



## Review

## An invitation to measure insect cold tolerance: Methods, approaches, and workflow



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## ARTICLE INFO

## Article history:

Received 25 September 2015

Received in revised form

28 October 2015

Accepted 2 November 2015

Available online 10 November 2015

## Keywords:

Chill coma

Critical thermal minimum

Supercooling point

Cold tolerance strategy

Lower lethal temperature

Rapid cold-hardening

Acclimation

Deacclimation

Phenotypic plasticity

Experimental design

## ABSTRACT

Insect performance is limited by the temperature of the environment, and in temperate, polar, and alpine regions, the majority of insects must face the challenge of exposure to low temperatures. The physiological response to cold exposure shapes the ability of insects to survive and thrive in these environments, and can be measured, without great technical difficulty, for both basic and applied research. For example, understanding insect cold tolerance allows us to predict the establishment and spread of insect pests and biological control agents. Additionally, the discipline provides the tools for drawing physiological comparisons among groups in wider studies that may not be focused primarily on the ability of insects to survive the cold. Thus, the study of insect cold tolerance is of a broad interest, and several reviews have addressed the theories and advances in the field. Here, however, we aim to clarify and provide rationale for common practices used to study cold tolerance, as a guide for newcomers to the field, students, and those wishing to incorporate cold tolerance into a broader study. We cover the 'tried and true' measures of insect cold tolerance, the equipment necessary for these measurement, and summarize the ecological and biological significance of each. Finally, we suggest a framework and workflow for measuring cold tolerance and low temperature performance in insects.

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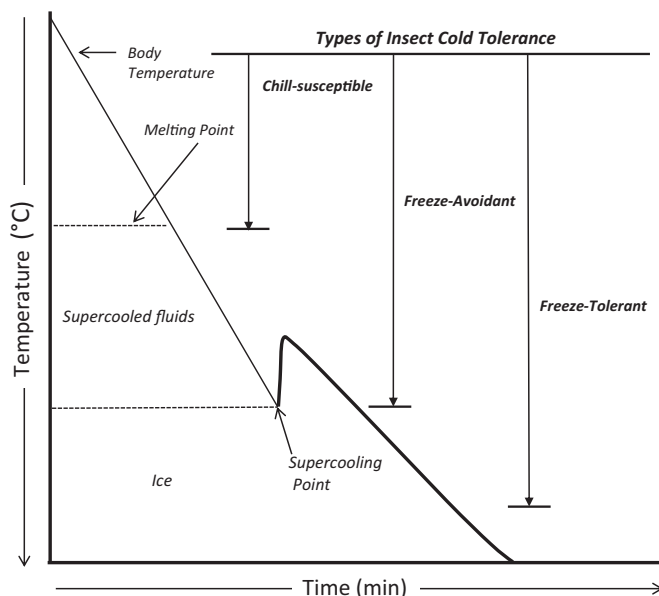
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## 1. Introduction

Temperature constrains the geographic distribution and seasonal activity of insects (Chown and Nicolson, 2004), and therefore can directly or indirectly affect the spread and impact of invasive pests, the success of species introduced for biological control, and the dynamics of native insect populations (Bale and Hayward, 2010). In temperate, polar and montane habitats, the majority of insects spend a large proportion of their life in an overwintering stage, and must survive the low temperatures and accompanying environmental stressors that are associated with winter (Leather et al., 1993; Williams et al., 2015). Similarly, insects in deserts and tropical high mountains can also be regularly exposed to potentially-lethal freezing conditions (Sømme, 1995; Sømme et al., 1996; Sømme and Zachariassen, 1981). Thus, low temperature biology is a key component of insect fitness, and one of the best determinants of insect distribution (Andersen et al., 2015b).

Most insects are ectotherms, and as such, their body temperatures are generally similar to the ambient microclimate temperature, and changes in ambient temperature can thus have drastic effects on the physiology of an insect. Thus, measuring low temperature performance (which we refer to loosely here as ‘cold tolerance’) is an excellent way to incorporate the pervasive effects of temperature in studies ranging from ecological (e.g. van Dooremalen et al., 2013) to molecular (e.g. Reis et al. (2011)). In the cold, many insects enter a reversible state of paralysis, called chill coma, at the critical thermal minimum ( $CT_{min}$ ) (MacMillan and Sinclair, 2011a). At sub-zero temperatures, insects risk freezing of the body fluids, as well as a host of other low temperature injuries (Denlinger and Lee, 2010). The ability of an insect to survive at low temperatures is referred to as its cold hardiness, and their responses to low temperature have generally been categorized as chill-susceptible, freeze-avoidant, and freeze-tolerant (Fig. 1, see Section 3) (Bale, 1993).



**Fig. 1.** Classifications of insect cold tolerance. Insect cold tolerance is divided into three main classifications, based on survival of low temperatures and extracellular ice formation. Chill-susceptible insects die of cold exposure unrelated to freezing, whereas freeze-avoiding insects maintain their body fluids in a supercooled state and die when ice formation occurs (i.e. at the supercooling point). Freeze-tolerant insect are able to withstand extracellular ice formation. Adapted from Lee (2010).

There is a long history of the study of insect cold tolerance (Ring and Riegert, 1991; Sømme, 2000), and the sub-discipline has consequently developed its own semantic and methodological traditions. Although there are many excellent reviews on the subject (e.g. Asahina (1969), Bale (2002), Block (1982a), Denlinger and Lee (2010), Lee (1991), Salt (1961), Sinclair et al. (2003b), Zachariassen (1985)), a unified summary of the methods and approaches used in insect cold tolerance is not available. Nevertheless, although care must be taken in the design and interpretation of experiments, measuring insect performance at low temperatures is by no means arcane, and many measurements require no specialist equipment. Our purpose in this review is to explain some of the common measures of insect low temperature biology from a methodological viewpoint, with the intention of making these methods more accessible. We try to identify some of the diverse measurements that are comparable among studies, and our ultimate goal is to reduce some of the trial-and-error inherent in learning a new set of techniques and measurement. Our intended audience is newcomers to the field, students, and (particularly) those who are interested in incorporating low temperature performance into their existing studies, and are looking for an overview of common practices. While there is an unavoidable bias towards the methods or approaches used over the past two decades by the first author, we have tried to encompass alternative approaches wherever possible, and also provide (hopefully) lucid explanations of why we favor one approach over others. In some cases, we give examples of equipment that has been used in these studies, but we do not intentionally endorse any manufacturer or model.

## 2. Technical and apparatus considerations

Studying insect low temperature performance requires some form of temperature control and measurement. We conclude this section by discussing how to identify and measure an insect's supercooling point (SCP), as measuring the SCP is fundamental to many other measures of insect cold tolerance and provides a useful application of measuring an insect's body temperature during cold exposure.

### 2.1. Temperature control

The simplest cold exposures involve constant low temperatures, and the equipment needed for these exposures is often readily available. For example, an ice–water slurry is at a constant (and precise) 0 °C, many laboratory and domestic refrigerators and cold rooms are held at approximately +4 °C, and domestic freezers are usually somewhere between –12 and –20 °C. Similarly, refrigerated incubators or refrigerated baths can be easily set to a single temperature. Ultra-low freezers (usually set somewhere between –70 and –90 °C), dry ice (–80 °C), a dry ice–acetone slurry (–78 °C) and liquid nitrogen (–196 °C) all provide constant low temperatures with reasonable precision. Although these latter temperatures are of limited biological relevance when studying living insects, they can be useful if extreme rapid cooling is required.

More commonly, insects are cooled using specialised cooling equipment, such as rate-controlled incubators (e.g. Ju et al. (2011)) or refrigerated circulators (e.g. Marshall and Sinclair (2011)). These are often programmable, allowing precise cooling, hold (‘soak’ in engineering terminology), and warming programmes. Refrigerated

circulators will require a bath liquid (e.g. glycol, alcohol, or a synthetic or mineral oil) for sub-freezing use. In our laboratory we use ethylene glycol (usually mixed 1:1 with water) for moderately low temperatures (c.  $-20^{\circ}\text{C}$ ) because it has a lower viscosity than the less-toxic propylene glycol and can be heated. We use methanol (either undiluted or mixed 1:1 with water) for lower temperatures. Samples (in containers) can either be placed directly in the bath, or the bath liquid can be circulated to a cooling stage; in our laboratory we circulate liquid to an insulated aluminum block that is milled with holes to accommodate various sample container sizes, from 1.5 mL microcentrifuge tubes to 35 mL standard *Drosophila* vials.

