising ice content should (by reducing cellular volume loss through dehydration) reduce shrinkage and molecular crowding in the (unfrozen) cells of the frozen insect, improving survival.

In practice, the link between ice content and survival is not entirely clear. For example, freeze-tolerant *E. blanchardi* appear uninjured when up to 65% of body water freezes, while freeze-sensitive morphs are injured at an ice content of only 55% (Zachariassen, Hammel & Schmidek, 1979*b*). Furthermore, frozen *H. maori* (Ramløv, 1999; Ramløv & Westh, 1993) and

H. borealis (Worland *et al.*, 2000) have ice contents above 80% (Fig. 5), which should lead to substantial dehydration stress. In addition, while proline accumulation correlates with lower ice content in *C. costata*, vitrification (rather than ice content) appears to be the strongest predictor of freeze tolerance at low temperatures (Rozsypal *et al.*, 2018). This suggests that ice content (and dehydration of cells below a critical MCV) is not the sole determinant of frozen insect survival. There are surprisingly few ice content studies, and no cell volume measurements, in freeze-tolerant insects. We suggest that additional data are required to critically evaluate the relationship between ice content, cell volume, and mortality of frozen insects.

An alternative to avoiding dehydration stress may be to mitigate it, for example by accumulating anhydroprotectant molecules (e.g. trehalose) that stabilise macromolecules and cells under low water activity (Crowe *et al.*, 1987). These anhydroprotectants could enhance freeze-tolerant insect survival at concentrations too low to alter ice content substantially. For example, the approximately 40–85 mM proline accumulated by overwintering *H. maori* (Neufeld & Leader, 1998; Ramløv, 1999) likely has only a minimal impact on ice content, but could facilitate survival by directly protecting macromolecules (see Section IV.3) under the (presumably intense) desiccating conditions associated with >80% ice content.

(3) Stabilising cells and macromolecules reduces cell damage and death

Like all ectotherms, insects can mitigate the cellular effects of low temperatures by altering their macromolecules (Fig. 4; Somero *et al.*, 2017; Storey & Storey, 2013; Tattersall *et al.*, 2012). These changes include alterations in membrane composition to maintain fluidity (and integrity) at low temperatures, e.g. higher concentrations of polyunsaturated phospholipids in *C. costata* (Koštál, Berková & Šimek, 2003). Insects may also accumulate more flexible protein

isoforms that retain function and are less likely to denature or aggregate at low temperatures. For example, *Cucujus clavipes* have winter-specific cytoskeletal protein isoforms (Carrasco *et al.*, 2011). While the cellular stresses associated with cold are common to both freeze-tolerant and freeze-avoidant insects, here we focus on how freeze-tolerant insects protect against the specific cellular stresses associated with internal ice formation, including low water availability, and dehydration-induced molecular crowding and cell shrinkage (Fig. 4).

Because freezing reduces water availability, freeze-tolerant insects must counteract the destabilising effect of dehydration on membranes (including organelle and vesicle membranes) and proteins. These macromolecules can be stabilised by direct or indirect (*via* the hydration shell) interaction with low molecular weight metabolites such as trehalose, proline, and other amino acids (Anchordoguy *et al.*, 1988; Arakawa & Timasheff, 1982, 1983; Crowe *et al.*, 1987; Rudolph & Crowe, 1985; Tsvetkova *et al.*, 1991), several of which have been reported in freeze-tolerant insects (Table 2).

The molecular crowding associated with ice formation could promote unfavourable interactions among macromolecules, e.g. aggregation of denatured proteins. Freeze-tolerant insects may therefore accumulate molecules that refold, remove, or isolate denatured proteins. Proline (Rudolph & Crowe, 1986) and arginine (Arakawa & Tsumoto, 2003; Das *et al.*, 2007) may reduce protein aggregation by forming chains/clusters to physically buffer proteins from each other (Koštál *et al.*, 2016). We speculate that freeze-tolerant insects may also accumulate intrinsically disordered proteins (Table 3), which prevent protein aggregation under cold and dehydrating conditions (Newman *et al.*, 2017; Toxopeus, Warner & MacRae, 2014). Several freeze-tolerant insects accumulate HSPs (Lu *et al.*, 2014; Rinehart *et al.*, 2006; Zhang *et al.*, 2011). These molecular chaperones (Table 3) can prevent denaturation and/or aggregation under

osmotic stress and low temperatures, and may refold denatured proteins (King & MacRae, 2015), which will both maintain cellular function and reduce macromolecular damage.

Freezing-induced cell shrinkage could place the cell membrane, cytoskeleton, and cell–cell junctions under shear or strain stress. Well-documented changes in membrane composition [e.g. increased proportion of phosphatidylethanolamine (Izumi *et al.*, 2009) or increased membrane sterol fraction (Koštál *et al.*, 2013)] likely increase membrane flexibility and prevent rupture during shrinkage. Proteomic studies of *B. antarctica* (Li *et al.*, 2009) and transcriptomic studies of freeze-intolerant insects (Clark *et al.*, 2009; Des Marteaux *et al.*, 2017; MacMillan *et al.*, 2016) suggest that changes in cellular/tissue modelling are imporant for stress tolerance. We speculate that freeze-tolerant insects accumulate alternative isoforms of cytoskeletal and cell adhesion proteins that withstand the tensions associated with cell dehydration, or accumulate regulators of these proteins. Conversely, freeze-tolerant insects may not prevent cytoskeleton depolymerisation at low temperatures, but rather accumulate chaperones that promote cytoskeleton reassembly post-freeze, as suggested by upregulation of chaperone T-complex protein 1 (TCP-1) in *E solidaginis* (Storey & Storey, 2013).

(4) Managing biochemical processes reduces damage from harmful metabolites

Because the frozen state is not static, freeze-tolerant insects may need to neutralise or prevent the production of harmful/toxic metabolites such as ROS and metabolic end products (e.g. lactate and uric acid; Fig. 4). The metabolic suppression accompanying diapause in many overwintering insects (Hahn & Denlinger, 2011), and specifically associated with freezing (Irwin & Lee, 2002; Marshall & Sinclair, 2012*b*), likely reduces production of these metabolites. To remove harmful metabolites, *B. antarctica* upregulates detoxifying cytochrome P450 enzymes

589 when recovering from dehydration (Lopez-Martinez *et al.*, 2009), and we expect to see similar
590 responses during thawing of freeze-tolerant insects (Table 3). Overwintering *E. solidaginis*
591 accumulate ion-scavenging proteins (e.g. ferritin) that likely reduce ROS formation, and also
592 glutathione and antioxidant enzymes (e.g. superoxide dismutase) that neutralise ROS once
593 produced (Table 3; Joanisse & Storey, 1998; Storey & Storey, 2010). Sirtuins are also important
594 in ROS detoxification (Merksamer *et al.*, 2013), but have not, to our knowledge, been explored
595 in association with freeze tolerance. Similarly, strategies to mitigate the effects of accumulated
596 anaerobic end products, such as lactate and alanine (e.g. lactate clearance in *E. solidaginis;*
597 Storey & Storey, 1985), in frozen insects remain to be explored.
598 We also anticipate disruption of non-metabolic processes with freezing, which freeze-
599 tolerant insects may prevent, or recover post-thaw. For example, high Ca^{2+} concentrations due to
600 cell dehydration will disrupt cell signalling (Zachariassen *et al.*, 2004*a*), which freeze-tolerant
601 insects may mitigate by accumulating ion chelators (Table 3). Other processes may be
602 unavoidably disrupted, such as loss of membrane potentials (e.g. due to reduced activity of ion
603 pumps; Overgaard & MacMillan, 2017) and reduced intracellular trafficking (e.g. due to high
604 cytoplasmic viscosity in the frozen state; Lee, 2010; Zachariassen, 1985). These processes must
605 instead be recovered during or post-thaw.
606
607 **(5) Repair and recovery of physiological function post-thaw**
608 During or after thawing, insects must restore physiological function. If the freezing and
609 thawing processes caused injuries, this damage must be repaired. Surprisingly, there is almost no
610 data on mechanisms of damage and repair in freeze-tolerant insects, so we are largely confined to

speculation on how they recover at the whole-animal and cellular level, as well as how they recognise and repair damage (Fig. 4).

