PART I PHYSIOLOGICAL AND MOLECULAR RESPONSES

1

A primer on insect cold-tolerance

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1.1 Introduction

Low temperature affects insects differently based on the severity of the cold and the duration of exposure. Life stage and acclimation state also have a major impact on an insect's response to low temperature. Many temperate and polar insects enhance their cold-tolerance seasonally in preparation for winter, as short, cool days in autumn trigger cold acclimatization, as well as entry into the metabolic depression of diapause. However, insects also have the capacity to make significant and rapid adjustments to even slight changes in environmental temperature, as would occur on a summer's day.

This introductory chapter seeks to provide a short primer on the physiology of insect cold-tolerance that will be useful to students and others new to the area of study. This overview of basic concepts in insect cold-tolerance intends to provide a context for later chapters providing in-depth reviews of specific areas. Specifically, this primer focuses on regulation of supercooling and ice nucleation, and basic adaptations promoting cold-tolerance. Suggestions for conducting and clearly reporting experimental results on insect cold-tolerance are also included. Since this volume is intended to update and complement our previous book, *Insects at Low Temperature* (Lee and Denlinger, 1991), this synoptic chapter will emphasize articles published during the past 20 years and topics not covered elsewhere in this volume.

1.2 Types of insect cold-tolerance

Chilling and cold are relative terms; consequently, the temperature ranges they represent vary depending on the species in question. For a tropical species,

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10 °C may be sufficiently low to cause chill coma (a loss of locomotory capacity due to impaired neuromuscular function), while a polar or alpine species may be able to walk normally at −5 °C or below (e.g. a Himalayan midge can walk at −16 °C (Kohshima, 1984)). Cold-hardening refers to the capacity of an insect to increase its level of cold-tolerance. It may result in a lowering of the chill-coma temperature, an increase in the ability to survive extreme cold, or the acquisition of the capacity to survive internal ice formation.

Numerous systems for categorizing types of insect cold-tolerance have been proposed, which have generated spirited discussion at numerous conferences and in the literature (Ramlov, 1998; Sinclair, 1999; Nedved, 2000; Chown and Terblanche, 2007; Hawes and Bale, 2007; Chown *et al.*, 2008; see Chown and Sinclair, Chapter 8). Though particular categories and names vary, it is generally agreed that there are three basic types of cold injury corresponding to the primary types of insect cold-tolerance (Fig. 1.1).

Chilling intolerant refers to species that succumb to the direct effects of low temperature without internal ice formation. Depending on the species, such chilling injury may occur at temperatures above or below 0 °C. This category may be usefully subdivided into direct and indirect chilling injury. Direct chilling injury, or cold-shock injury, results from brief exposures to cold (on the order of minutes to hours) that damage cell membranes by causing a phase transition from the liquid-crystalline to the gel state, and the lateral separation of membrane proteins (Levitt, 1980; Larcher, 2001). Cold-shock injury is a major problem for the cryopreservation of various mammalian cells and tissues, particularly spermatozoa, and recent evidence indicates similar injury occurs in insects, as Lacoume *et al.* (2007) reported that cold-shocked (1 h at −18 °C) males of the parasitoid wasp, *Dinarmus basalis,* had markedly reduced sperm stores and fertilized fewer females than control wasps. (See Lee and Denlinger, Chapter 2, for a review of cold-shock injury and the rapid cold-hardening response that protects against it.)

Indirect chilling injury occurs over extended periods of days to weeks and is found in a wide range of plants and animals. This type of injury occurs at higher temperatures, from a few degrees below zero to 10–15 °C. Indirect chilling injury is important commercially because it determines the shelf life of fruits and vegetables, particularly those of sub-tropical and tropical origin. It is a major problem for storage of chilled human tissues and organs for transplantation (Taylor *et al.*, 2007). Understanding long-term chilling injury is also important for predicting the establishment of non-native pests, storing biological control agents for mass release, and the use of cold for quarantine treatment of imported produce and other commodities (Hallman and Denlinger, 1998; Bale, Chapter 14).

