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Review

Intracellular ice formation in insects: Unresolved after 50 years?☆

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ABSTRACT

Many insects survive internal ice formation. The general model of freeze tolerance is of extracellular ice formation (EIF) whereby ice formation in the haemocoel leads to osmotic dehydration of the cells, whose contents remain unfrozen. However, survivable intracellular ice formation (IIF) has been reported in fat body and certain other cells of some insects. Although the cellular location of ice has been determined only *in vitro*, several lines of evidence suggest that IIF occurs *in vivo*. Both cell-to-cell propagation of intracellular ice and inoculation from the haemocoel may be important, although the route of ice into the cell is unclear. It is unclear why some cells survive IIF and others do not, but it is suggested that the shape, size, and low water content of fat body cells may predispose them towards surviving ice formation. We speculate that IIF may reduce water loss in some freeze tolerant species, but there are too few data to build a strong conceptual model of the advantages of IIF. We suggest that new developments in microscopy and other forms of imaging may allow investigation of the cellular location of ice in freeze tolerant insects *in vivo*.

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1. Introduction

The strategies that insects use to survive sub-freezing temperatures are usually grouped into freeze avoiding (those that keep their body fluids supercooled and die at or above the temperature where they freeze) and freeze tolerant (those that withstand ice formation in the body) (Sinclair, 1999; Nedved, 2000; although the classifications can be more complex, see Bale, 2002). Since freezing tolerance was first described in caterpillars by Réaumur (1736), many biochemical correlates to freezing have been extensively studied (Duman et al., 1991; Storey, 1997; Sømme, 2000; Zachariassen and Kristiansen, 2000; see, e.g., Bale, 2002; Zachariassen et al., 2004). However, in spite of an overwhelming literature on cold tolerance, the processes of ice formation, and the location of ice in the frozen insect are not as well-understood as might be assumed. Although the general model of insect freezing survival assumes extracellular ice formation (EIF) (Asahina, 1969; Zachariassen, 1985), there are scattered reports of intracellular ice formation (IIF) in an array of insects.

Around the time of the founding of *Comparative Biochemistry and Physiology* (which, incidentally, published an extensive and influential series of reviews on insect cold tolerance in 1982 (Zachariassen, 1982)), R.W. Salt first published a series of three papers on intracellular ice formation (IIF) in insects (Salt, 1959, 1961, 1962). Surprisingly, few studies on IIF in insects have been conducted subsequently. Here, we

Abbreviations: IIF, intracellular ice formation; EIF, extracellular ice formation.
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will briefly review the evidence for IIF in insects, and the mechanisms of freeze tolerance in general. We will then put this into the context of recent research on insects' and other cells, and identify some outstanding questions and the recently-developed techniques that may allow them to be addressed.

Cells have a finite volume; the formation of ice is accompanied by expansion of the water, potentially leading to rupture of cellular membranes either during freezing, or due to recrystallisation leading to redistribution of ice during thawing (Muldrew et al., 2004). In addition, the propagation and presence of ice itself in cells have been shown to cause damage to membranes in mammalian cells (Acker and McGann, 2001; Muldrew et al., 2004) and IIF at ecologically relevant temperatures has thus generally been considered lethal for all organisms. IIF has long appeared to be lethal in intact plant tissues (Asahina, 1956), and most other IIF studies (which lead to the almost unequivocal conclusion that IIF is lethal, even at ecologically relevant temperatures) have been conducted on mammalian cells (see Muldrew et al., 2004 for review). In suspensions of mammalian cells in the context of cryopreservation, IIF has been identified as a likely source of mortality (Mazur, 1984) and cryopreservation biologists view temperatures from −15 to −60 °C as a danger zone — the temperatures where IIF and recrystallisation are most likely during both cooling and rewarming (e.g. Mazur, 1984), indeed, some IIF appears to be survivable only if cells are warmed through this zone at very fast rates (>c. 650 °C min^{-1} Mazur, 1977; Salinas-Flores et al., 2008). However, these danger zone temperatures are encountered in cold temperate, sub-polar, polar and alpine regions; regions which also have a large complement of terrestrial insects (Danks, 2000).