Many physiological processes depend on appropriate ion gradients across cell membranes and epithelia, including neuroendocrine function (neuron membrane potentials), locomotion (muscle membrane potentials), and digestion and excretion (ion gradients across gut and Malpighian tubule epithelia; Bradley, 2009). Thus, if ion and water balance were disrupted during freezing (as suggested by Boardman *et al.*, 2011; Kristiansen & Zachariassen, 2001; Štětina *et al.*, 2018; but not Williams & Lee, 2011), recovery from freezing may parallel recovery from chill coma (MacMillan *et al.*, 2012). That is, we predict that ATP-motivated ion transport is required to re-establish ion balance post-thaw, a process that is likely energetically demanding.

Because organismal integration is likely disrupted in frozen insects, we expect recovery processes to be regulated locally (e.g. by intracellular changes in $[Ca^{2+}]$; Teets *et al.*, 2013) until integration of those systems (e.g. *via* neuroendocrine function) are restored. Although intracellular signalling is likely important for this regulation, freeze-tolerant insects may have to blunt/moderate signalling during thawing, when cell volume increases rapidly modify intracellular ion (and other signalling molecule) concentrations (Zachariassen *et al.*, 2004*a*). Other processes that may be involved in restoring cellular homeostasis include refolding and/or reassembling denatured proteins [using chaperones such as HSPs (Štětina *et al.*, 2018; Storey & Storey, 2013)], and clearing metabolites accumulated during/prior to freezing, which may include harmful end products [e.g. in *C. costata* (Štětina *et al.*, 2018) and *E. solidaginis* (Storey & Storey, 1985)], as discussed above, as well as cryoprotectants [e.g. glucose in the wood frog *L. sylvaticus* (Costanzo & Lee, 2013)]. We hypothesise that freeze-tolerant insects may remove

these metabolites *via* catabolism, as well as whole-organism processes such as excretion and/or storage.

If damage occurred during freezing, recognition and repair of damage will be required (Fig. 4). The recovering insect may identify cellular damage *via* several markers, including accumulation of damaged proteins, disturbance of the cellular redox state, and alterations in ion concentrations (e.g. Ca^{2+}) that alter signal transduction (Korsloot *et al.*, 2004). Irreparably damaged cells may undergo apoptosis (programmed cell death) or necrosis (unregulated cell death) during recovery (Korsloot *et al.*, 2004). Post-thaw, this cellular injury may activate immune responses (Sinclair *et al.*, 2013*a*), and stimulate cell proliferation to repair tissue (Smith, Howes & Treherne, 1990). Alternatively, insects may repair damaged cells, for example by removing damaged macromolecules to the proteasome, or organelles *via* autophagy (Štětina *et al.*, 2018; Teets & Denlinger, 2013). These cellular components will need to be replaced, and we speculate that recovery from freezing could involve several waves of prioritised repair.

We expect both recovery and repair to be energetically costly. The beetles *Hydromedion sparsutum, Perimylops antarcticus* (Block, Worland & Bale, 1998*b*) and *E. blanchardi* (Zachariassen *et al.*, 1979*b*) appear to have elevated metabolic rates post-thaw, whereas larvae of the lepidopteran *P. marioni* (Sinclair *et al.*, 2004) and *Pyrrharctia isabella* (Marshall & Sinclair, 2011) do not. This discrepancy could arise from differences in methods (earlier studies used closed-system respirometry, whereas later studies used more sensitive open-flow systems), from a phylogenetic signal (beetles *versus* moths), or because repair and recovery is metabolically costly in some species but not in others. If repair and recovery are energetically costly, then we expect freeze-tolerant insects to manage those energy demands by ensuring an adequate energy supply (Sinclair, 2015; Sinclair & Marshall, 2018), and reducing overall energy demand during

the freezing and thawing processes, by suppressing metabolism (e.g. in diapause; Irwin & Lee, 2002) and minimising the need to replace/repair cells and macromolecules by sufficiently protecting them during freezing and thawing.

V. ECOLOGY AND EVOLUTION OF FREEZE TOLERANCE

Sinclair & Chown (2010) identified freeze-tolerant Orthoptera, Blattaria, Coleoptera, Hymenoptera, Lepidoptera and Diptera, to which we can now add Phasmatodea (Dennis *et al.*, 2015) and Plecoptera (Walters *et al.*, 2009*b*). Making this summary at the Order level is somewhat misleading, since there is ample evidence that freeze tolerance has evolved multiple times within most of these orders (Sinclair & Chown, 2010). Within the radiation of New Zealand stick insects, for example, freeze tolerance has evolved at least twice (and freeze avoidance the same number of times; Dennis *et al.*, 2015), and within one species (*Niveaphasma annulata*) only five of the six populations studied were freeze tolerant. Here, we discuss selective pressures and potential routes that allow insects to evolve freeze tolerance, and the implications for freeze tolerance under climate change.

(1) Pressures that select for freeze tolerance: three non-exclusive hypotheses

(*a*) *Extreme low temperatures*

The physical limit for maintaining aqueous solutions in a supercooled (i.e. liquid) state is around –58 °C (e.g. Miller, 1982). Some alpine habitats, and continental sub-Arctic and temperate habitats [notably the Yukon (Danks *et al.*, 1997), Siberia (Li, 2016) and interior Alaska (Miller, 1982)] that have high insect abundance and diversity regularly experience air temperatures below this limit. While some insects likely survive by selecting buffered microhabitats [e.g. *Cossus*

cossus caterpillars in soil, under snow cover (Li, 2016)], others are clearly exposed to ambient temperatures (e.g. *Upis ceramboides* overwinters under tree bark, above the snow line, exposed to –50 °C; Miller, 1978). This imposes strong selection pressure for either cryoprotective dehydration (e.g. *B. antarctica*; Elnitsky *et al.*, 2008), vitrification (e.g. *C. clavipes*; Sformo *et al.*, 2010) or freeze tolerance. Bale (1996) inferred that freeze tolerance was the most extreme form of cold tolerance (i.e. at one end of the continuum), and many insects from these extremely cold environments are indeed freeze tolerant (Li, 2016; Miller, 1982; Turnock & Fields, 2005). However Sinclair (1999) showed that the range of lower lethal temperatures in freeze-tolerant insects was similar to that of freeze-avoidant insects, implying that freeze tolerance is not the only viable strategy for surviving extremely low temperatures.

(*b*) *High risk of freezing*

We expect selection for freeze tolerance in species at high risk of inoculative freezing. Insects exposed to ice in their microhabitats (e.g. those encased in ice, or exposed to frozen soil) are susceptible to inoculative ice formation (Pedersen & Holmstrup, 2003; Ramløv, 1999; Zachariassen & Kristiansen, 2000). In these circumstances, freeze tolerance may be advantageous compared to either cryoprotective dehydration (Elnitsky *et al.*, 2008; Holmstrup, 2014) or resisting ice formation through an impermeable cuticle and AFPs (Crosthwaite *et al.*, 2011). Aquatic insects might be particularly exposed to these conditions: at least one aquatic insect (*Nemoura arctica*) is freeze tolerant (Walters *et al.*, 2009*b*), and we expect that Odonata (Sawchyn & Gillott, 1975) and Trichoptera (Olsson, 1981) that overwinter encased in ice are also freeze tolerant. Freeze tolerance may also be prevalent in wetland insects in the Arctic and sub-Arctic, where there is a high diversity of Diptera whose cold tolerance has been only

sparsely investigated (Danks, Kukal & Ring, 1994; Ring, 1982). Similarly, insects with gut floras that produce ice nucleators (e.g. gut bacteria of *H. sparsutum*; Worland & Block, 1999), or that do not clear their gut of food prior to cold exposure (e.g. *Hemideina* spp., Sinclair *et al.*, 1999) may be prone to inoculation from the gut.

Sinclair, Addo-Beddiako & Chown (2003) proposed that environments with unpredictable, year-round likelihood of freezing events select for insects to remain active, e.g. to take advantage of warm spells between freezes. In turn, this selects against significant preparation for winter, such as entering diapause and/or clearing the gut. If the gut remains full, then there is a strong likelihood of ice nucleation, leading to selection for freeze tolerance. Alpine and sub-Antarctic environments in the Southern Hemisphere meet the criteria for frequent, unpredictable freeze events, and have a correspondingly high proportion of freeze-tolerant species (Sinclair *et al.*, 2003; Sinclair & Chown, 2005*a*). This situation also applies to the strong daily variations in temperature on tropical high mountains, where there is also an apparent preponderance of freeze-tolerant insects (Sømme, Davidson & Onore, 1996; Sømme & Zachariassen, 1981).