Like direct chilling injury, indirect chilling injury appears to be caused, at least in part, by thermotropic damage to cell membranes causing metabolic imbalance

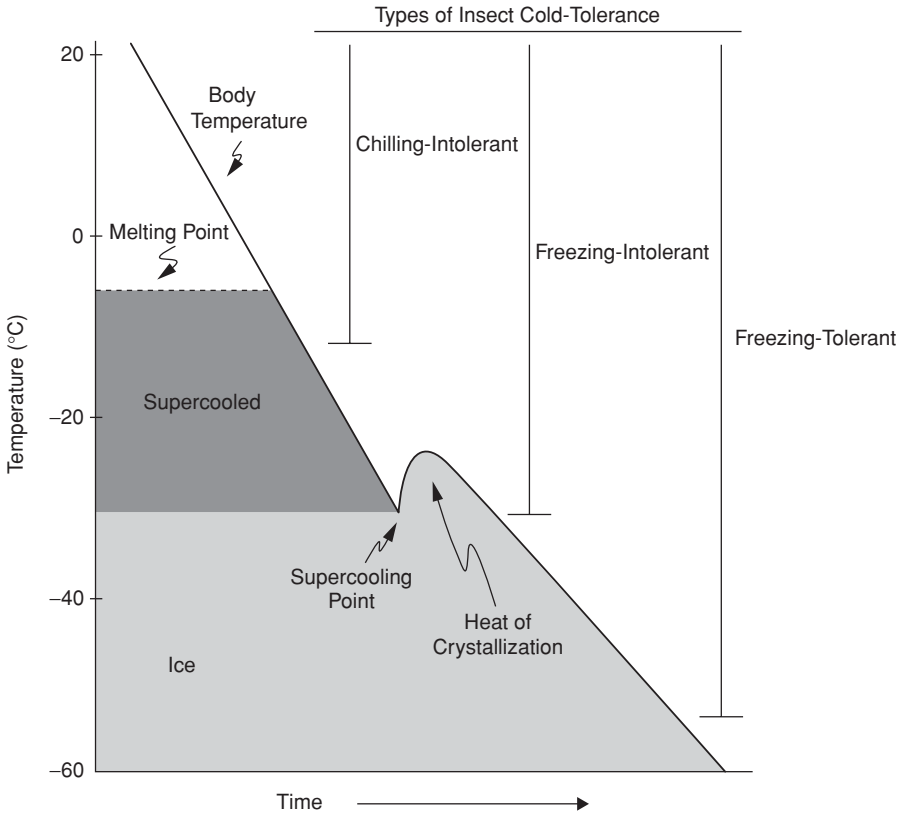

Figure 1.1 Insect responses to low temperature. Body temperature (bold line) in relation to hemolymph melting point, supercooling point (or temperature of crystallization), and release of the heat of crystallization as body water freezes. Bars on the right indicate representative ranges for different types of insect cold-tolerance. (Adapted from Lee, 1989.)

and loss of selective membrane permeability. The magnitude of chilling injury is closely associated with a loss of ion homeostasis, particularly increases in potassium ions and decreases in sodium and magnesium ions in the hemolymph, and loss of electrochemical potentials across cell membranes (Kostal *et al.*, 2004; Kostal *et al.*, 2006; Kostal *et al.*, 2007). Cryoinjury to the cell membrane is consistent with the observed decreases in membrane potential of nerves and muscles, and impaired coordination within the neuromuscular system (Denlinger and Lee, 1998). Chilling injury may also result from oxidative stress during cold storage (Rojas and Leopold, 1996; Storey and Storey, Chapter 6).