Thermoelectric cooling via Peltier devices can be used to construct efficient cooling devices (especially if the cooling modules are stacked and liquid-cooled), and we have used these in the past (e.g. Sinclair et al. (2003a), Sinclair and Sjørnsen (2001)); unfortunately, neither of these devices (nor designs for them) are commercially available, so they must be custom-designed and -built (Wharton and Rowland, 1984). However, they are light and robust and use little power, so are ideal for field situations. Another field alternative is to use an endothermic reaction to provide cooling. For example, Sømme et al. (1993) used a  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ /crushed ice mixture to determine SCPs of mites at a field camp in Antarctica. This has the disadvantage of producing chemical waste, but greatly reduces the electricity requirements for cooling in the field. Another approach to control cooling rate is to use a set-temperature incubator or freezer, and to moderate the cooling rate (if not the final temperature) using insulation, such as a Styrofoam box (e.g. Hao and Kang (2004)).

Different methods of cold exposure will have different levels of among-sample and time-related variance. For example, there can be spatial variation among wells in the aluminum block mentioned above, and also within incubators. Thermal fluctuations in incubators (especially at temperatures where compressors may be starting and stopping) can be quite substantial. It is important to view these fluctuations in the context of biological relevance – for example, fluctuations of  $1^{\circ}\text{C}$  may be unimportant if among-treatment differences are in the order of tens of degrees, but if differences among treatments are subtle (or fluctuations are large), the noise may overpower the signal. Immersing samples in a liquid medium can be used successfully to significantly dampen fluctuations in incubators (e.g. MacMillan et al. (2009)). In addition, the temperature actually experienced by an insect may differ from that to which the instrument has been set. Thus, good practice is to always measure the temperature which an animal or group of animals experiences, and to use that as the treatment temperature in analyses.

Investigations of insect cold tolerance initially adopted a uniform cooling rate of approximately  $1^{\circ}\text{C}/\text{min}$  to standardize results (Salt, 1966). Although this rate is convenient, it is significantly faster than the cooling rates experienced in nature (e.g. Sinclair (2001) measured a maximum cooling rate of  $0.01^{\circ}\text{C}/\text{min}$  in alpine microhabitats in New Zealand – 100-fold slower than used in these laboratory studies) and several studies have shown that fast cooling rates lead to additional cold shock (e.g. Bale et al. (1989), Cloudsley-Thompson (1973), Miller (1978)). In particular, directly plunging an insect into an extreme temperature can significantly decrease survivorship, compared to that achieved during a ramping regime (Nguyen et al., 2014). Conversely, slow cooling rates can make experiments impractically long, and may allow insects to mount a physiological response to cold, such as that observed during rapid cold-hardening (Kelty and Lee, 1999; Nguyen et al., 2014). Most authors currently choose cooling rates that compromise between ecological relevance and time investment; generally  $0.1$  to  $0.5^{\circ}\text{C}/\text{min}$  (e.g. Boardman et al. (2012), Crosthwaite et al. (2011), Renault et al. (2004), Sformo et al.

(2011)). It is clear that the rate of temperature change does affect the response of the insect (this has also been vigorously discussed in the context of high temperature tolerances, e.g. Santos et al. (2011), Terblanche et al. (2007), Terblanche et al. (2011)). Thus, it is important to maintain consistent cooling rates among sites or species for comparisons, and to be cognizant of the assumptions inherent in comparing thermal limits among studies that used different exposure conditions.

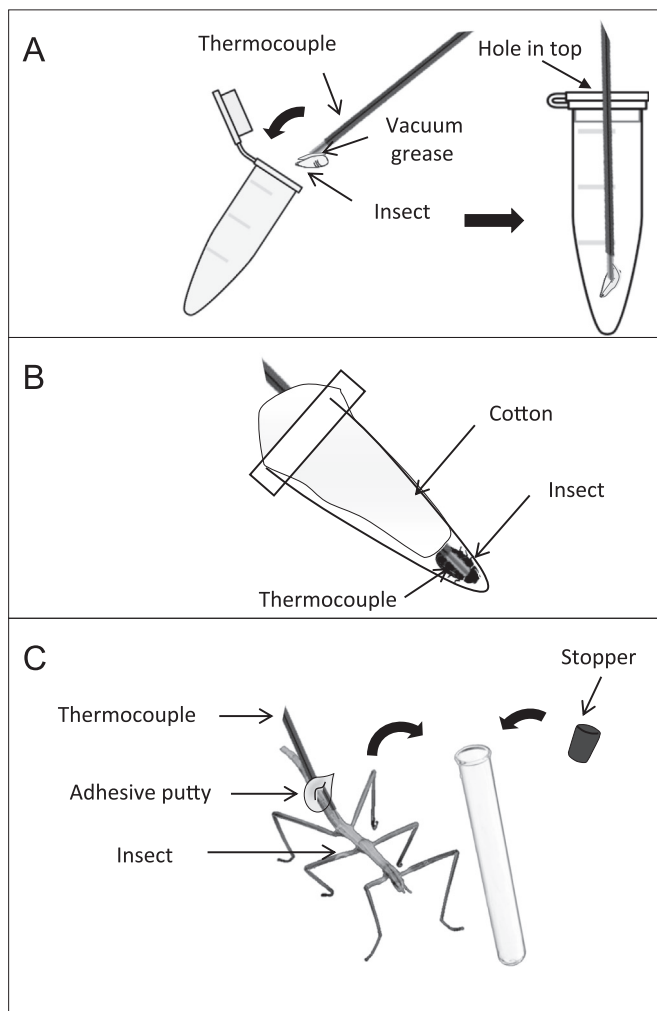
## 2.2. Temperature measurement

Thermocouples are the most common way to measure insect temperatures during cooling. Thermocouples are thin wires that measure temperature via the changes in the voltage output of a bimetallic junction. Usually one thermocouple is used per insect, although it is also possible to increase throughput by carefully placing more than one (small) insect on a thermocouple (e.g. springtails; Sinclair et al. (2006a)), or by connecting multiple thermocouples in series (Nedvĕd et al., 1995). Thermocouples differ in the types of metal used, and in the diameter of the wire; any thermocouple type is acceptable if it returns valid measurements over an appropriate range of sub-zero temperatures and is compatible with the recording device. Types K (chromel–alumel) and T (copper–constantan) are most common in insect cold tolerance studies. Smaller-diameter thermocouples will yield a faster response (but are more fragile); we have found 36 AWG thermocouples to be a suitable compromise. Pre-made thermocouples can be purchased in various lengths, or thermocouple wire can be purchased and used to make thermocouples by stripping the wires and twisting them together to create a thermocouple junction (damaged pre-made thermocouples can also be repaired this way). Thermistors can be used in an identical manner to thermocouples for SCP measurements (provided they are small enough), but require specific data acquisition apparatus.

To detect freezing, thermocouples must be close enough to (or in contact with) the insect during cooling so they can detect the latent heat of crystallization that indicates ice formation. The lowest temperature that precedes this exotherm is termed the supercooling point (the SCP; Fig. 1). Small insects and eggs can be attached to thermocouples by coating the tip of the thermocouple with a thin film of petroleum jelly, heat sink compound, or vacuum grease, which will adhere to the insect with a light touch (Fig. 2A). This has been particularly successful with springtails and mites (Block, 1982b; Block and Young, 1979). For larger insects (e.g. crickets and beetles), thermocouples may be maintained in contact with the cuticle by placing (or chasing) the insect into a microcentrifuge tube or pipette tip, and packing cotton wool in to hold the animal and a thermocouple in place (Fig. 2B). Removable adhesive putty (such as the sticky tack used to attach pictures to walls) may also be used to hold the tip of the thermocouple in contact with an insect such as a phasid (Fig. 2C). Exotherms can often be detected from large organisms even if the thermocouple is not in direct contact with the animal. This approach – placing the thermocouple on the outside of a sealed respirometry chamber containing the animal – has worked for large caterpillars (e.g. Sinclair et al., 2004) and frogs (Sinclair et al., 2013).

Data from thermocouples can be acquired directly by a computer using commercially-available thermocouple interfaces, such as the Sable Systems International T2000 ([www.sablesys.com](http://www.sablesys.com)) and the Pico Technology TC-08 ([www.picotech.com](http://www.picotech.com)). Alternately, thermocouple data loggers can be used to record data (many will interface directly to a computer). For example, the Hobo UX120 ([www.onsetcomp.com](http://www.onsetcomp.com)), the Grant Squirrel ([www.grantinstruments.com](http://www.grantinstruments.com)), or the Campbell range ([www.campbellsci.ca](http://www.campbellsci.ca)).