(*c*) *Physiological advantages of being frozen*

Many insects overwintering in temperate and polar environments do not feed (although see Sinclair & Chown, 2005*a*), so energy and water stores over winter may be non-renewable. A cold-tolerance strategy that reduces energy drain or water loss should therefore be advantageous. Frozen insects lose less water to the dry winter environment than unfrozen insects at the same temperature (Danks, 2000; Irwin & Lee, 2002; Ring & Danks, 1994). Similarly, there is some evidence that frozen insects have lower metabolic rates than their unfrozen counterparts (Irwin &

Lee, 2002; Sinclair *et al.*, 2004), and that this metabolic suppression allows frozen insects to save energy over an entire winter (Marshall & Sinclair, 2012*b*).

Overwintering insects can experience significant mortality from pathogens and parasites, or can bear the eggs or larvae of parasitoids. If freeze-tolerant insects can withstand being frozen better than their pathogens, parasites, or parasitoids, then freeze tolerance may be a strategy to reduce pathogen or parasite loads. There is ample evidence of immune activation or modification during winter (Ferguson & Sinclair, 2017), and at least one freeze-tolerant insect (*P. isabella*) has greater resistance to fungal pathogens after freezing exposure, implying an activation of the immune system (Marshall & Sinclair, 2011). A survey of cold-tolerance strategies of Arctic sawflies and their hymenopteran parasitoids concluded that both the hosts and parasitoids are freeze tolerant (Humble, 2006). In addition, Tyrrell *et al.* (1994) showed that nematode intestinal parasites can survive freezing of their *H. maori* host (and other nematodes are also significantly cold tolerant, see, e.g. Wharton, 1995). However, there is variation in the freeze tolerance of entomopathogenic nematodes that could make host freeze tolerance advantageous in some instances (Shapiro-Ilan, Brown & Lewis, 2014). To our knowledge, the effect of freezing (rather than just cold) on fungal or bacterial pathogens has not been explored in freeze-tolerant insects. Thus, there is considerable scope for investigating the role of pathogens in the evolution of freeze tolerance.

(2) Routes to evolve freeze tolerance

The evolutionary path to freeze avoidance is intuitive: insects that freeze die, those with subtle improvements in maintaining their body fluids in a liquid state will survive, and if that improvement is heritable, then it will be passed onto their offspring. To make the transition to

749 withstanding internal ice formation is more difficult: many insects are killed by even a small
750 amount of internal ice (Sinclair & Chown, 2010). Here we examine two (non-mutually
751 exclusive) routes towards evolving freeze tolerance: pre-adaptation to related stresses and partial
752 freeze tolerance.
753
754 (*a*) *Pre-adaptation to cold and/or desiccation*
755 Desiccation tolerance, freeze avoidance and freeze tolerance share many protective molecules
756 (Tables 2 and 3), both large (e.g. TH proteins, HSPs) and small (e.g. glycerol, proline). Similarly,
757 freeze tolerance and desiccation tolerance share many characteristics, at both the organismal
758 level (Ring & Danks, 1994), and at the cellular level, where our model of extracellular freezing
759 implies intracellular desiccation (Fig. 3). Thus, insects already adapted to dry and/or cold
760 environments could co-opt mechanisms of desiccation or cold tolerance to facilitate survival of
761 internal ice.
762 Many terrestrial insects are physiologically adapted to dry environments (Sømme, 2012).
763 At the extremes, there are insects which can withstand complete loss of body water (Sakurai *et*
764 *al.*, 2008), as well as those that exploit dehydration as a freeze-avoidance strategy [*B. antarctica*
765 (Elnitsky *et al.*, 2008); and *C. clavipes* (Sformo *et al.*, 2010)]. Thus, it is plausible that the
766 biochemical and cellular mechanisms for freeze tolerance evolved *via* cross tolerance for
767 desiccation (Sinclair *et al.*, 2013*a*). For example, *B. antarctica* can employ both freeze tolerance
768 and cryoprotective dehydration (Elnitsky *et al.*, 2008; Lee *et al.*, 2006), which supports a link, at
769 least in this case. However, although anhydrobionts tolerate extensive cellular dehydration
770 (Sakurai *et al.*, 2008), increased desiccation tolerance at the organismal level is not necessarily
771 associated with increased desiccation tolerance at the cellular level. Among-species variation in

desiccation tolerance in *Drosophila* is largely driven by changes in water loss rates (Rajpurohit, Parkash & Ramniwas, 2008) or initial water content (Gibbs & Matzkin, 2001). In addition, dehydrated insects preferentially lose haemolymph volume to preserve cellular volume (Barton-Browne, 1964). Clearly, more work comparing the capacity of insect cells to tolerate dehydration (we would predict that freeze-tolerant species should have high capacity) is necessary to understand the relationship between cold and desiccation tolerance.

Alternately, freeze tolerance could arise by co-opting adaptations, such as polyol cryoprotectants and antifreeze proteins, present in already cold-hardy lineages. If the cryoprotectants in freeze-avoidant animals provide adequate protection from internal ice formation, then this transition appears relatively straightforward. This may explain the shift from freeze avoidance to freeze tolerance in species like *C. costata* that rely on inoculative freezing (Shimada & Riihimaa, 1988), and the strategy shift in the other direction in *D. canadensis* (Horwath & Duman, 1984). Freeze-avoidant species with very high haemolymph cryoprotectant concentrations may survive internal ice formation because their low content of freezable water should minimise ice content. This property could explain the existence of freeze-tolerant species with very low supercooling points (Ring, 1982) and provide another route of transition to freeze tolerance (K.E. Zachariassen, personal communication). Under this hypothesis, we might predict that some set of freeze-tolerant species have very cold-tolerant freeze-avoidant ancestors; unfortunately, there has been relatively little exploration of the evolution of cold tolerance in phylogenies with temperate or polar (rather than tropical or sub-tropical) ancestries, so we cannot yet evaluate the strength of this hypothesis.

(*b*) *Partial freeze tolerance*

Many insects are partially freeze tolerant; that is, they will survive the initiation of ice formation (and consequently a small amount of ice in their body), but are then killed if that ice formation progresses beyond some threshold (Sinclair, 1999). Sinclair (1999) and Voituron *et al.* (2002) suggest that this partial freeze tolerance could be an evolutionary route to freeze tolerance: individuals that are exposed to brief periods in the cold (e.g. the variable habitats of the southern hemisphere or tropical high mountains; Sinclair *et al.*, 2003) might have differential mortality, allowing those that are better able to withstand ice formation a selective advantage, leading to the evolution of freeze tolerance. Partial freezing can only occur when environmental cold exposure is shorter than the duration of ice formation. We therefore hypothesise that this evolutionary pathway to freeze tolerance is more likely in (large) species where ice formation takes a long time [e.g. stick insects (Dennis *et al.*, 2015); *Hemideina* spp. (Sinclair *et al.*, 1999)], making partial freezing likely in nature.

This pattern of partial freeze tolerance begetting freeze tolerance is supported in New Zealand stick insects, in which partial freeze tolerance is a widespread – and possibly ancestral – trait (Dennis *et al.*, 2015). However, there is relatively little evidence that partial freeze tolerance is heritable. Morey, Venette & Hutchison (2013) attempted to select for partial freeze tolerance by removing *Epiphyas postvittana* (light brown apple moth) larvae at their SCP. Although the SCP distribution shifted slightly (suggesting that SCP is heritable), there was no significant change in freeze tolerance. Damage caused by partial ice formation has not been well explored, but could include mechanical damage from ice, as well as damage caused by dehydration/osmotic stress (see Section III.3). We are unaware of any other selection

experiments on freeze tolerance in insects, although this approach could yield valuable information about the evolution of physiological traits (Gibbs, 1999).