Interestingly, mortality caused by indirect chilling may be prevented, or damage caused reversed or repaired, by a brief warming episode in the midst of weeks of cold exposure (Chen and Denlinger, 1992) or by brief periods of daily warming

(Turnock and Bodnaryk, 1993; Renault *et al.*, 2004; Colinet *et al.*, 2006). During these brief intervals of warming some insects can re-establish ion gradients (Kostal *et al.*, 2007) or up-regulate proteins involved in energy metabolism, protein chaperones (Hsp70/Hsp90), or cytoskeletal components (Colinet *et al.*, 2007; Michaud and Denlinger, Chapter 4). Other reports suggest these warming periods may allow for recharging depleted energy reserves (Chen and Denlinger, 1992) or for removal of accumulated toxic metabolites (Leopold *et al.*, 1998; Colinet *et al.*, 2007).

Freezing intolerant or *avoidant* species can survive cold as long as ice does not form within their bodies. These insects are not injured by cold-shock and can tolerate chilling to sub-zero temperatures. By far, most insects exposed to sub-zero cold in nature are freezing intolerant. Although *freezing susceptible* is sometimes used to describe this type of cold-tolerance, this name is somewhat ambiguous because it can be interpreted to mean the organism has the potential to freeze internally (which, of course, all organisms do), instead of indicating that internal ice formation is lethal to it. In winter, freezing-intolerant species rely on mechanisms by which they seasonally increase their cold-tolerance and capacity to remain unfrozen by supercooling – in some cases, to extremely low temperatures of −40 °C or even below −60 °C (Ring and Tesar, 1981). The capacity to supercool increases as body size and water content decrease, and with the accumulation of low-molecular-mass solutes.

Freezing-tolerant species can survive the freezing of their body water. Insects that survive freezing typically do so only over a specific temperature range from the initiation of ice formation to some lower temperature (Fig. 1.1). As ice forms in the extracellular space, only water molecules join the growing ice lattice and dissolved solutes are excluded. Consequently, as freezing continues, solutes become increasingly concentrated (termed freeze concentration) in the hemolymph, causing an osmotic outflow of water from surrounding cells. As cells are dehydrated, intracellular solutes are freeze concentrated until osmotic equilibrium is re-established.

1.2.1 Cryoinjury due to freeze concentration

There is general agreement that the freeze concentration of solutes results in freezing injury; however the actual mechanism remains unclear (Mazur, 2004; Muldrew *et al.*, 2004). One hypothesis, sometimes referred to as solution-effects injury, posits that extracellular freeze concentration causes an excessive concentration of extracellular and intracellular electrolytes that damages the cell membrane and leads to cytolysis. Alternative explanations focus on the specific effects of cellular dehydration. Meryman (1968) suggested that excessive osmotic shrinkage may exceed a minimum critical cell volume, from which the cells cannot recover. Membrane destabilization or loss of membrane materials during dehydration may also occur (Steponkus and Lynch, 1989). These explanations for freezing injury are

primarily based on studies with red blood cells, plant protoplasts and other isolated cell suspensions from humans or other mammalian models that do not naturally experience severe cold. Since few studies have been done with freeze-tolerant insects or other ectotherms, some caution should be used in extending explanations to naturally cold-hardy animals.

1.3 Mechanical injury

The growing ice lattice can also damage tissues and organs as ice forms between adjacent cell layers. As the ice mass grows, it pulls tissue layers apart, causing mechanical injury that remains after thawing. Mechanical injury may also result from recrystallization within frozen tissues. In this scenario, small ice crystals shrink as they lose water molecules to growing crystals. Recrystallization occurs especially rapidly at high sub-zero temperatures – the range in which many freeze-tolerant ectotherms winter. Ice-binding proteins can inhibit recrystallization and, thereby, prevent its damaging effects within body tissues (Duman *et al.*, Chapter 3).