Thermocouples can nucleate ice formation, particularly for soft-bodied or moist animals, or if the thermocouple cools more



**Fig. 2.** Attaching thermocouples to insects. (A) For small insects, thermocouples are secured to the insect using a thin layer of vacuum grease. (B) For medium-sized and/or active insects, a piece of cotton can be inserted into the vessel (pipette tip, microtube) to immobilize the insect near the tip and secure the thermocouple in contact with the insect. (C) For large and highly active insects, the thermocouple can be secured in contact with the insect using adhesive, such as adhesive putty.

quickly than the insect. For example, Sinclair et al. (2009) found that ice nucleation appeared to begin at the thermocouple attached to drosophilid larvae. Although this impact of thermocouple contact on the SCP is probably negligible for most insects, care should be taken with moist, soft-bodied animals that may be susceptible to inoculative freezing. One way to avoid this problem is to use differential scanning calorimetry (DSC) or infrared thermography (see below), or to place a very fine (and therefore sensitive) thermocouple near, but not in contact with, the insect.

Differential scanning calorimetry measures heat flow into or out of a sample, usually for chemical analysis. However, the sensitivity and precision of DSC means it can be used to measure freezing in small insects placed in the cooling chamber. Many DSC instruments are cooled by liquid nitrogen, and can therefore be used to measure very low SCPs (e.g. below  $-40^{\circ}\text{C}$ ). Differential Scanning Calorimetry likely avoids artifacts from thermocouple contact, and can be used for multiple small insects at once (e.g. Worland and Convey (2001)). Infrared thermography is another approach to detecting and measuring SCPs without physical contact. This approach has been used successfully to detect SCPs (Palmer et al., 2004), to analyze the ice formation process (Sformo et al., 2009), and to separate freezing of insects from ice formation in their substrate (Košťál et al., 2012). It is now possible to

purchase infrared cameras that interface with a smartphone, so the availability of this technology may increase rapidly. Infrared ‘thermocouples’ are also commercially available, but have not, to our knowledge, been used for measuring insect cold tolerance in the published literature.

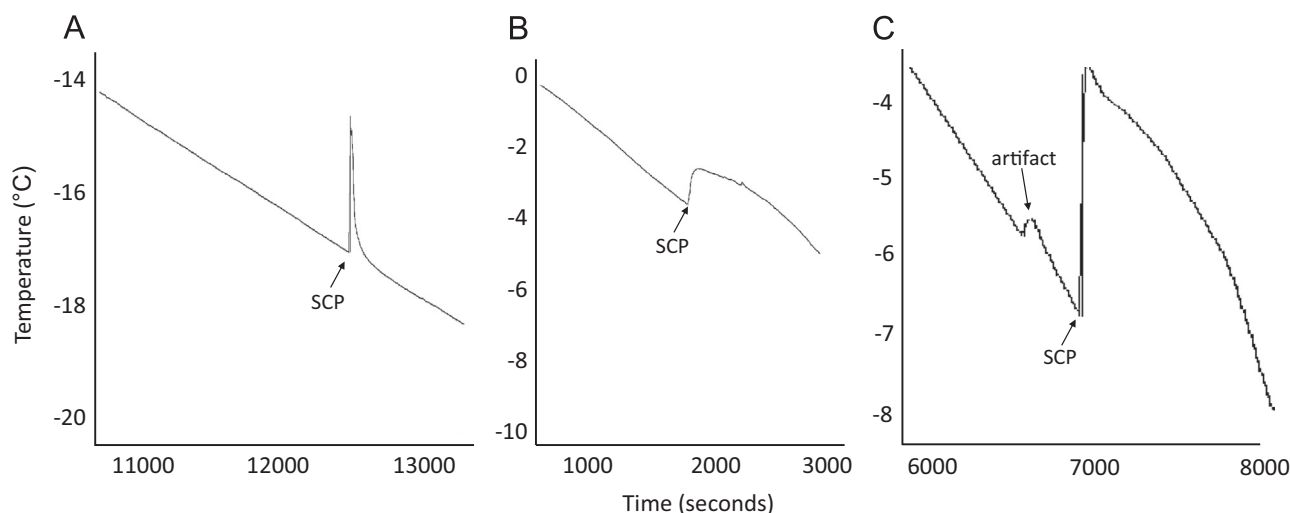
### 2.3. Measuring supercooling points

The supercooling point is the temperature at which freezing begins, and ice formation will usually then proceed from the site of nucleation to other parts of the insect's body (Lee and Lewis, 1985). Many investigations of insect cold tolerance begin with preliminary measurements of the SCP because this provides an anchor point about which the cold tolerance strategy can be determined: an initial step is to determine whether insects survive freezing or not (see Section 3.1). Additionally, changes in the SCP with treatment or season can indicate biochemical or physiological changes, even if the SCP is not a good measure of survival of low temperatures (Coleman et al., 2014). Because the SCP does not necessarily equate to cold-hardiness, it must be interpreted with caution, and in many cases has limited ecological relevance (Bale, 1987; Baust and Rojas, 1985; Renault et al., 2002). However, for freeze tolerant and freeze-avoidant insects (see below), the SCP does represent an important physiological threshold or lower lethal limit, respectively, and can indicate ecologically-relevant variation in cold tolerance in the latter (e.g. Crosthwaite et al. (2011), van der Merwe et al. (1997), Worland et al. (2006)).

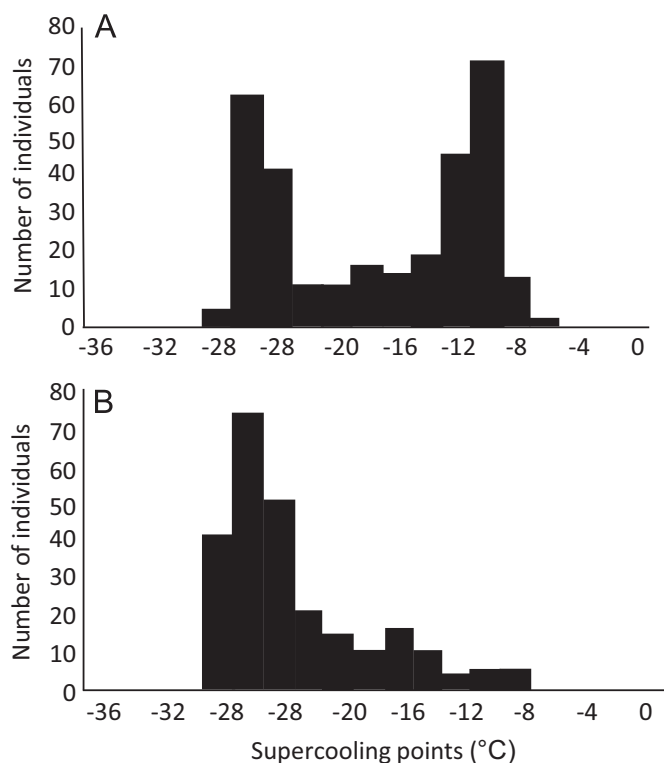
Measuring the SCP is as simple as placing an insect in contact with a thermocouple, and cooling it at a reproducible rate to a temperature at which all of the individuals have frozen. The exotherm released by the latent heat of crystallization is easily detected (Fig. 3). The SCP is defined as the lowest temperature recorded prior to the initiation of the exotherm, the duration and shape of which will depend on the size of the insect. Larger insects (e.g. crickets and caterpillars) have more water, and the freezing process takes longer than in small insects (*Drosophila* and springtails) yielding distinctly different shapes (Fig. 3). In terms of data acquisition, higher sampling rates may be required to capture the SCP of small insects (e.g. springtails) with exotherms that occur rapidly releasing a small amount of heat. In general, a sampling rate of  $< 1\text{ Hz}$  is suitable for larger insects, and (from experience)  $10\text{ Hz}$  should be sufficient for even small insects, insect eggs, Collembola, and mites. If the cooling method results in fluctuations in temperature, or there is a large amount of condensation present, it is sometimes possible to detect artifactual freezing events that are not the result of the insect freezing (Fig. 3C). A simple solution to this is to include a thermocouple without an insect attached, to allow interpretation of any artifacts.