(3) Insect freeze tolerance in a changing climate

Climate change will affect the frequency and intensity of extreme temperature events, as well as the duration of winter and the timing of these extreme cold events (see Williams, Henry & Sinclair, 2015, for a comprehensive review). An increase in the frequency of extreme cold events (which may be a result of increased weather variability or of reduced snowpack leading to more exposure to freeze–thaw; Marshall & Sinclair, 2012*a*; Williams *et al.*, 2015) could have contrasting implications for freeze-tolerant insects. If repeated freezing comes with a substantial cost (Bale, Worland & Block, 2001; Brown, Bale & Walters, 2004; Marshall & Sinclair, 2011; Sinclair & Chown, 2005*b*), then increased frequency of freezing events will be detrimental to freeze-tolerant insects compared to their freeze-avoidant counterparts. Conversely, if unpredictable freezing events favour freeze tolerance, then freeze tolerance may remain advantageous, as it appears to be for insects in the low alpine zone of New Zealand (Sinclair, 2001). The energy savings associated with freeze tolerance could hypothetically increase survival if increased precipitation leads to longer winters; however, microclimate temperatures beneath snow are too warm for insects to freeze in most habitats (Williams *et al.*, 2015), so freeze-tolerant insects may not gain any advantage. Thus, the role of cold-tolerance strategy in predicting insect responses to changing climate will likely be both species and habitat specific.

VI. NEW HYPOTHESES AND RELEVANT TOOLS

Molecules associated with freeze tolerance can be identified by comparing freeze-tolerant and freeze-intolerant individuals from different species (Joanisse & Storey, 1996), populations (Lee & Lewis, 1985), seasons (Baust & Lee, 1981) or laboratory treatments (Koštál *et al.*, 2011). The advent of untargeted -omics approaches has (at least in theory) facilitated the identification of a wider range of molecules (Courteau *et al.*, 2012; Dennis *et al.*, 2015; Poupardin *et al.*, 2015). While continuing to identify and describe cryo- and cytoprotectants in freeze-tolerant insects is useful, lists of molecules or putative pathways do not lead automatically to an understanding of mechanisms. Indeed, it is unclear whether there is functional convergence of biochemically unrelated cryoprotectants (e.g. can proline and trehalose fill the same roles?) or whether unique cryoprotectants are required in different mechanisms. We argue that a thorough understanding of the mechanisms of freeze tolerance requires a better characterisation of the processes and challenges associated with cooling, freezing, and thawing, and a critical assessment of how cryoprotectants modify those processes and mitigate the challenges.

(1) Understanding the processes and challenges of freezing

Most of our empirical data about processes and challenges associated with cooling and rewarming are derived from studies on chill-susceptible insects (Overgaard & MacMillan, 2017), and mammalian cell cryopreservation (Pegg, 2010). However, cryopreservation conditions (vitrified cells in suspension) do not reflect those that insects experience in nature (e.g. cooling rates of <1 °C min^{-1}; Sinclair, 1997, 2001). Similarly, freeze-tolerant insects appear to resist the challenges observed in chill-susceptible insects; for example, freeze-tolerant *Cyphoderris monstrosa* do not enter chill coma, but instead remain active at low temperatures until they

freeze (Toxopeus *et al.*, 2016). Thus, there is opportunity to focus efforts on how cooling, freezing and thawing alter biological processes and challenge survival in freeze-tolerant insects.

Internal ice formation is the most obviously unique feature of freeze tolerance, yet there have been only a few direct studies of the dynamic process of ice formation in real time. The existing studies on ice propagation have not identified the initial and final location of ice, or the size or quality of ice crystals, whether studied *in vivo* (e.g. synchrotron X-ray visualisation; Sinclair *et al.*, 2009) or *ex vivo* (e.g. live cell imaging; Sinclair & Wharton, 1997). *In silico* models of ice formation (e.g. Haji-Akbari & Debenedetti, 2017) are not yet scalable to whole animals (Li & Liu, 2010), but could be used to explore cellular dehydration and IIF (see. Botkin, Hoffmann & Turova, 2011; Fadda, Cincotti & Cao, 2011), and ice propagation (e.g. Lee *et al.*, 1993; Worland *et al.*, 2004) in real time (Table 5).

Given the difficulty in studying ice formation in real time, an alternative approach is to take 'snapshots' throughout the freezing process, and recreate the dynamic nature and responses. For example, freeze-substitution (e.g. Wharton *et al.*, 2005) could be used to pinpoint the location of ice at different time points in the freezing process (Table 5). In addition, snapshots of ice content, determined by calorimetry, can be used to quantify the amount of ice at different temperatures and times in the freezing process (Table 5; Koštál *et al.*, 2012; Lee & Lewis, 1985; Ramløv & Westh, 1993). This 'snapshot approach' could be combined with -omics approaches to characterise dynamic responses by the insect to cooling, freezing, thawing, and recovery.

As an adjunct to understanding the ice formation process and cellular responses to it, we need to determine the cause of injury in insects that do not survive ice formation. To understand the challenges associated with freezing better, we encourage moving beyond binary measurements of freeze injury (e.g. cell death or survival), to document damage during the

freeze–thaw process at the ultrastructural and macromolecular level (e.g. using techniques listed in Table 5). It should also be possible to detect responses to damage, including apoptosis and autophagy (Table 5). We should then determine the causes of this damage. For example, it would be valuable to test whether high ice contents (Fig. 5) are associated with cell/organismal death in freeze-tolerant insects (e.g. Lee & Lewis, 1985). Similarly, it should be possible to distinguish freeze-induced cellular damage caused by osmotic stress (e.g. high ion concentrations – as measured by fluorescent dyes, similar to Teets *et al.*, 2013) or dehydration stress (e.g. membrane fusions determined by electron microscopy; Collins *et al.*, 1997), rather than mechanical damage. By describing processes and challenges better, especially those associated with ice, we expect to be in a much better position to test how cryo- and cytoprotectants contribute to freeze tolerance by altering processes or mitigating challenges

(2) Models and experimental manipulations for understanding freeze tolerance

Although the repeated evolution and complexity of freeze tolerance makes it difficult to draw generalisations about mechanisms and processes, within- and among-species comparisons are a potential tool to test mechanisms underlying freeze tolerance. However, we identify several caveats: (1) within-species comparisons of freeze-tolerant and freeze-intolerant morphs can be confounded by life history. For example, freeze-tolerant stages of *E. solidaginis* and *C. costata* are in diapause (Table 1) while freeze-intolerant stages are not. Thus, care must be taken to disentangle mechanisms associated with diapause from those associated with surviving internal ice formation. For example, upregulation of storage proteins in a freeze-tolerant insect is more likely to be an important component of diapause (Hahn & Denlinger, 2011), but unlikely to protect against low temperatures or ice. In species that are freeze tolerant only when in deep

diapause, it may not be possible to disentangle diapause and freeze tolerance. For example, metabolic rate suppression may be important for developmental arrest (diapause) as well as for preventing metabolic dysregulation when frozen. (2) Among-species comparisons may be confounded by phylogeny. For example, the differences in post-thaw metabolic rate between *H. sparsutum*, *P. antarcticus* (Block *et al.*, 1998*b*) and *E. blanchardi* (Zachariassen *et al.*, 1979*b*) relative to *P. marioni* (Sinclair *et al.*, 2004) and *P. isabella* larvae (Marshall & Sinclair, 2011) may reflect a phylogenetic signal (Coleoptera *versus* Lepidoptera; Table 1), rather than different post-thaw recovery processes. Correcting for phylogeny is theoretically straightforward (Garland, Bennett & Rezende, 2005), but practically difficult, since it requires freeze-tolerant species to be placed within a resolved phylogeny of many species for which cold tolerance has been explored (e.g. Dennis *et al.*, 2015; Sinclair *et al.*, 1999). With these caveats in mind, we can use within- and among-species comparisons to continue to identify putative cryo- and cytoprotectants, and generate hypotheses concerning the mechanisms by which they contribute to freeze tolerance.