The textbook model for ice formation in freeze tolerant insects (e.g. Chown and Nicolson, 2004; Hill et al., 2008) also assumes that IIF leads to the organism's death. The textbook model suggests that ice formation is initiated in the haemocoel, leading to osmotic dehydration of cells which prevents them from freezing at a given temperature (Fig. 1). There is microscopical evidence for this extracellular ice formation in Malpighian tubule cells of the alpine weta, *Hemideina maori* (Orthoptera: Anostostomatidae) (Sinclair and Wharton, 1997). Survivable IIF is well-documented in the Antarctic nematode *Panagrolaimus davidi* (Wharton and Ferns, 1995), but IIF appears to be uniformly lethal in mammalian cells (Muldrew et al., 2004).

2. Evidence for IIF in insects

R.W. Salt (1959) observed IIF in fat body cells of the goldenrod gall fly *Eurosta solidaginis* (Diptera: Tephritidae) through observation of opaque, hard cells when larvae were dissected frozen, as well as their change in appearance when thawing and the coalescence of lipid droplets inside the cells post-thaw. Importantly, he also notes the presence of lipid coalescence in individuals collected from the field after cold events, which suggests that IIF does occur in the intact organism in nature. Taking a more experimental approach, Salt (1961) used electrical discharges (from the spark coil of a Model T Ford!) to nucleate freezing in larvae of the cephid wasp *Cephus cinctus*. By manipulating nucleation temperature, he was able to initiate slow freezing at relatively high sub-zero temperatures (which resulted in extracellular freezing and osmotic dehydration of cells), or let larvae freeze at lower temperatures after supercooling, which resulted in IIF. Although survival was better in extracellularly frozen animals, particularly at lower temperatures, there was measurable survival of IIF (c. 30% at −20, compared to 52% with EIF). Furthermore, the rate of water loss from the thawed larvae was reduced if larvae were frozen intracellularly, which implies that IIF reduces osmotic perturbation in this species.

Salt (1962) was clear that survivable IIF is not present in all tissues, even in insects where he observed it. Indeed, IIF appeared to be largely confined to the larger cells (e.g. in the fat body), while smaller cells tended toward extracellular freezing. Following Salt's discoveries, subsequent observations of IIF in insect cells have typically been made using cryomicroscopy of tissue harvested from the insect. So far, to our knowledge, IIF has been observed in fat body cells of Diptera and Hymenoptera (Salt, 1961, 1962; Davis and Lee, 2001). In addition, cells in the midgut and fat body of the cockroach *Celatoblatta quinquemaculata* freeze intracellularly when nucleated at −4 but not at −2 °C (Worland et al., 2004), which is consistent with Salt's (Salt, 1961) observations in *C. cinctus*. Worland et al. (2004) did not observe IIF in Malpighian tubules of *C. quinquemaculata*, but shrinkage associated with extracellular ice formation was not observed, leading them to conclude that IIF had occurred, contrasting with observations in *H. maori* (Sinclair and Wharton, 1997). Berger and Uhrik (1996) observed IIF *in vitro* in salivary gland cells of the chironomid *Chironomis thummii*, but the cells did not survive this stress. In contrast to insects, IIF has been reported in all cell types of the Antarctic nematode, *Panagrolaimus davidi* (Wharton and Ferns, 1995; Wharton, 2003; Smith et al., 2008b). Wharton et al. (2005) have described ice-active proteins associated with this phenomenon, but the mechanisms of IIF survival remain unclear. By contrast, Asahina et al. (1954) found that heart muscles of the slug caterpillar *Cnidocampa flavescens* did not survive IIF, and Sinclair and Wharton (1997) observed EIF in Malpighian tubules of *H. maori*. Both Asahina and Aoki (1958) and Losina-Losinsky (1963) demonstrated survival in Lepidoptera frozen to very low (e.g. liquid nitrogen or liquid helium) temperatures after a pre-freezing at a higher temperature (e.g 30 °C). Losina-Losinsky (1963) was able to demonstrate using vital staining that ice crystals formed inside the cells at these ultra-low temperatures, whereas Asahina and Aoki (1958) interpreted the pre-freezing period as allowing substantial dehydration of the cells (see also Asahina, 1969).