Supercooling points are usually measured on 20–30 individuals, which provides a robust sample size with which to assess the shape of the distribution of SCPs. Although cold-hardy populations of insects often have unimodal SCP distributions, some species (and also microarthropods like springtails and mites) have bimodal SCP distributions (Cannon and Block, 1988; Sinclair et al., 2006a; Fig. 4). Bimodal SCP distributions can arise due to variation in the presence of ice nucleators or differences in body composition. For example, springtails that have recently fed (introducing ice nucleators into the gut leading to a high group of SCPs; Sømme (1982)), and/or molted (removing nucleators; Worland et al. (2006)) have very different SCPs. Starved adult *Alphitobius diaperinus* beetles also had a bimodal SCP distribution, but this was due to a starvation  $\times$  sex interaction such that the SCPs of males decreased with starvation, but the SCPs of females did not (Salin et al., 2000). Although techniques do exist to determine breakpoints in bimodal distributions (e.g. Aldrich (1987)), many authors simply decide on an *a priori* high group–low group divider





**Fig. 3.** Exotherms from insects of different sizes. (A) An exotherm from a small insect, a c. 1.6 mg fruit fly (*Drosophila suzukii*). (B) An exotherm from a larger insect, a c. 153 mg cricket (*Gryllus veletis*). (C) an exotherm from *Gryllus veletis* (135 mg) preceded by an artifact caused by condensation in or on the chamber. All measurements were made using 36 AWG type-T thermocouples.

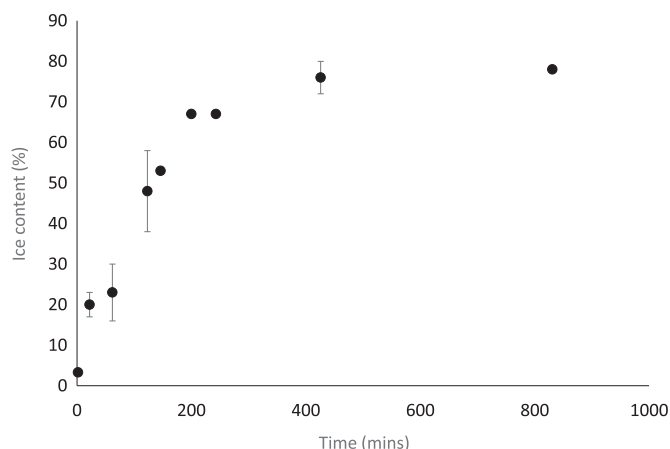


**Fig. 4.** Supercooling point distributions from two species of Antarctic Collembola. (A) A bimodal distribution of supercooling points of *Cryptopygus cisantarcticus* (Isotomidae). (B) A unimodal distribution of supercooling points of *Friesea grisea* (Neonuridae). Redrawn from Sinclair et al. (2006a).

by collating all SCPs and visually assessing a histogram for an obvious break and then compare the frequency, means or medians of the high and low groups among treatments using standard methods (e.g. Sinclair et al. (2006a)). Because supercooling point distributions are seldom normal, and abnormally high or low values from one or two individuals can skew the entire distribution, care must be taken in using parametric statistics when comparing SCPs among samples or treatments. In addition, because larger insects contain more water, they have a higher probability of freezing, and therefore higher SCPs. One approach to account for this potential confounding factor is to incorporate body size (e.g.

as mass) into statistical model (or conduct analyses on residuals of a regression of body size on SCP), demonstrated by Hahn et al. (2008). This may be necessary if (e.g.) dietary treatments yield individuals of markedly different body sizes. An alternate approach (when possible) is to use broadly size-matched individuals for comparison.

Ice formation can take only a few seconds in small animals, particularly at low SCPs. By contrast, freezing can be a slow process in larger insects. For example, the peak of the exotherm in 3–5 g alpine weta, *Hemideina maori* (Orthoptera: Anostostomatidae) occurs approximately 1.5 min after the SCP, but at that point only 5% of the body water is converted into ice. Ice content reached an equilibrium 70–80% after c. 8 h (Fig. 5; Ramløv and Westh, 1993). Because the amount of heat released is proportional to the quantity of water converted to ice, it is tempting to use the exotherm area to estimate ice content. However, a thermocouple does not capture the heat release accurately enough to quantify ice formation from its measurements (or to usefully interpret the highest temperature the thermocouple records), so the amount of ice formed must be measured calorimetrically (e.g. Block et al. (1998), Layne and Blakeley (2002), Ramløv and Westh (1993)).



**Fig. 5.** Time course of ice accumulation in adult *Hemideina maori* held at  $-5^{\circ}\text{C}$ . Redrawn after Ramløv and Westh (1993).

### 3. Cold tolerance strategy

An insect's cold tolerance strategy describes how it survives temperatures where its body fluids might be expected to freeze. **Chill susceptible** species are killed by cold in the absence of internal ice formation (Bale, 1993). Chill susceptibility is sometimes referred to as chilling- or cold-intolerance. For example, the larvae of the false codling moth, *Thaumatotobia leucotreta* (Lepidoptera: Tortricidae), freeze between  $-13^{\circ}\text{C}$  and  $-22^{\circ}\text{C}$  but are killed by brief exposures between  $-8^{\circ}\text{C}$  and  $-12^{\circ}\text{C}$  (Boardman et al., 2012). Chill susceptibility is not a strategy *per se*, but represents the impact of cold on a majority of insects, particularly those that are not at all cold-hardy, and including many insects from the tropics or in temperate areas during the growing season. **Freeze avoidance** (sometimes referred to as freezing-intolerance) refers to species that survive cold in the absence of internal ice formation, but that are killed by any internal ice. Most of these insects maintain their body fluids in a supercooled state (i.e. remain liquid below the melting point) via accumulation of cryoprotectants and prevention of ice nucleation (Salt, 1961). For example, prepupae of the emerald ash borer, *Agrilus planipennis* can survive prolonged exposures to subzero temperatures, provided they do not freeze, and in the winter have SCPs below  $-25^{\circ}\text{C}$  (Crosthwaite et al., 2011). Cryoprotective dehydration is a special case of freeze avoidance, whereby ice formation is prevented via removal of freezable water to environmental ice through a permeable cuticle. This dehydrates the animal, increasing the concentration of solutes to an equilibrium point that is unfreezeable at a given temperature (Holmstrup et al., 2002). For example, larvae of the Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae), overwinter in the soil matrix, which dehydrates them to the point that they cannot freeze (Elnitsky et al., 2008). Finally, **Freeze-tolerant** insects tolerate the formation of ice in their body tissues and fluids. For example, the pre-pupae of the golden rod gall fly *Eurosta solidaginis* (Diptera: Tephritidae) freeze around  $-8^{\circ}\text{C}$  but can survive with more than 60% of their body converted to ice, with mortality beginning below  $-25^{\circ}\text{C}$  (Morrissey and Baust, 1976). Many temperate species switch from one strategy to another at the onset of winter (Salt, 1961), and the presence or absence of external ice can also determine cold tolerance strategy (e.g. Shimada and Riihimaa (1988)).

Defining a species' cold tolerance strategy is essential for interpreting the SCP, and for determining the subsequent approach to measuring lethal temperatures (see Section 4). However, while the cold tolerance strategy helps explain how insects respond to and survive exposure to low temperatures, it does not predict or quantify survival in a particular winter or overwintering site, nor does it designate absolute levels of cold tolerance (Bale, 1993; Sinclair, 1999). For example, in temperate southwestern Ontario, Canada, winter temperatures regularly drop below  $-25^{\circ}\text{C}$ , yet freeze avoidant (e.g. Crosthwaite et al., 2011), freeze-tolerant (e.g. Marshall and Sinclair (2011)) and chill-susceptible (Udaka and Sinclair, 2014) species are all abundant. Thus, cold tolerance strategy is not a substitute for understanding lethal limits, and cannot be used on its own to predict survival. Despite its inability to predict cold tolerance, the cold tolerance strategy can indicate potential prediction and control methods (see Leather et al. (1993), for a comprehensive review). For example, freeze-avoidant species may be susceptible to conditions that promote ice formation, such as the application of ice nucleators (e.g. Fields (1993), Lee et al. (1994)).

#### 3.1. Determining cold tolerance strategy

Cold tolerance strategy is best inferred from two pieces of information gathered from the same individual during a cold

**Table 1**

Cold tolerance strategies of insects as determined by survival of internal ice formation. Chill-susceptible insects die of injuries unrelated to freezing, freeze-avoidant insects die upon internal ice formation, and freeze-tolerant species are able to survive internal ice formation.

	Internal ice formed	No internal ice formed
<b>Alive</b>	Freeze-tolerant	Chill-susceptible or freeze-avoidant
<b>Dead</b>	Chill-susceptible or freeze-avoidant	Chill-susceptible

exposure: (1) whether or not the insect froze, and (2) whether or not it survived (Table 1; Salt, 1961). Cold tolerance strategy can be determined with a small number of individuals cooled, in contact with thermocouples, to a temperature close to the SCP. By re-warming all of the individuals once half of them have frozen (evident from exotherms), it is possible to generate a population of insects that includes individuals that did and did not freeze, but that were all exposed to the same low temperature. The cold tolerance strategy can then be determined from the survival of these individuals (Tables 1 and 2; see Section 4.1 for a discussion of survival metrics).