To test hypotheses about the mechanisms underlying freeze tolerance, we suggest identifying Krogh models that allow laboratory manipulations, and the disentangling of confounding factors such as life stage and diapause. Many putative cryo- and cytoprotectants are associated with freeze tolerance (Tables 2 and 3), yet their function remains in the realm of hypothesis. Experimental manipulation of these cryoprotectants is the most powerful approach to understanding their function, such as reducing cryoprotectant synthesis [e.g. *via* CRISPR/Cas9 technology (Gratz *et al.*, 2013) or RNA interference (RNAi; Scott *et al.*, 2013)] or increasing cryoprotectant abundance [e.g. by feeding (Koštál *et al.*, 2016) or injection (Benoit *et al.*, 2009; Rosendale *et al*., 2016)]. If cryoprotectant manipulations reduce or enhance freeze tolerance

(loss- and gain-of-function, respectively), we can conclude that they contribute to the mechanisms underlying freeze tolerance. We can also use these experiments to test whether different cryoprotectants contribute to similar mechanisms underlying freeze tolerance. As a caveat, these manipulative experiments cannot be applied to understand all mechanisms; for example, it would be challenging to modify the abundance of acetylated triacylglycerols in *E. solidaginis* (Marshall *et al.*, 2014) because their synthesis is unknown, and manipulating the composition of (intracellularly) stored triacylglycerols is difficult, if not impossible. Because there is no 'magic bullet' molecule conferring freeze tolerance these loss- and gain-of-function experiments will also need to unravel synergisms among cryo- and cytoprotectants, in the context of both cellular-level processes and whole-organism survival.

Of the many freeze-tolerant insects we have highlighted in this review, we identify several that are particularly promising as emerging model systems (denoted by asterisks in Table 1). A good model for within-species comparisons that avoid confounding effects of life history is *G. veletis*, which has both freeze-tolerant and freeze-intolerant morphs at the same life stage (late instar nymph; McKinnon, 2015), without any apparent diapause. Both New Zealand stick insects (Dennis *et al.*, 2015) and weta (Sinclair *et al.*, 1999) are good systems for examining freeze tolerance among species: they have well-resolved phylogenies, and include freeze-tolerant, partially freeze-tolerant, and freeze-intolerant lineages. There is also extensive diversity in cold tolerance of Diptera and Coleoptera, which could be amenable to among-species comparisons. For example, there are both freeze-tolerant and freeze-avoidant species in the Holarctic carabid genus *Pterostichus* (Miller, 1969; Rossolimo, 1997). To manipulate cryo- and cytoprotectants experimentally, we must work with species that can be reared in laboratories, such as *C. costata* and *G. veletis*. *Chymomyza costata* larvae are amenable to cryoprotectant manipulation *via*

feeding (Koštál *et al.*, 2011), and, once the in-progress genome sequencing is completed (V. Koštál, personal communication), CRISPR-Cas9 (e.g. Newman *et al.*, 2017). *Gryllus veletis* are likely responsive to RNAi (as are other *Gryllus* spp.; Meyering-Vos *et al.*, 2006), and are large enough (> 100 mg) to allow cryoprotectant concentrations to be manipulated easily *via* injection, and to perform tissue/cell-specific assays. In addition, we can advance our understanding of freeze tolerance by identifying mechanisms by which freeze-intolerant model organisms can be converted to freeze-tolerant insects. This has been done with some success by feeding proline to *D. melanogaster* (Kostal *et al.*, 2012), and could represent a gold standard for confirming the sufficiency of putative cryoprotectants or processes in freeze tolerance. Continued characterisation of cold-tolerance strategies will likely reveal additional potential models, which will be critical in unravelling the complexity and diversity of mechanisms underlying freeze tolerance.

VII. CONCLUSIONS

(1) Freeze tolerance facilitates survival of low temperatures and unpredictable climates, and has evolved repeatedly across insects. However, we have limited understanding of the mechanisms that underlie survival of internal ice.

(2) Many molecules (cryo- and cytoprotectants) are associated with insect freeze tolerance, but none appear to be necessary or sufficient to support this cold-tolerance strategy.

(3) Freeze tolerance requires surviving a process (cooling, freezing, thawing), and mitigating the associated challenges. There are several mechanisms by which freeze-tolerant insects may control ice, prevent or repair damage to cells and macromolecules, manage biochemical processes while frozen/thawing, and restore physiological processes post-thaw.

(4) Freeze tolerance likely evolved to facilitate survival in environments with extreme low temperatures and/or high risk of freezing, and in cases where freezing offers a physiological advantage (e.g. energy reserve management). This cold-tolerance strategy may have evolved in insects that were partially freeze-tolerant, or those already well adapted to stresses associated with low temperatures and dehydration.

(5) We encourage a more concentrated effort to characterise better the dynamic processes and challenges associated with cooling, freezing, and thawing in emerging laboratory models (e.g. *C. costata*, *G. veletis*). This, along with the framework presented herein, will improve our understanding of how insects survive internal ice.

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Table 1. Examples of freeze-tolerant (FT) insects described herein, grouped by Order (bold) and Family. Asterisks denote model species or candidate model species for studying the mechanisms and/or evolution of freeze tolerance.

Insect	FT stage	Example of:	References
Blattaria			
Blattidae			
Celatoblatta quinquemaculata	Nymph	Year-round freeze tolerance, survivable intracellular ice formation (IIF)	Sinclair (1997); Worland *et al.* (2004)
Cryptocercidae			
Cryptocercus punctulatus	Adult	Long lethal time (Lt) (205 days)	Hamilton *et al.* (1985)
Coleoptera			
Carabidae			
Pterostichus spp.	Adult	Genus with FT and freeze-avoidant members	Miller (1969); Rossolimo (1997)
Cucjidae			
Cucujus clavipes	Larva	Multiple cold-tolerance strategies	Duman (1984); Sformo *et al.* (2010)
Promecheilidae			
Hydromedion sparsutum	Larva	Elevated metabolic rate post-thaw	Block *et al.* (1998*b*)
Perimylops antarcticus	Larva	Elevated metabolic rate post-thaw	Block *et al.* (1998*b*)
Pyrochroniae			
Dendroides canadensis	Larva	Antifreeze protein (AFP) accumulation	Duman (1980)
Pythidae			
Pytho deplanatus	Larva	Very low supercooling point (SCP)	Ring (1982)
Scarabidae			
Osmoderma eremicola	Larva	Ice content	Storey *et al.* (1993)
Tenebrionidae			
Eleodes blanchardi	Adult	Relating SCP and ice content to mortality	Zachariassen *et al.* (1979*b*)
Upis ceramboides	Adult	Extreme low-temperature survival	Miller (1978)
Diptera			
Chironomidae			
Belgica antarctica	Larva	Freeze tolerance, cryoprotective dehydration	Elnitsky *et al.* (2008)
Drosophilidae			
Chymomyza amoena	Diapausing larva	Ice formation	Sinclair *et al.* (2009)
*Chymomyza costata**	Diapausing larva	Inoculative freezing, very low lower lethal temperature (LLT; –196 °C)	Koštál *et al.* (2011)
Drosophila melanogaster	Quiescent larva	Proline confers freeze tolerance	Koštál *et al.* (2012)
Heleomyzidae			
Heleomyza borealis	Dormant larva	Survive high ice contents (>80%)	Worland *et al.* (2000)
Mycetophilinae			
Exechia nugatoria	Adult	Compartmentalised freezing	Sformo *et al.* (2009)

Tephritidae			
*Eurosta solidaginis**	Diapausing prepupa	Ice-formation dynamics, microRNA (miRNA) synthesis when frozen	Lee & Lewis (1985); Courteau *et al.* (2012)
Tipulidae			
Tipula trivittata	Larva	Cryoprotectant accumulation	Duman *et al.* (1985); Knight & Duman (1986)
Hymenoptera			
Cephidae			
Cephus cinctus	Larva	Survivable IIF	Salt (1961)
Vespidae			
Vespula maculata	Adult (queen)	Haemolymph ice-nucleating agents (INAs)	Duman *et al.* (1984)
Orthoptera			
Anostostomatidae			
*Hemideina maori**	Adult	High ice content (>80%), osmotic cellular dehydration	Ramløv & Westh (1993); Sinclair & Wharton (1997)
Gryllidae			
*Gryllus veletis**	Late-instar nymph	Short Lt (7 days)	McKinnon (2015)
Prophalangopsidae			
Cyphoderris monstrosa	Late-instar nymph	Avoid chill coma during freezing	Toxopeus *et al.* (2016)
Phasmatodea			
Diapheromeridae			
Niveaphasma annulata	Adult	Variance in FT among populations	Dennis *et al.* (2015)
Lepidoptera			
Cossidae			
Cossus cossus	Larva	Buffered overwintering microhabitat	Li (2016)
Crambidae			
Chilo suppressalis	Diapausing larva	Aquaporin (AQP) function	Izumi *et al.* (2007)
Erebidae			
Pyrrharctia isabella	Diapausing larva	Low metabolic rate, immune activity post-thaw	Marshall & Sinclair (2011)
Tineidae			
Pringleophaga marioni	Larva	Respiration while frozen	Sinclair *et al.* (2004)
Plecoptera			
Nemouridae			
Nemoura arctica	Nymph	Aquatic insect; makes glycerol while frozen	Walters *et al.* (2009*b*)

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1632 Table 2. Putative cryoprotectants associated with insect freeze tolerance.