Thus, of the freeze tolerant insects that have been examined so far, the majority actually display IIF in one or more cell types, and this is spread across several insect orders. As well as suggesting that the textbook model of freeze tolerance in insects needs to recognise this plurality, the prevalence of IIF in insects raises a number of questions: Does IIF occur *in vivo* as well as *in vitro* in each of these cases? How does ice get into and between the cells? And why are some cells killed by IIF and others not? Finally, what are the advantages and disadvantages of IIF? We will address each of these questions.

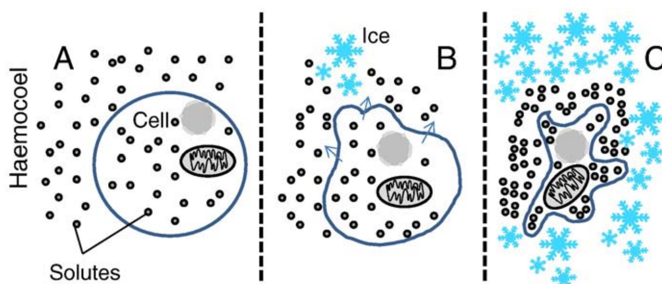

Fig. 1. The extracellular ice formation model for freeze tolerance in insects, as described by Asahina (1969). Under normal conditions, the cell is isosmotic with the surrounding haemolymph (A). When ice begins to form, exclusion of solutes from the crystals results in an increase in solute concentration in the haemolymph, and water is lost from the cell via osmosis (indicated with arrows). Once ice formation is complete, the cell is in osmotic equilibrium with the unfrozen portion of the haemolymph, and should therefore have an internal melting point at or below the current temperature. Additional cooling will cause the extracellular ice to grow, resulting in additional dehydration of the cell to maintain osmotic equilibrium.

3. Does IIF occur in vivo?

Unlike nematodes, which are transparent and have allowed the observation of IIF in intact, living individuals (Wharton and Ferns, 1995), seeing inside frozen insects and determining the distribution of ice is a technological challenge. (Salt, 1961) presented indirect evidence that he was inducing IIF in some larvae, and EIF in others

based upon the external appearance of the larvae — clear in the latter case, milky white in the former. In *E. solidaginis*, IIF in fat body cells is accompanied by coalescence of lipids (Salt, 1962; Lee et al., 1993), which can act as a marker for the occurrence of IIF in intact animals. Salt (1959) noted that this lipid coalescence is observed in *E. solidaginis* larvae collected from the field after likely freezing events. Other studies have examined only excised tissue (e.g. Sinclair and Wharton, 1997; Worland et al., 2004). There are several techniques to examine internal structures of intact insects and thus potentially determine the location of ice. For example, a Magnetic Resonance Imaging (MRI) study of frozen *E. solidaginis* resolved individual fat body cells (Mietchen et al., 2008), while synchrotron x-ray imaging may also hold promise for investigating internal structures of intact, frozen insects (Westneat et al., 2008).

However, the necessity for freezing survival of IIF in *E. solidaginis* fat body cells remains uncertain, in spite of evidence that IIF occurs *in vitro* and possibly *in vivo* (Salt, 1959; Lee et al., 1993). Mietchen et al.'s (2008) MRI observations may indicate that fat body cells are unfrozen in intact, frozen *E. solidaginis.* Because of the large proportion of lipid in the fat body cell, it is possible that IIF escapes detection using this technique, although an explicit ice signal appears to be absent from these cells (Mietchen et al., 2008). In addition, inhibiting aquaporins (thought to be necessary to allow egress of water from the cell in EIF) prevents freezing survival in *E. solidaginis* fat body cells (Philip et al., 2008), suggesting that some movement of water is necessary. Although IIF does not necessarily preclude a need for water redistribution during freezing, the requirement is clearly greater for EIF (Izumi et al., 2006).