Many studies (e.g. Bemani et al. (2012), Slabber and Chown (2004)) derive cold tolerance strategy from separate measurements of SCP and lower lethal temperature (reasoning that  $LT_{50} < \text{SCP} = \text{freeze tolerant}$ ;  $LT_{50} = \text{SCP} = \text{freeze avoidant}$ ; and  $LT_{50} > \text{SCP} = \text{chill susceptible}$ ). While this indirect method is suitable if there are very large differences between SCP and LLT (e.g. *Drosophila melanogaster* dies at approximately  $-5^{\circ}\text{C}$ , but has an SCP below  $-15^{\circ}\text{C}$  (Czajka and Lee, 1990)), it is imprecise if SCP and LLT are in close proximity. In particular, it can be difficult to distinguish between freeze avoidance and chill-susceptibility if the latter insects are cold-hardy.

Ice formation is a slow process, particularly in larger insects (Fig. 5). Thus, survival of brief exposures below the SCP may not reflect survival of 'complete' freezing (i.e. freezing to an equilibrium ice content). While it is possible to measure the ice content of insects (e.g. Block et al. (1998), Layne and Blakeley (2002), Ramlöv and Westh (1993)), holding insects below the SCP for a longer period (e.g. overnight) can indicate whether the animal is tolerant of equilibrium ice content (Dennis et al., 2015; Sinclair et al., 1999). Insects that do not survive complete ice formation (termed 'partially freeze tolerant' by Sinclair (1999)) may nevertheless use freeze tolerance as a component of their cold tolerance strategy, depending on the environmental conditions, particularly if sub-freezing conditions are brief (e.g. Sømme et al. (1996), Sømme and Zachariassen (1981)), although this has not been well-explored. Voituron et al. (2002) hypothesized that this partial freeze tolerance may be an important intermediate step in the evolution of freeze tolerance. Similarly, although some insects may survive short periods of acute cold exposure if they remain unfrozen, freeze-avoidant insects must remain unfrozen when supercooled for long periods (Sømme, 1996). The ability to remain supercooled is easily tested by cooling insects to close to the SCP, and holding them at that temperature, for example, overnight (e.g. Crosthwaite et al. (2011)).

Some species of insect tolerate freezing only if ice formation is initiated (inoculated) at relatively high subzero temperatures (Lee, 2010). For example, larvae of *Chymomyza costata* (Diptera: Drosophilidae) were initially classified as freeze-avoidant, but if freezing is inoculated by contact with ice crystals, then diapausing larvae will survive below  $-80^{\circ}\text{C}$  (Shimada and Riihimaa, 1988). This is most relevant to species expected to overwinter in moist conditions, and ice formation can be inoculated by placing an animal in contact with a moist substrate (e.g. Boardman et al.

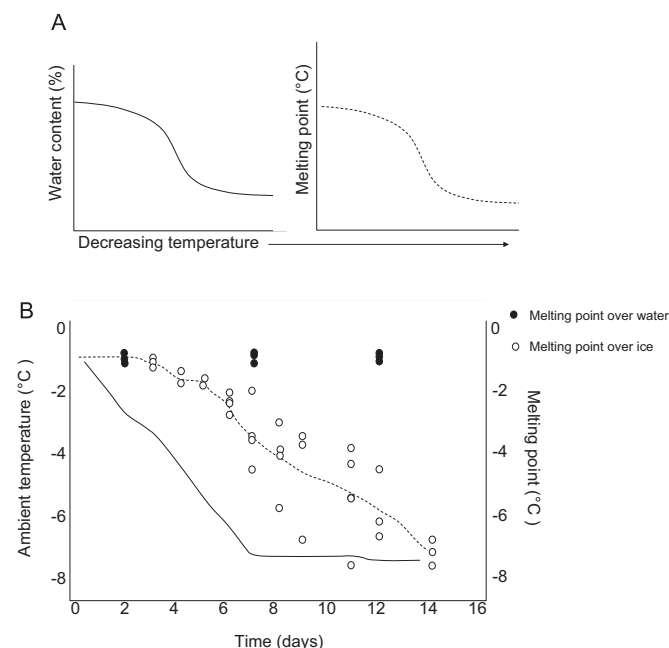
**Table 2**

Cold tolerance strategies of insects as determined by survival following exposure to the supercooling point. When half of the insects have reached their supercooling point during cooling, all insects are returned to warm conditions to monitor survival. This provides a group of insects that experienced freezing, and a group that did not, while maintaining exposure to approximately the same low temperature. Survival following freezing indicates freeze-tolerance, whereas survival until freezing is reached indicates freeze-avoidance. Mortality before the supercooling point is reached indicates chill-susceptibility. *Drosophila suzukii*: Jakobs et al. (2015); *Reticulitermes flavipes* (a termite): Clarke et al. (2013); *Perisphaeria* sp. (a cockroach): Sinclair and Chown (2005).

Species	<i>Drosophila suzukii</i>		<i>Reticulitermes flavipes</i>		<i>Perisphaeria</i> sp.	
Physiological state	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen
Survival	0%	0%	100%	0%	100%	100%
Strategy	Chill-susceptible		Freeze-avoidant		Freeze-tolerant	

(2012); Košťál and Havelka (2000)), applying ice (e.g. Layne et al. (1990)), or applying a known ice nucleator, such as silver iodide (e.g. Strachan et al. (2011)). Cold tolerance strategy can then be assessed as described above.

To identify cryoprotective dehydration, insects need to be exposed to low temperatures in the presence of (but not actually in contact with) ice. In the field, this occurs in frozen soil or moss (Holmstrup, 2014), and the most common laboratory method is to confine insects in a mesh or perforated container within a larger sealed container that contains crushed ice (e.g. Elnitsky et al. (2008), Sørensen and Holmstrup (2011), Udaka and Sinclair (2014)), which is exposed to cold, usually in an incubator or other large chamber. The hallmarks of cryoprotective dehydration are a decrease in water content (usually measured gravimetrically), such that the concentration of solutes in the body fluids increases to yield a melting point approximately equivalent to the ambient temperature, rendering the insect essentially unfreezable (Fig. 6; see Holmstrup (2014) for an extensive review). Simultaneously, the insect may also accumulate low molecular weight cryo/anydroprotectants, such as trehalose. Thus, insects using cryoprotective dehydration will have both reduced water content and depressed supercooling points, and these should decrease in concert with decreasing temperature.



**Fig. 6.** Hallmarks of cryoprotective dehydration in insects. (A) Characteristically, insect body water content and the melting point of the insect will decrease as the ambient temperature decreases. Redrawn from Sinclair et al. (2003b). (B) In the collembolan *Onychiurus arcticus*, the melting point of the body fluids does not change over time if the insect is held over water, whereas the melting point decreases over time and mimics that of the ambient temperature when the insect is held over ice. The dotted line represents the average melting point over ice and the solid line represents the ambient temperature. Redrawn from Holmstrup et al. (2002).

Cold tolerance strategy is not necessarily fixed. First, many species only express their winter-relevant cold-tolerance strategy after appropriate seasonal cues. For example, *Eurosta solidaginis* is chill-susceptible in the summer, but freeze-tolerant in the winter (Lee and Hankison, 2003), and *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae) is chill-susceptible in the summer, but freeze-avoidant in the winter (Košťál and Simek, 2000). Second, there may be variation in cold tolerance strategy among populations, for example *Pieris rapae* (Lepidoptera Pieridae) is freeze-avoidant in northern Europe, but freeze tolerant in central Siberia (Li and Zachariassen, 2007). Third, some species may switch strategy entirely. For example, overwintering larvae of the beetle *Dendroides canadensis*, (Coleoptera: Pyrochroidae) switched from freeze tolerant in the winter of 1978–1979 to freeze avoidant in 1981–1982 (Horwath and Duman, 1984). We will discuss approaches used to elicit this plasticity in Section 6.

#### 4. Lower lethal limits

Lower lethal limits quantify the temperatures that kill an insect population or species under specified conditions. Lethal temperatures are usually expressed as a proportional lethal temperature ( $LT_x$ ), the point where  $x\%$  of exposed individuals die. For example, the  $LT_{50}$  is the median lethal temperature, expected to kill 50% of a population. The Lower Lethal Temperature (LLT) is the temperature at which all individuals are killed (the  $LT_{100}$ ; functionally, this is often expressed as the highest temperature at which measured survival was zero). The lowest temperature at which no individuals are killed has been called the Upper Limit of the Cold Injury Zone (ULCIZ; Nedvéd (1998)). Note that any assessment of the lower lethal temperature is dependent also on the exposure time (see Section 4.1).