Cryoprotectant	Hypothesised function	Example in freeze-tolerant insect(s)
Low molecular weight metabolites		
Polyols (e.g. glycerol, sorbitol)	Increase 'bound' water, colligatively reduce ice content (Lee, 2010), and reduce probability of intracellular ice formation (IIF)	*Pyrrharctia isabella* accumulate over 800 mM haemolymph glycerol (Marshall & Sinclair, 2011)
Sugars (e.g. trehalose)	Stabilise macromolecules *via* direct interaction with them/their hydration shell (Crowe *et al.*, 1984)	*Hemideina maori* accumulate up to 300 mM haemolymph trehalose in the winter (Neufeld & Leader, 1998)
Amino acids (e.g. proline, arginine)	Stabilise macromolecules *via* direct interaction with them/their hydration shell (Arakawa & Timasheff, 1983) Prevent protein aggregation by physical buffering (Rudolph & Crowe, 1986)	High *in vivo* concentrations of proline and arginine increase freeze tolerance of *Chymomyza costata* (Koštál *et al.*, 2011) and confer freeze tolerance on *Drosophila melanogaster* (Koštál *et al.*, 2012, 2016)
Lipids		
Antifreeze glycolipids	Prevent recrystallisation (Duman, 2015)	*Upis ceramboides* accumulate glycolipid with antifreeze properties (Walters *et al.*, 2009*a*)
Acetylated triacylglycerols (acTAGs)	Improve survival of IIF (Marshall *et al.*, 2014)	*Eurosta solidaginis* accumulate acTAGs prior to winter (Marshall *et al.*, 2014)
Ice-binding proteins		
Ice-nucleating agents (INAs)	Control ice formation (Zachariassen, 1985)	*H. maori* (Wilson & Ramløv, 1995) have haemolymph INAs
Recrystallis-ation inhibitors (RISs)	Control ice crystal size and shape (Duman & Horwath, 1983; Zachariassen & Kristiansen, 2000)	Antifreeze proteins (AFPs) from *Dendroides canadensis* inhibit ice recrystallisation *in vitro* (Knight & Duman, 1986)
Transport proteins		
Aquaporins (AQPs)	Facilitate water movement out of cells during freezing, reducing IIF (Storey & Storey, 2013); facilitating water movement into cells during thawing	AQP inhibition increases freeze injury of *E. solidaginis* (Philip *et al.*, 2008) and *Chilo suppressalis* (Izumi *et al.*, 2007) tissues
Cryoprotectant transporters	Facilitate cryoprotectant redistribution during freezing, improving cellular survival (Storey & Storey, 2013)	Glycerol movement into *C. suppressalis* fat body cells during freezing minimises freeze injury (Izumi *et al.*, 2006)

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1634 Table 3. Cytoprotective proteins predicted to facilitate insect freeze tolerance. n/a indicates molecules for which we are unaware of
1635 any studies in freeze-tolerant insects.

Proteins	Hypothesised function	Example in freeze-tolerant insect(s)
Antioxidants	Reduce oxidative damage (Storey & Storey, 2010)	Antioxidant enzymes in *Belgica antarctica* (Lopez-Martinez *et al.*, 2008), *Eurosta solidaginis* (Joanisse & Storey, 1994)
Cell adhesion proteins	Maintain tissue integrity (Des Marteaux *et al.*, 2017); reduce ice formation between cells	n/a
Chaperones (e.g. heat shock proteins, HSPs)	Protect macromolecules; prevent protein aggregation (Storey & Storey, 2013)	HSPs in *Chilo suppressalis* (Lu *et al.*, 2014), *E. solidaginis* (Zhang *et al.*, 2011), *B. antarctica* (Rinehart *et al.*, 2006)
Chelators	Reduce damage due to high ion concentrations (ion binding; Storey & Storey, 2010)	Ferritin (iron chelator) in *E. solidaginis* (Storey & Storey, 2010)
Cytochrome P450s	Reduce oxidative damage (Poupardin *et al.*, 2010); general detoxification/repair (Scott & Wen, 2001)	Several cytochrome P450s in *B. antarctica* (Lopez-Martinez *et al.*, 2009)
Cytoskeletal protein isoforms, and cytoskeletal regulators	Maintain cell structure/integrity at low temperatures (resist depolymerisation; Storey & Storey, 2013)	T-complex protein 1 (cytoskeletal chaperone) in *E. solidaginis* (Zhang *et al.*, 2011), altered expression of *B. antarctica* cytoskeletal isoforms (Li *et al.*, 2009)
Disordered proteins (e.g. dehydrins)	Reduce dehydration stress (e.g. late embryogenesis abundant (LEA) proteins; Toxopeus *et al.*, 2014)	Putative cryoprotective dehydrin in *E. solidaginis* (Pruitt *et al*., 2007)
Sirtuins (Sir2 proteins)	General stress resistance (Jung *et al.*, 2016; Preyat & Leo, 2013), e.g. reduce oxidative stress	n/a

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Table 4. Hypotheses (H) concerning the potential causes of lethal limits in freeze-tolerant insects. Arrows, ↑ and ↓, indicate increase(s) and decrease(s), respectively. Both ↓ lower lethal temperature (LLT) and ↑ lethal time (Lt) imply increased freeze tolerance.

Challenge	Hypothesis	Prediction
H1. Low temperature		
Low temperatures destabilise proteins and membranes	Death at the LLT occurs at a critical level of macromolecule/cell damage	Mechanisms that stabilise macromolecules (e.g. accumulating proline, trehalose, chaperones); ↓ LLT
H2. Ice content		
High ice content increases osmotic/ dehydration stress	Death at the LLT occurs when dehydrate below a minimum critical volume (MCV)	Mechanisms that reduce ice content (e.g. accumulating colligative cryoprotectants) reduce cell dehydration; ↓ LLT
High ice content increases mechanical damage	Death at the LLT occurs when ice content above a critical threshold causes mechanical damage	Mechanisms that reduce ice content (e.g. accumulating colligative cryoprotectants) reduce mechanical damage from ice; ↓ LLT
H3. Ice quality		
Recrystallisation mechanically damages cells	Death at the Lt occurs at a critical level of mechanical damage due to ice crystal growth while frozen	Mechanisms that control ice size [e.g. recrystallization inhibitors (RIs)] reduce mechanical damage from ice crystals; ↑ Lt (e.g. at moderate subzero temperatures)
H4. Ice location		
Intracellular ice formation (IIF) and extracellular ice formation within tissues cause mechanical damage	Death at the LLT occurs when ice propagates into (weak link) cells or tissues, which may occur at high ice content	Mechanisms that reduce ice content (e.g. accumulating colligative cryoprotectants) and control ice location [e.g. ice-nucleating agents (INAs), tight intercellular junctions] improve cell and insect survival; ↓ LLT
H5. Accumulation of harmful metabolites		
Accumulation of harmful metabolic wastes while frozen damages cells/tissues	Death at the Lt occurs at a critical waste [e.g. reactive oxygen species (ROS), lactate] accumulation threshold, defining the Lt	Metabolic suppression reduces ROS and other waste production, ↑ Lt Mechanisms that remove/neutralise these products (e.g. antioxidants, buffers) improve cell survival; ↑ Lt
H6. Metabolic activity		
Depletion of energy reserves in the frozen state precludes recovery/repair	Death at the Lt occurs at a critical energy reserve (e.g. adenylate charge) depletion	Accumulating energy reserves, metabolic suppression and/or overwintering at low temperatures (lower metabolic rate) slows energy reserve depletion; ↑ Lt

1642 Table 5. Techniques and tools for measuring parameters that will inform our understanding of
1643 processes, challenges, and mechanisms of insect freeze tolerance.

