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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*.

Contents

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.

☆ This Perspective is a contribution to the *Comparative Biochemistry and Physiology* Fiftieth Anniversary Celebration.

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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.

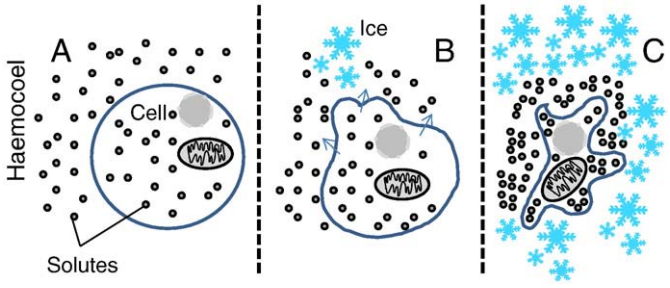

Fig. 1. The extracellular ice formation model for freeze tolerance in insects, as described by Asahina (1969). Under normal conditions, the cell is isosmotic with the surrounding haemolymph (A). When ice begins to form, exclusion of solutes from the crystals results in an increase in solute concentration 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.

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.

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