The LLT estimates an absolute limit to low temperature survival and, as such, can provide information about likelihood of survival under a given set of conditions (e.g. Hatherly et al. (2005)), and allow survival to be compared under circumstances where the SCP is uninformative about survival (e.g. for chill-susceptible or freeze-tolerant species; Baust and Rojas, 1985). Other thresholds, such as the  $LT_{90}$ , indicate thresholds associated with specified levels of probability, and are therefore useful for assessing the risk of mortality or survival under a given set of conditions. Used in conjunction with the supercooling point, lower lethal temperatures can be used to indirectly estimate cold tolerance strategy (see Section 3.1). However, estimates of LLT are sensitive to exposure conditions, especially cooling and rewarming rates. For example, survival of *Chymomyza costata* larvae exposed to  $-40^\circ\text{C}$  decreases from  $>90\%$  to zero when the cooling rate is increased from 0.1 to  $1^\circ\text{C}/\text{min}$  (Shimada and Riihimaa, 1988). Thus, if the laboratory conditions do not reflect the salient features of the conditions in nature, laboratory-derived LLT estimates may over- or under-estimate the probability of mortality in the field.

#### 4.1. Estimating lower lethal limits

Lower lethal limits are usually determined by exposing individual insects (or more commonly, groups of individuals) to a pre-determined range of test temperatures, each for the same fixed period of time. Approximately five temperatures spanning the range of 0 to 100% mortality are usually sufficient to determine the  $LT_{50}$ , although additional temperatures are sometimes added to obtain a finer-scale estimate of  $LT_{50}$ . After cold exposure, insects are returned to recovery conditions (e.g. room temperature or the rearing temperature), and allowed to recover before survival is assessed.

The choice of exposure time is dictated by both the temperatures encountered in nature, and convenience. Lower lethal temperature estimates that examine responses to short cold exposures (1–2 h; e.g. Clarke et al. (2013)) estimate survival of an acute, transient extreme temperature. In many habitats, these might reflect the minimum temperature experienced over a winter. However, a case can be made for longer exposures, such as overnight treatments, that may be more ecologically-relevant (Sømme, 1996). Ideally these measurements (or assessment of the risk of survival) will be interpreted in the context of known overwintering or exposure conditions, such as temperature records from microclimate monitoring (e.g. Udaka and Sinclair (2014)). A similar approach can be used to measure high temperature tolerance (ULT) and lethal time at a low temperature (LLT). The latter is particularly relevant for risk assessment for insects that may be exposed to prolonged cold during refrigerated shipping (e.g. Beaudry et al. (1998)) or while overwintering under snow (Pauli et al., 2013), or to extensive periods below freezing (Sømme, 1996). To estimate LLT, a similar approach to LLT is taken, but with exposures to a set temperature across a range of pre-determined times. Ultimately, LLT and LLT are both important information: for most species, thermal tolerance is a temperature–time surface (see Nedv d (1998), Nedv d et al. (1998)), which has seldom been fully parameterized.

Determining LLT is, by necessity, an iterative process. Ideally, survival metrics, SCP, and cold tolerance strategy have already been determined, and can help to determine a starting point, with the goal of anchoring the analysis with exposures to temperatures that yield 0% and 100% survival, with additional replicates to fill the intervening space (e.g. Udaka and Sinclair (2014)). An alternative approach is to expose groups of insects to a pre-determined set of temperatures (e.g. at 5 °C intervals; Bouchard et al. (2006), Kleynhans et al. (2014)). This approach has advantages in design and execution; in particular, it can allow random assignment of insects to treatments, allow all of the exposures to be made on a single day, and is more suitable for using longer-term measures of survival.

Assessing mortality after cold exposures requires a consistent (and ecologically-relevant) estimate of whether or not an insect is alive, and an appropriate time period to capture mortality caused by cold, but exclude mortality from other causes. Ideally, survival to reproduction indicates that an insect has retained fitness after cold exposure (Baust and Rojas, 1985), but this is surprisingly rare as a metric. Survival to the reproductive stage for immature insects (i.e. successful eclosion as an adult) is an ecologically-relevant estimate of mortality that is more common (e.g. Strachan et al. (2011), Williams et al. (2014)). However, for species that do poorly in captivity, those with long life cycles, or because of pressure to obtain data, most researchers choose simple metrics of survival that can be rapidly assessed. These metrics are often a coordinated movement (standing or a righting response, e.g. MacMillan and Sinclair (2011b), Nyamukondiwa et al. (2011), Tomcala et al. (2006)), although resumption of feeding, or a more ‘athletic’ ability (running, jumping, or flying) would also be appropriate. More

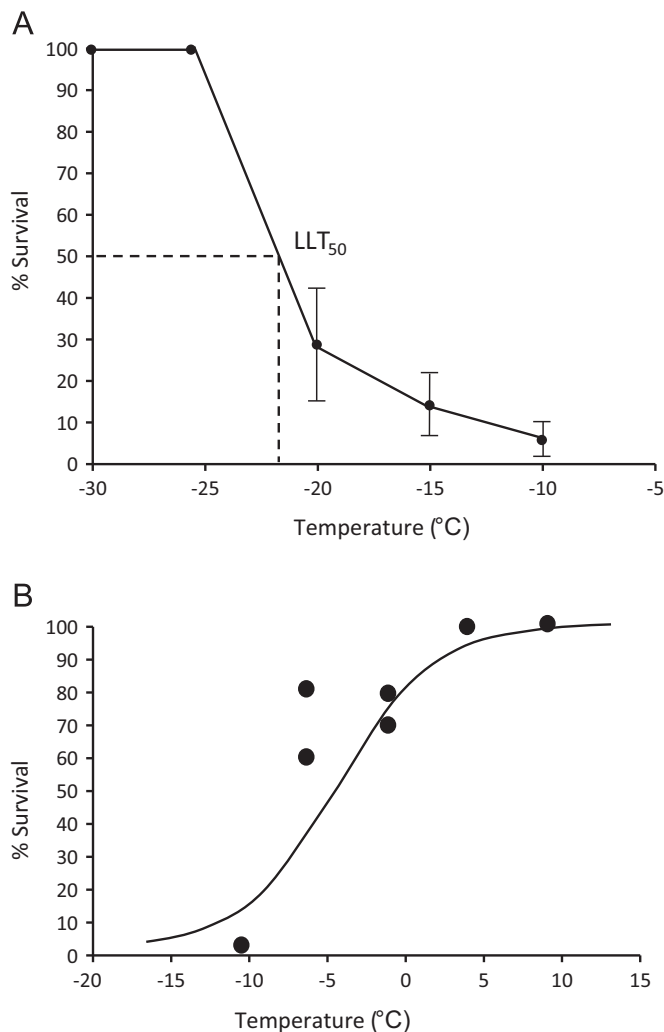
simple measures can be problematic, as they can lack an assessment of biologically-relevant performance. For example, cold-exposed insects can be injured and able to move in an uncoordinated fashion, but unable to perform more complex functions. Such individuals have been characterized as ‘injured’ (Ko  t l et al., 2006; MacMillan and Sinclair, 2011b) or ‘moribund’ (Sinclair, 1997). These individuals are usually considered ‘dead’ in analyses on the basis that since they could not reproduce, their fitness is zero.

Mortality is usually assessed within the first 24 h to a few days after cold exposure. Thus, lethal temperature estimates may ignore mortality that occurs in the longer term, and also disregard sub-lethal effects on behavior or reproductive capacity (Baust and Rojas, 1985). For example, adults of the freeze-tolerant carabid *Pterostichus brevicornis* will survive for a few days when frozen and thawed under sub-optimal conditions, but die after a week (Miller, 1969). By contrast, it is sometimes possible to significantly reduce the recovery period needed for a survival assessment. For example, Udaka and Sinclair (2014) found no difference in survival estimates of cold-exposed *Curculio glandium* (Coleoptera: Curculionidae) larvae made 15 min and 24 h after rewarming. Choosing a timeframe and metric for assessment of mortality will thus depend on the goal of the study; when assessing potential for overwintering survival, longer-term survival and reproductive-capacity measures may be most appropriate.