1.4 Supercooling

During cooling, the body temperature of most insects closely tracks that of their immediate environment due to their small size and limited ability to generate heat (Fig. 1.1). A few notable exceptions are some winter-active moths and a few other endothermic insects that can generate sufficient heat to remain active, and can even fly at low winter temperatures (Heinrich, 1993). As insects are cooled to temperatures below 0 °C, ice does not form immediately in their body tissues for two reasons. First, ions, sugars, amino acids, proteins and other dissolved solutes in an insect's hemolymph colligatively depress the melting point (MP) by 1.86 °C per osmole of solute. For example, if an overwintering insect had a hemolymph osmolality of 1.5 osmoles, its MP would be −2.79 °C and no ice could form until the temperature dropped below this value.

Second, once cooled to temperatures below its hemolymph MP, an insect usually enters a supercooled state, in which its body water remains unfrozen. The capacity to supercool is inversely related to water volume (Angell, 1982). A few microliters of water readily supercool to −15 to −20 °C, while under special conditions supercooling continues to its limit, near −40 °C. Most insects behave as though they are small vessels of water and, whether collected in summer or winter, supercool at least a few – if not, many – degrees before ice forms spontaneously in their tissues. The temperature at which ice forms is termed the supercooling point (SCP) because it denotes the limit of supercooling (Fig. 1.1). The SCP is readily

determined using thermocouples to detect the heat of crystallization, which is released as body water freezes. For this reason, the SCP is sometimes referred to as the temperature of crystallization (T_c).

Supercooling capacity is the difference between the hemolymph MP and the SCP. So an insect with an MP = −1.5 °C and an SCP = −22.5 °C would have a supercooling capacity of 21 °C. Note that an insect with a low SCP would have a high supercooling capacity, and vice versa. Freezing-intolerant insects enhance their supercooling capacity in winter, while freezing-tolerant ones generally diminish this capacity. Extensive lists of SCP values for diverse arthropods are available (Sømme, 1982; Lee, 1991; Sinclair, 1999; Turnock and Fields, 2005).

For both freezing-intolerant and freezing-tolerant insects, the SCP represents a major transition point. For freeze-intolerant species it represents the lower limit of their potential survival, while for species that tolerate freezing it marks the beginning of a radical change in their physiological state, characterized by anoxia, cellular desiccation, build-up of metabolic wastes and depressed metabolic activity (Storey and Storey, 1988). However, even in the frozen state some complex physiological processes continue, including cryoprotectant synthesis (Storey *et al.*, 1981; Walters *et al.*, 2009) and diapause development (Irwin *et a*l., 2001). When assigning an insect to one of these categories, it is critical to keep in mind the conditions and temperatures the insect would experience in nature.

Using ecologically relevant cooling (often $< 0.5\ ^\circ\text{C min}^{-1}$) rates and appropriate durations of exposure are especially important in laboratory experiments (Lee, 1991). For example, if a wintering insect is naturally exposed to severe cold for weeks at a time, then laboratory exposures lasting only minutes to hours may reveal little about tolerance in the field. Freeze-tolerance should not be determined by thawing insects shortly after the SCP is reached and then assessing survival. In laboratory determinations of the SCP, the freezing exotherm is often very brief – lasting only seconds to minutes – due to relatively high rates of cooling and the rapid removal of the heat of crystallization by the surrounding heat sink. Even though the freezing exotherm has dissipated, an equilibrium amount of ice in the insect has not necessarily formed. In freeze-tolerant larvae of the gall fly (*Eurosta solidaginis*), only 47% of body water froze during the first 6 h of exposure to −23 °C, while at least 48 h were required to reach an equilibrium level of ice formation under these conditions (Lee and Lewis, 1985). Furthermore, slow rates of freezing may be critical to allow time for the mobilization of cryoprotective responses as ice forms within the body (Holmstrup *et al.*, 1999). Consequently, it is often necessary to hold an insect for extended periods at temperatures near the SCP before it can be judged freezing-tolerant.