Technique/tool	Measurement	Reference
Processes: characterising the freezing process		
Synchrotron X-ray imaging	Whole-body ice formation (real time)	Sinclair *et al.* (2009)
Cold stage microscopy	Intracellular ice formation (real time)	Sinclair & Wharton (1997)
Freeze substitution	Ice location	Wharton *et al.* (2005)
Calorimetry (e.g. differential scanning calorimetry, DSC)	Ice content	Rozsypal *et al.* (2018)
Transcriptomics and (phospho-)proteomics	Gene expression (and regulation thereof)	Courteau *et al.* (2012)
Metabolomics	Cryoprotectant composition/location	Koštál *et al.* (2011)
Challenges: measuring damage/responses to damage		
Live–dead staining	Cell death or survival	Yi & Lee (2003)
Electron microscopy	Ultrastructural changes/damage	Collins *et al.* (1997)
Protein misfolding assays	Protein aggregation	Gregoire *et al*. (2012)
Comet assay	DNA damage	Olive & Banáth (2006)
Membrane lipid peroxidation assay	Oxidative damage	Lopez-Martinez *et al.* (2008)
Protein carbonylation assay	Oxidative damage	Lopez-Martinez *et al.* (2008)
TUNEL assay	Apoptosis	Yi *et al*. (2007)
Monodansylcadaverine (MDC) staining	Autophagy	Wu *et al.* (2011)
Fluorescent ion chelators	Intracellular ion concentrations (osmotic pressure)	Teets *et al.* (2013)
Mechanisms: cryoprotectant manipulations		
Injection/feeding	Increase cryoprotectant concentrations *in vivo*	Koštál *et al.* (2016)
RNA interference	Knockdown protein synthesis	Scott *et al.* (2013)
CRISPR-Cas9	Generate knockout mutants	Gratz *et al.* (2013)

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FIGURE LEGENDS

Fig. 1. Insect body temperature, and physical processes in an insect as the environmental cools and rewarms.

Fig. 2. Documented sites of ice nucleation in freeze-tolerant insects, with examples (denoted by superscripts) from: 1, Sinclair *et al.* (1999); 2, Mugnano *et al.* (1996); 3, Zachariassen & Hammel (1976); 4, Duman (1980); 5, Duman (1984); 6, Duman *et al.* (1985); 7, Hamilton *et al.* (1985); 8, Wilson & Ramløv (1995); 9, Tsumuki & Konno (1991); 10, Shimada & Riihimaa (1988); 11, Walters *et al.* (2009*b*).

Fig. 3. Model of extracellular freezing in freeze-tolerant insects. Ice is nucleated extracellularly, osmotically dehydrating cells and preventing internal ice formation.

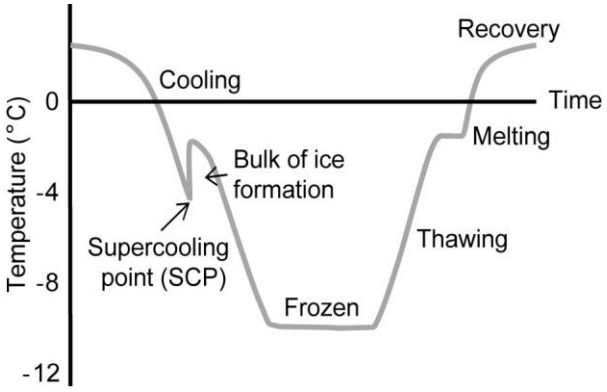
Fig. 4. Summary of the challenges associated with cooling, freezing and thawing, and mechanisms for addressing those challenges. ↑ indicates high (physical effects, challenges) or increase (mechanisms); ↓ indicates low (physical effects, challenges) or reduce (mechanisms); ROS, reactive oxygen species. These hypotheses are supported by the following references, indicated by superscript numbers on the figure: 1, Crowe *et al.* (1984); 2, Dias *et al.* (2010); 3, Doelling *et al.* (2014); 4, Hazel (1995); 5, Irwin & Lee (2002); 6, Koštál *et al.* (2007); 7, Lalouette *et al.* (2011); 8, Marqués (2006); 9, Miguel *et al.* (2013); 10, Pegg (2010); 11, Ramløv (2000); 12, Scholander *et al.* (1953); 13, Sinclair (2015); 14, Sinclair & Renault (2010); 15, Somero *et al.* (2017); 16, Štětina *et al.* (2018); 17, Storey & Storey (1985); 18, Storey & Storey (2010); 19, Tattersall *et al.* (2012); 20, Teets & Denlinger (2013); 21, Zachariassen (1985); 22,

Zachariassen *et al.* (2004*a*); 23, Table 2 (and references therein) for examples of cryoprotectants that are hypothesised to contribute to controlling ice formation; 24, Tables 2 and 3 (and references therein) for examples of how low molecular weight metabolites (Table 2), and chaperones and disordered proteins (Table 3) are hypothesised to contribute to stabilising macromolecules.

Fig. 5. Relationship between haemolymph osmolality and ice content. Values of equilibrium ice content and haemolymph osmolality are from: 1, *Heleomyza borealis* (Worland *et al.*, 2000); 2, *Celatoblatta quinquemaculata* (Block *et al*., 1998*a*); 3, *Osmoderma eremicola* (Storey *et al*., 1993); 4, *Hemideina maori* (Ramløv, 1999; Ramløv & Westh, 1993); 5, *Eurosta solidaginis* (Lee & Lewis, 1985; Morrissey & Baust, 1976); 6, *Pyrrharctia isabella* (Layne & Blakeley, 2002). Most ice contents were measured at temperatures between –8 °C and –10 °C, with the exception of *E. solidaginis* (–25 °C). Filled circles, haemolymph osmolality measured by osmometry; open circles, haemolymph osmolality calculated from sum of haemolymph concentrations of sugars and polyols. When multiple osmolality measurements were available, the range is indicated by a solid line. Dashed line indicates regression line through the data ($r^2 = 0.62$, $P = 0.06$).