Lower lethal temperature experiments are usually conducted with sequential exposure of different groups of individuals to a range of temperatures (e.g. Bouchard et al. (2006); Fig. 7). The response variable is percent or proportion mortality (or survival, for the optimistic). Thus, the unit of replication in an LLT study is a group of individuals, not the individuals themselves; five groups of ten individuals gives  $n=5$ , whereas one group of fifty (the same number of animals) gives  $n=1$ . Because survival is expressed as a proportion, groups of fewer than five are less useful than larger groups, but the balance between resolution and replication usually leads most researchers to use groups of 5–10. There are statistical power advantages to treating each individual’s survival separately (using, for example, survival or failure time analyses, e.g. Jakobs et al. (2015)), however, researchers rarely take this approach, perhaps because most exposures are conducted in groups. Even if individuals are individually housed, a single exposure treatment should probably be considered a group, to avoid pseudoreplication (McArdle, 1996). When using multiple experimental groups of individuals (e.g. 3–5) at each temperature, 3–5 independent LLT curves do not need to be generated; instead, the order of exposure temperatures can be randomized to yield replication, and all the data considered in the same model. Because lethal limits are estimated using a regression approach, it is not necessary to expose insects to precisely determined temperatures; it is sufficient to have an accurate measurement of the temperature to which each group was exposed.

Lethal temperatures are often estimated graphically by plotting the survival proportions by temperature and ‘eyeballing’ the lethal limits (Fig. 7A). However, a formal statistical analysis of survival is relatively simple, and can yield values for  $LT_{50}$  and LLT (and any other level of mortality), and also variance or confidence intervals around those estimates. Because survival has a binomial error distribution (individuals are either alive or dead), linear models, such as ANOVA, are inappropriate for determining LLT (although ANOVA may be suitable for comparing among models, particularly in R, where this is a common approach – e.g. Crawley (2005)). A logistic regression (Fig. 7B) with logit link and binomial error distribution (i.e. a probit analysis) is, *a priori*, most appropriate (e.g. B r gi and Mills (2010), Sinclair (1997)). Alternative forms of survival analysis (e.g. Ransberry et al. (2011)) are inappropriate for LLT analyses, because they assume that an individual is followed throughout an exposure until it dies – clearly this is not the case when the individual has to be rewarmed to ascertain survival.





**Fig. 7.** Determining lethal temperatures. (A) Estimate of the lower lethal temperature of *Diamesa mendotae* based on survival of subzero temperatures. Flies were cooled to subzero temperatures and immediately re-warmed upon reaching the designated temperature. Survival was recorded 24 h following exposure. The lower lethal temperature was estimated to be  $-21.5^{\circ}\text{C}$ . Redrawn from Bouchard et al. (2006). (B) Survival of *Eldana saccharina* larvae exposed to low temperatures. Larvae were directly exposed to temperatures ranging from  $10^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for 2 h. Solid line represents the fitted logistic regression model. Re-drawn from Kleynhans et al. (2014).

## 5. Chill coma

When cooled, a majority of insects will slow down and eventually lose the ability to move. At a specific temperature, an insect will cross a threshold (the critical thermal minimum,  $\text{CT}_{\min}$ ), into a reversible state of paralysis, known as chill coma. (Hazell and Bale, 2011). Because insects cannot move, feed, reproduce, or evade predators while in chill coma, the  $\text{CT}_{\min}$  provides a useful lower limit to insect function (Andersen et al., 2015b; David et al., 1998; Gibert et al., 2001; MacMillan and Sinclair, 2011a), and is often used to approximate insect cold tolerance because it is broadly correlated to lethal limits (Andersen et al., 2015b). Resumption of movement after rewarming (chill coma recovery, CCR) is usually measured as the amount of time it takes insects to recover from a standardised period in chill coma (David et al., 1998; Gibert et al., 2001; MacMillan and Sinclair, 2011a). Additionally, because chill coma is usually non-lethal, it is possible to use insects for further experiments, or even breed from them, allowing for selection experiments (e.g. Bertoli et al. (2010); Franke et al. (2012), Mori and Kimura (2008), Telonis-Scott et al., 2009).

Although insects may accumulate injuries (or die) while in chill coma (MacMillan and Sinclair, 2011a),  $\text{CT}_{\min}$  and CCR are not measures of cold tolerance, but rather of resistance to the effects of cold (although it provides a useful estimate of thermal biology for comparative purposes; Andersen et al. (2015b)).  $\text{CT}_{\min}$  is also limited as a measurement of resistance to cold, because it can only be measured in mobile life stages; specifically, monitoring when an insect enters chill coma requires identifying and timing the loss or recovery of some form of movement. Some insects become immobile when disturbed (e.g. some species of stick insects and beetles), making it difficult to determine the  $\text{CT}_{\min}$ . Finally, not all species have a  $\text{CT}_{\min}$ . For example, freezing occurs before chill coma in the freeze-avoidant Antarctic springtail, *Isotoma klovstadi* (Sinclair et al., 2006a) and in some freeze-tolerant cockroaches (Sinclair and Chown, 2005).

### 5.1. Chill coma onset ( $\text{CT}_{\min}$ )

The  $\text{CT}_{\min}$  is the most common measure of the threshold at which chill coma onset (CCO) occurs. A similar measure of maintenance of performance at low temperatures, knockdown time, could be used to determine the amount of time at a predetermined temperature after which coordination is lost (cf. Hoffmann et al. (1997)). However, it is unclear whether these two approaches measure the same physiological phenomenon (MacMillan and Sinclair, 2011a), so  $\text{CT}_{\min}$  and knockdown time should not be used interchangeably. Because most literature currently uses  $\text{CT}_{\min}$  we suggest that this provides the measure of chill coma onset that is most readily comparable among studies (and is the approach we will discuss further below), assuming assessments have been made under comparable conditions.

To measure the  $\text{CT}_{\min}$ , the point at which an insect loses its ability to move needs to be identified. This could be through direct observation during cooling (e.g. Klok and Chown (1997)), or through identification of failure to remain in a chamber, or on a perch (e.g. Huey et al. (1992)). These observations need to be coupled to a measurement of temperature in the chamber (or even at the surface of the insect). When direct observations are being made, an ideal response will be repeatable among researchers and individual animals, involuntary, and easily discerned, although stimulus is often necessary at low temperatures because insects move more slowly (MacMillan and Sinclair, 2011a). Motor responses include the righting response (standing after being placed on back or knocked over; e.g. David et al. (1998)), response to a stimulus such as prodding with a probe (e.g. Klok and Chown (1997)), or coordinated standing (e.g. Košťál et al. (2004)); an appropriate species-specific response can be identified via a pilot study. We note (from experience) that some species are simply not tractable for  $\text{CT}_{\min}$  studies; for example, stick insects are immobile when threatened – this is it hard to tell if a stick insect is not moving because it cannot move, or because it will not move.

The cooling rate used for  $\text{CT}_{\min}$  measurement is a balance between being slow enough to ensure that the insect's body temperature does not lag behind the chamber (leading to inaccurate estimation of  $\text{CT}_{\min}$ ), and being fast enough to avoid substantial physiological (e.g. acclimation) responses during cooling (Huey et al., 1992). Thus, most  $\text{CT}_{\min}$  protocols require less than 2 h to complete the entire assay. Most studies use cooling rates between  $0.1$  and  $0.25^{\circ}\text{C}/\text{min}$  for determining  $\text{CT}_{\min}$  (e.g. Kleynhans et al. (2014), MacMillan and Sinclair (2011b), Terblanche et al. (2006)). Note that there has been considerable recent debate on the 'correct' rate for measuring high temperature tolerances (Rezende et al., 2011; Santos et al., 2011; Terblanche et al., 2007; Terblanche et al., 2011), and it is therefore imperative to clearly report the conditions when describing  $\text{CT}_{\min}$  experiments. To expedite measurements, it should be possible to rapidly reduce the temperature

from the rearing temperature to an intermediate temperature (e.g. 10 °C), allowing equilibration before cooling at the pre-determined rate. To our knowledge, the consequences of different equilibration times in such protocols have not been explored.

A variety of methods can be used to cool individual insects for manual  $CT_{min}$  determination, including controlled-temperature incubators (e.g. Hu and Appel, 2004), immersing insects in tubes or containers in the bath of a refrigerated circulator (e.g. Klok and Chown (1997)), or using purpose-built chambers cooled by fluid circulated from refrigerated circulators (e.g. MacMillan and Sinclair (2011b), Sinclair et al. (2006b)). Higher throughput has been achieved by placing a population of insects into a column with baffles/perches (Huey et al., 1992; Ransberry et al., 2011; Renault and Lalouette, 2012). The insects lose coordination at the  $CT_{min}$  as the column is cooled, and can be collected at intervals (or counted electronically; e.g. Shuman et al. (1996)) as they fall out the bottom of the column, with each interval representing individuals that entered chill coma over a specific time period.