In describing experimental treatments or reporting results, care should be taken to clearly distinguish between whether an insect was simply exposed to

a sub-zero temperature or whether actual freezing of its body water occurred. For example, if it was stated that an insect was exposed to freezing, it is unclear whether the author meant the insect was cooled to a sub-zero temperature or whether ice formed internally. Since many insects readily supercool for extended periods, especially at high sub-zero temperatures, it is important to report whether the insects actually froze internally by determining the SCP. This distinction may also become confusing when an insect has a low hemolymph freezing point (FP); for example, in an insect with an FP of −4 °C, exposure to −3 °C could not induce internal ice formation. Clearly distinguishing whether an insect remains supercooled or freezes is essential for correctly categorizing the type of cold-tolerance.

1.5 Initiation of freezing and ice nucleation

Despite the innate tendency of insects to supercool, ice eventually forms. In biological systems it is generally believed that ice forms by a heterogeneous mechanism in which a non-water substrate provides a template that stabilizes aggregations of water molecules, leading to ice formation at higher temperatures than would occur otherwise (see reviews by Vali 1995; Wilson *et al.*, 2003). However, Zachariassen and colleagues (2004) recently challenged this view in freeze-intolerant insects. They argue that supercooling capacity is closely correlated with an insect's water volume, and the pattern of freezing is more similar to homogeneous mechanisms of nucleation.

The most efficient ice-nucleating agent in an organism will determine the temperature at which ice begins to form. As the ice lattice grows, the heat of crystallization often increases the body temperature by several degrees or more (Fig. 1.1). Due to this increase in body temperature and because freeze concentration of solutes further depresses the hemolymph MP, it is unlikely that other less active, endogenous nucleators will induce freezing at other locations in the body.

Several distinct classes of ice-nucleating agents have been identified in insects: (1) ice-nucleating proteins, (2) crystalloid compounds and (3) ice-nucleating microorganisms (Table 1.1). The ice-nucleating activity of these endogenous nucleators ranges from highly efficient, in the range of −2 to −5 °C, to ones with little activity (<−18 °C).

1.5.1 Ice-nucleating proteins

Zachariassen and Hammel (1976) first reported the presence of an ice-nucleating agent in the hemolymph of freeze-tolerant beetles, which led to the discovery of ice-nucleating active proteins. Since then, ice-nucleating proteins with activity in the range of −6 to −10 °C have been reported from the hemolymph

Table 1.1 *Classes and efficacy of endogenous ice-nucleating agents compared to inoculative freezing by environmental ice*

Type	Ice-Nucleating Activity (°C)	Reference
Ice-nucleating proteins	−6 to −9	(Zachariassen and Hammel, 1976)
Crystalloid compounds	−7 to −10	(Mugnano *et al.*, 1996)
Ice-nucleating bacteria	−2 to −10	(Kaneko *et al.*, 1991a,b; Lee *et al.*, 1991, Lee *et al.*, 1993a)
Ice-nucleating fungi	−5	(Tsumuki *et al.*, 1992)
Inoculation by environmental ice	−1 or <MP of hemolymph, little or no supercooling	(Tursman *et al.*, 1994)

of various freeze-tolerant insects. (See Chapter 3 by Duman *et al.* for additional information on ice-nucleating proteins.)

In freezing-tolerant species these unusual proteins function to insure extracellular ice formation and prevent extensive supercooling and the resulting rapid propagation of ice that would be more likely to cause cryoinjury (Zachariassen, 1985; Zachariassen, 1992; Duman, 2001). By insuring freezing at a high sub-zero temperature, the ice lattice forms more slowly as environmental temperatures decrease, thereby lessening the osmotic stress caused by the freeze concentration of solutes in the extracellular space. Cellular dehydration also increases the capacity of cells to supercool and prevents lethal intracellular freezing. Also, by freezing at a high sub-zero temperature, ice formation and growth occur more slowly allowing insects and other freezing-tolerant animals to make physiological adjustments, such as mobilization of cryoprotectant, as ice is formed in their bodies (Storey and Storey, 1988; Lee and Costanzo, 1998; Holmstrup *et al.*, 1999). Note, however, that ice nucleation at temperatures near 0 °C is not required for freezing-tolerance; some insects supercool extensively and still survive freezing (Ring and Tesar, 1980).