FIGURES

Figure 1



1689 **Figure 2**

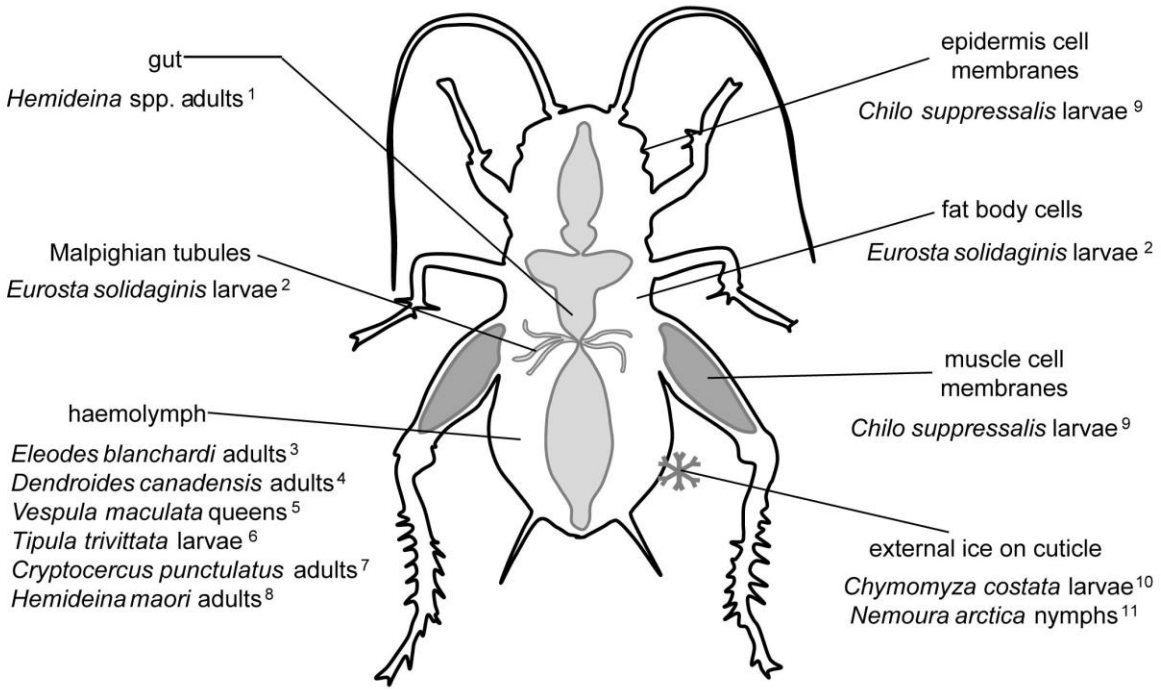


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Figure 3

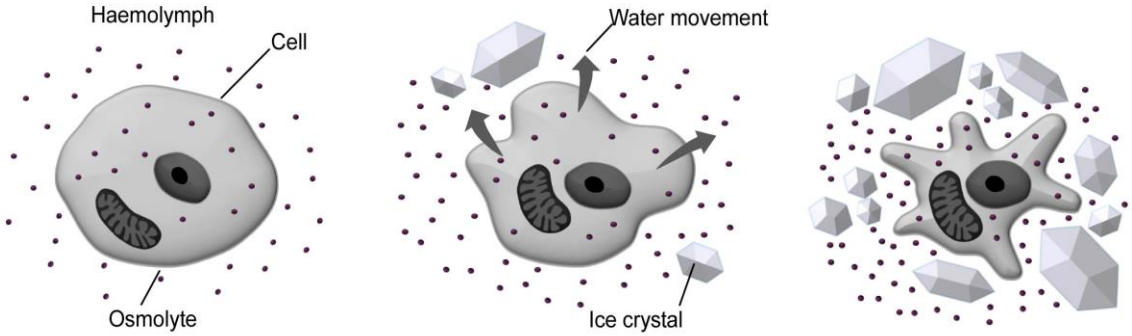


Figure 4

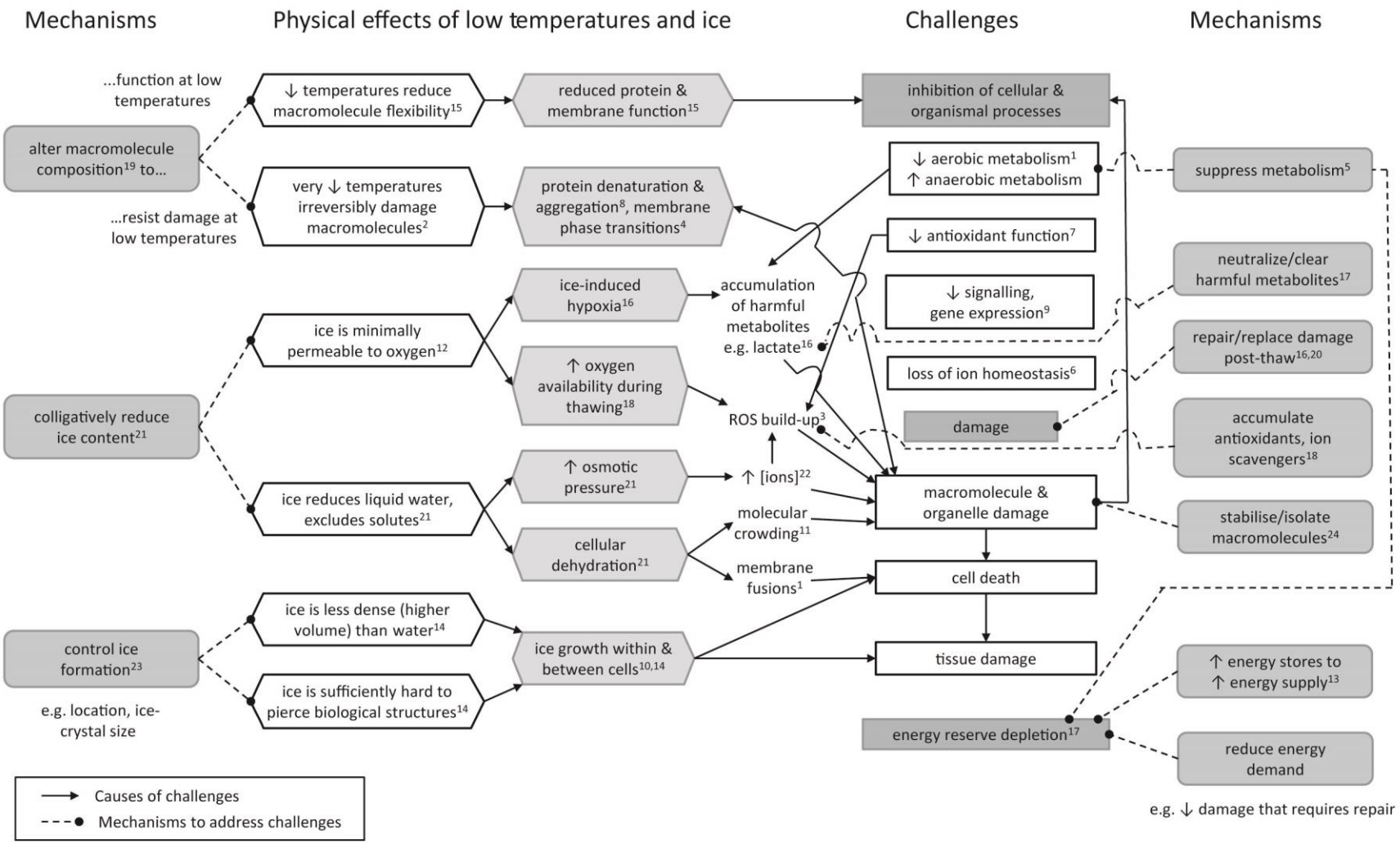
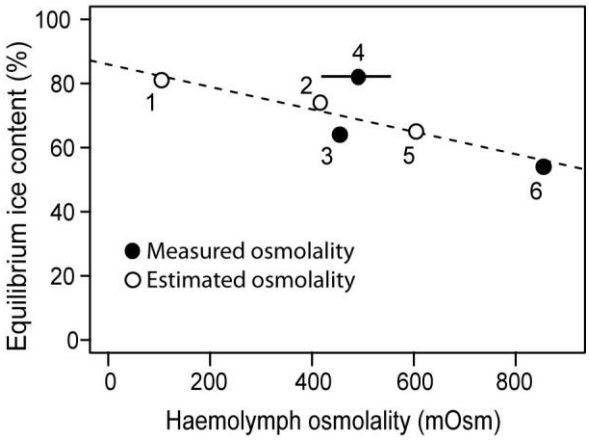

Mechanisms
Physical effects of low temperatures and ice
Challenges
Mechanisms
...function at low temperatures
alter macromolecule composition[19] to...
...resist damage at low temperatures
↓ temperatures reduce macromolecule flexibility[15]
very ↓ temperatures irreversibly damage macromolecules[2]
reduced protein & membrane function[15]
protein denaturation & aggregation[8], membrane phase transitions[4]
inhibition of cellular & organismal processes
↓ aerobic metabolism[1]
↑ anaerobic metabolism
↓ antioxidant function[7]
↓ signalling, gene expression[9]
loss of ion homeostasis[6]
damage
suppress metabolism[5]
neutralize/clear harmful metabolites[17]
repair/replace damage post-thaw[16,20]
accumulate antioxidants, ion scavengers[18]
stabilise/isolate macromolecules[24]
colligatively reduce ice content[21]
ice is minimally permeable to oxygen[12]
ice-induced hypoxia[16]
↑ oxygen availability during thawing[18]
accumulation of harmful metabolites e.g. lactate[16]
ROS build-up[3]
ice reduces liquid water, excludes solutes[21]
↑ osmotic pressure[21]
cellular dehydration[21]
↑ [ions][22]
molecular crowding[11]
membrane fusions[1]
macromolecule & organelle damage
cell death
tissue damage
control ice formation[23]
e.g. location, ice-crystal size
ice is less dense (higher volume) than water[14]
ice is sufficiently hard to pierce biological structures[14]
ice growth within & between cells[10,14]
energy reserve depletion[17]
↑ energy stores to ↑ energy supply[13]
reduce energy demand
e.g. ↓ damage that requires repair
Causes of challenges
Mechanisms to address challenges

Figure 5


Equilibrium ice content (%)
100
80
60
40
20
0
0
200
400
600
800
Haemolymph osmolality (mOsm)
1
2
3
4
5
6
Measured osmolality
Estimated osmolality