Intracellular ice formation may also be occurring *in vivo* in insects exposed to extremely low (<−55 °C) sub-zero temperatures (summarised by Asahina, 1969). Insects that are equipped to survive extreme cold tend to have large quantities of cryoprotectants (Zachariassen, 1985), and while it is possible that this leads to vitrification at low temperatures (Block, 2002), there is also the possibility that the remaining aqueous solutions in the previously EIF-dehydrated cells could freeze. Most insects that survive exposure to liquid nitrogen temperatures or cooler require pre-conditioning after freezing at a much higher temperature (e.g. −30 °C for *Papilio machaon*), which likely removes the majority of the water from the cells (Asahina, 1969).

4. How does ice get into and between cells?

Much of what we know about the effects of ice on isolated cells (and cells in tissue) comes from the mammalian cryopreservation literature (reviewed in Muldrew et al., 2004). Because of the apparent lethality of IIF, several studies have examined the formation and propagation of intracellular ice in mammalian cells and tissues (Berger and Uhrik, 1996; Acker et al., 1999, 2001; Acker and McGann, 2000). Although the hydrophobic properties of the cell membrane should provide a barrier to nucleation (and do so in most cases), inoculation of IIF occurs in mammalian cells (Acker et al., 2001). In insects, Davis and Lee (2001) observed that fat body cells of *E. solidaginis* appear highly susceptible to inoculative freezing, which supports the notion of both cell–cell propagation of ice and inoculation (rather than osmotic dehydration) caused by ice in the haemocoel. In addition, work on IIF in tissues shows that intracellular ice can readily propagate among connected mammalian (Acker et al., 2001) and insect salivary gland cells (Berger and Uhrik, 1996). The latter study implicated gap junctions as the site of ice propagation (which is supported by Acker et al., 2001). However, intercellular propagation of ice is also observed in tissues composed of cells that don't form cell–cell pores (Muldrew et al., 2004), and, Toner et al. (1990) show that IIF could also be nucleated by surface-catalysed nucleation, whereby the plasma membrane itself becomes a nucleating agent when in contact with ice. The latter mechanism is a likely route for external inoculation — gap junctions are only found at cell–cell junctions. Aquaporins, whose role in water movement (and freezing tolerance — see Izumi et al., 2006; Philip et al., 2008) make them an attractive candidate, but the small internal diameter – about 2 Å for the *Plasmodium falciparum* aquaglyceroporin (Newby et al., 2008) – make propagation of ice via aquaporins unlikely.

Ice formation is thought to begin in the gut of some freeze tolerant species, and then spread into the rest of the body (Worland et al., 1997; Sinclair et al., 1999; Zachariassen and Kristiansen, 2000; e.g. Humble, 2006). Because the gut is tightly closed to the haemocoel, there are few routes for ice propagation from the gut fluids to the haemolymph: either via nucleation across the tight junctions, perhaps analogous to surface-catalysed nucleation (Toner et al., 1990), or through the cells themselves. Worland et al. (2004) describe intracellular freezing in isolated gut cells of the alpine cockroach *C. quinquemaculata*, a species in which nucleation is thought to happen in the gut. This species also has high levels of ice-active proteins in extracts of the gut and Malpighian tubules (Wharton et al., 2009), which further implies that the gut tissue experiences significant freezing stress compared to other tissues, and may indicate an intracellular route of ice propagation in this species. Many insect species survive freezing only via external inoculation through the cuticle, mouth or anus (Zachariassen and Kristiansen, 2000), and may also utilise this mechanism. We predict that they would have high concentrations of ice-active proteins in the epidermis and/or gut cells.

5. Why are some cells killed by IIF, when others are not?

The causes of mortality due to intracellular freezing have been vigorously debated in the cryopreservation literature (reviewed by Muldrew et al., 2004). The consensus appears to be that the primary cause of injury is damage to the plasma membrane (Mazur, 1977), with concentration effects also playing a role, although Muldrew (2008) has recently re-established the idea that mortality could also stem from post-thaw hypertonicity (at least in mammalian cells). It appears that – in cryopreserved systems, at least – recrystallisation during rewarming may be a significant cause of mechanical damage during thawing (Muldrew et al., 2004). These stresses are remarkably similar to the stresses that insect cells suffer regardless of whether freezing is extracellular or intracellular. For example, antifreeze proteins are thought to inhibit recrystallisation *in vivo* (Knight and Duman, 1986), and the EIF model clearly suggests that cells can tolerate extensive concentration of their contents.