These nucleators may also function to conserve energy and water during winter, since frozen insects have lower metabolic rates (Scholander *et al.*, 1953) and lose less water than ones that remain supercooled (Zachariassen, 1992). Frozen larvae of the gall fly, *E. solidaginis,* have a metabolic rate 47% lower and a water loss rate 35% lower, than supercooled larvae (Fig. 1.2; Irwin and Lee, 2002).

Crystalloid inorganic compounds comprise another category of endogenous ice-nucleating agents. Larvae of *E. solidaginis* often contain more than 30 calcium phosphate spherules in their Malpighian tubules (Mugnano *et al.*, 1996). Many of these spherules exhibit ice-nucleating activity between −8 to −10 °C that closely matches the larval T_c of −9.4 °C. During the larval-to-pupal transition these spherules

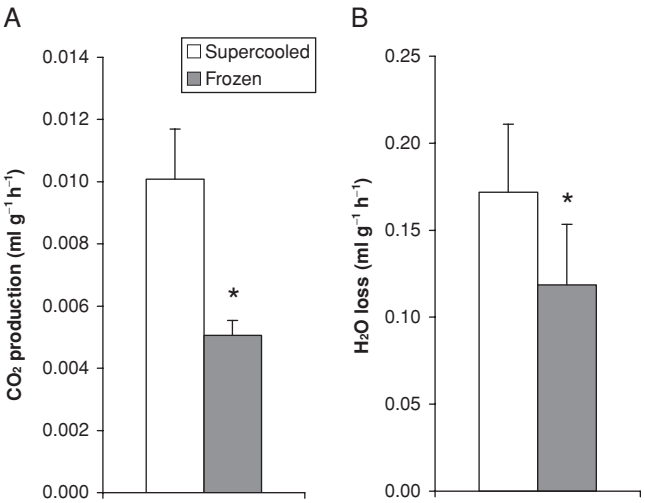

Figure 1.2 Metabolic rate (CO_2 production) (A) and water loss (B) of frozen and supercooled goldenrod gall fly larvae (*Eurosta solidaginis*) at −5 °C. Frozen larvae had significantly lower metabolic rates and water loss. (From Irwin and Lee, 2002)

disappear concomitant with a decrease in T_c to −18.3 °C. Since the larval hemolymph and most other tissues lack efficient nucleators, as evidenced by their capacity to supercool extensively, these spherules appear to determine the larval T_c. Uric acid, calcium carbonate, potassium phosphate and other crystalloids are known from various overwintering insects; commercial preparations of these compounds have ice-nucleating activity (Mugnano *et al.*, 1996). Consequently, it seems likely additional freeze-tolerant species will be discovered that use these nucleators to insure freezing at high temperatures.

Ice-nucleating active (INA) micro-organisms possess the unique trait of catalysing ice nucleation at temperatures as high as −1 °C (see reviews in Lee *et al.*, 1995c). In INA bacteria, aggregations of proteins in the outer membrane function as templates on which embryonic seed crystals can form and induce freezing at very high sub-zero temperatures. These epiphytic, and frequently plant pathogenic, bacteria are well known for their role in promoting frost damage to crops. By promoting freezing damage, the ice-nucleating phenotype may function to facilitate bacterial invasion of the plant tissues and access to nutrients (Lindow, 1983).

In the early 1990s, two research groups independently isolated INA bacteria from the gut of insects (Kaneko *et al.*, 1991a; Lee *et al.*, 1991). Some of the bacterial isolates exhibited maximal INA thresholds near −2 °C. In 1992, Tsumuki and colleagues isolated an ice-nucleating active fungus (*Fusarium* sp.) from freeze-tolerant larvae of the rice stem borer. When sterile larvae were fed a suspension of fungal