Although survivable IIF has been described in a number of insects, it has been confined largely to fat body cells. Fat body cells are generally globular, and thus more likely to nucleate internally (Asahina, 1969), have a relatively low water content, and are distinctive for the inclusion of large stores of energy reserves, particularly carbohydrates and (in larvae) lipids and triglycerides, as well as being the main site for intermediary metabolism (Wigglesworth, 1965; Nation, 2002). Thus, they are not 'normal' cells, and perhaps these specific features either make nucleation more likely (resulting in strong selection for survival of intracellular freezing), or make the cells more likely to survive. Fat body cells are also very large, and it is possible that there is limited scope for rapid osmotic dehydration below some threshold surface area:volume ratio. By contrast, gut cells, which survive freezing in *C. quinquemaculata* under the right conditions (Worland et al., 2004), are not globular and have few of the features that might be ascribed to the ability of fat body cells to withstand IIF. Thus, the key features of cells that allow survival of IIF remain to be determined.

6. Is IIF advantageous?

The insect cold tolerance literature contains much speculation (but few data) about the relative advantages (or the evolution of) of the freeze tolerance and freeze avoidance cold hardiness strategies (e.g.

Lee, 1989; Block, 1991; Renault et al., 2002; Vernon and Vannier, 2002; Sinclair et al., 2003). Any discussion of the advantages and disadvantages of IIF must be similarly speculative, and may follow similar lines, since IIF is essentially a cellular case of freeze tolerance, while EIF is analogous to freeze avoidance. Central to the potential advantages of IIF is the reduction of cellular (and organismal) dehydration, a key corollary to low overwinter temperatures (Ring and Danks, 1994). If mortality due to freezing in cells is caused by mechanical damage from cellular shrinkage, then IIF would certainly be advantageous. Acker and McGann (2003) show that mammalian cells still in tissues have better survival with IIF (which was not the case with isolated cells), and speculate that this increase in survival may result from a reduction in water loss. Salt (1961) observed a reduced rate of water loss in thawed *C. cinctus* that had been frozen intracellularly, compared to extracellularly frozen individuals, suggesting that IIF may confer water balance advantages in *C. cinctus*, although the intracelluarly frozen individuals had overall worse survival, especially at lower temperatures.

We speculate that survivable IIF may be achieved through different mechanisms in the gut and fat body cells. Furthermore, given the wide phylogenetic spread of survivable IIF in insects, we hypothesise that survivable IIF has arisen independently in the species that display it. This latter hypothesis suggests that insects might evolve freeze tolerance first, and then survival of IIF. However, given the paucity of data that might allow the among-species prevalence of survivable IIF, even in fat body cells, to be determined, it is just as likely that the ability of fat body cells to survive IIF may be a prerequisite to the evolution of freeze tolerance.

7. Conclusions

Although extracellular ice formation remains the most parsimonious explanation for freeze tolerance in insects, there is evidence dating back 50 years that some insects can survive intracellular ice formation in some cells or tissues. However, the prevalence of IIF across taxa remains to be determined, as do the mechanisms and consequences of this strategy. Currently, there is no adequate way to follow ice formation in real time with the resolution necessary to determine the processes of IIF, but advances in microscopy (e.g. Giepmans et al., 2006; Hsieh et al., 2006; Ishiguro and Horimizu, 2008; Smith et al., 2008a; Stott and Karlsson, 2009) and other imaging techniques (e.g. Socha et al., 2007; Mietchen et al., 2008) could well provide the means to examine IIF *in vivo*. Thus, although many fields of comparative physiology (including insect cold tolerance) have advanced considerably since the birth of CBP, many questions, like that of IIF, remain unanswered, providing plenty of material for the journal's next half-century.

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