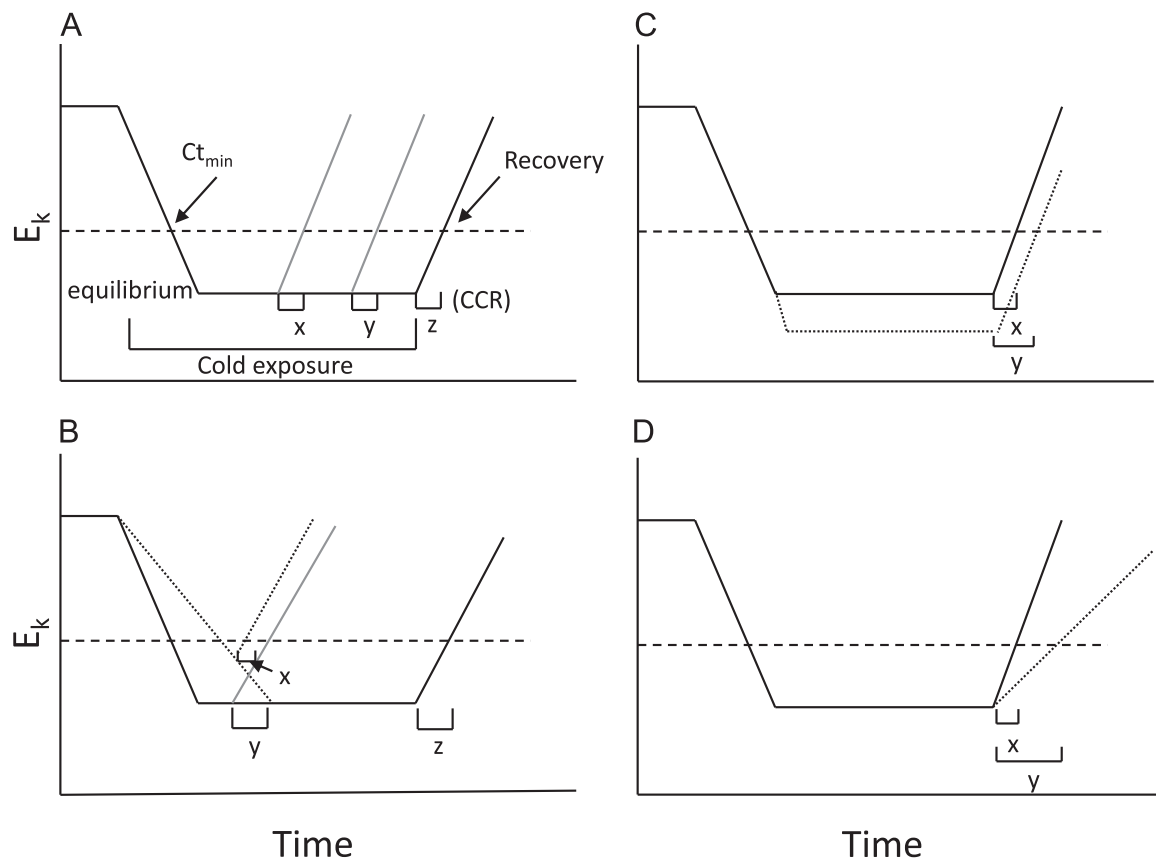
Other methods to observe the  $CT_{min}$  have also been explored. Renault et al. (1999) used a microbalance to detect activity during declining temperature to identify the  $CT_{min}$  in a beetle. Similarly, Hazell et al. (2008) describe making a video recording as an insect is cooled, and then extracting from the video the point at which the last voluntary movement was made (Everatt et al. (2013) describe a means to automate this measurement). Alternately, physiological measurements may be plausibly developed that allows the  $CT_{min}$  to be identified from a physiological threshold. Such a measure could use heart function (Andersen et al., 2015a), metabolic rate (Lighton and Turner, 2004; Sinclair et al., 2004), or automated activity monitoring (MacMillan et al., 2012a), although most of these approaches are low-throughput.

Individual- or group-based studies of insect  $CT_{min}$  usually use 10–20 individuals per treatment (e.g. Clarke et al. (2013), Jumbam et al. (2008), Klok and Chown (1997), Renault and Lalouette (2012)), but the knockdown column approach allows for larger sample sizes (as many as 263 flies in Ransberry et al. (2011)). Critical thermal minima can be compared among treatments by treating each individual as a replicate, and using conventional statistics (e.g. ANOVA; Klok and Chown (1997)), or non-linear approaches such as Generalized Linear Models, if not normally distributed. Critical thermal minima may also satisfy the assumptions of survival analyses such as Accelerated Failure Time models (e.g. Ransberry et al. (2011)).

## 5.2. Chill coma recovery

Because chill coma is reversible and movement is easy to detect in mobile insects, recovery from chill coma is also used as a measure of cold tolerance. Although some authors have measured this as a temperature at which movement reinitiates (e.g. Sinclair et al. (2006a)), current research suggests that chill coma recovery is a complex interplay between the rate of loss of homeostasis, the low temperature equilibrium attained, and the rate and threshold for recovery (Fig. 8). Thus, the most-used metric tends to be time until recovery under standardised conditions (Chill Coma Recovery time; CCRT), since this minimizes confounding variables (e.g. confounding the rate of recovery versus a temperature threshold for recovery). Here we will therefore discuss only CCRT, although we note that although it is simple to measure under standardised conditions, it is difficult to interpret in ecological context.

To measure CCRT, insects are exposed to a temperature–time combination that induces chill coma, and returned to a recovery



**Fig. 8.** Variation in chill-coma recovery time depending on entry (A, B) and exit (C, D) from chill coma. (A) CCRT of insects x, y, and z are equal, because equilibrium is reached during time spent in chill coma. (B) CCRT of x is less than y (and z), because the rate of entry into chill coma did not allow for equilibrium to be reached before recovery began. (C) CCRT of y is greater than x, despite the same time spent in chill coma, because the equilibrium of y is lower than that of x. (D) The CCRT of y is greater than x because recovery occurs at a slower rate.  $E_k$  = membrane equilibrium potential of  $K^+$ .

temperature (often the rather vague ‘room temperature’ – we recommend at least reporting and measuring that temperature). The insects are observed, and the time taken to resume a pre-determined behavior or activity is recorded. Movements and behaviors include coordinated leg movement (Halle et al., 2015), standing or walking (e.g. Macdonald et al. (2004)), righting response, or abdominal contractions (e.g. Macmillan et al. (2012b)). It should be possible to use video analysis to automate CCRT (e.g. by modifying the method described by Hazell et al. (2008) for  $CT_{min}$ ). Insects can be cold-exposed via refrigerated baths or incubators (see Section 2.1); however, because many insects enter chill coma around 0 °C, an ice–water slurry is often sufficient (e.g. Gibert et al., 2001; Nilson et al., 2006; Ransberry et al., 2011). Recovery can be observed in any clear container; a convenient approach for smaller insects, such as *Drosophila* is to transfer the insect to 6- or 12-well cell culture plates, which allows multiple individuals to be observed simultaneously. The recovery temperature will likely influence the CCRT, however, this has not been well-explored (Macmillan et al., 2012b).

Ideally, cold exposures for CCRT are convenient (e.g. overnight, or within the span of a working day), and yield chill coma recovery times within a convenient range (we suggest 20–30 min). It is important that the resolution of observation is compatible with the expected variation in CCR. For example, the difference between 15 and 20 min (a 33% increase) is easily distinguished, but the difference between 3 and 4 min may not be if each individual is observed at 30 s intervals. There is often a bi-phasic relationship between exposure time (or temperature) and CCRT (David et al., 2003; Macdonald et al., 2004; Macmillan et al., 2012b), and in the plateau region (which may be narrow or broad), the loss of ion and water balance is putatively at an equilibrium (Macmillan and Sinclair, 2011a; Macmillan et al., 2012b); this ensures that differences among individuals, treatments or populations are primarily due to variation in the recovery processes (Fig. 8). Thus, comparing CCR across a combination of temperatures and times is a useful first step to ensure that the exposure conditions used to induce chill coma result in a recovery time that is convenient, appropriate for distinguishing between groups, and within the plateau region.

Because of its simplicity, sample sizes for CCRT can be larger than those for  $CT_{min}$ , typically 10–20 individuals/treatment (e.g. Andersen et al. (2015b), David et al. (2003), Findsen et al. (2013), Gibert et al. (2001), Macdonald et al. (2004), Ransberry et al. (2011)). Because only a limited number of individuals can be observed at a time (typically < 20, if the observation interval is 20–30 s), we recommend that the treatments in each observation set be randomised. Analysis approaches mirror those of  $CT_{min}$ , although because the CCRT has a lower bound (an insect cannot take a negative time to recover), survival analyses or generalized linear models might be more appropriate than linear parametric statistics such as ANOVA. Chill coma recovery time is often compared among treatments measured together as a way to examine relative differences; however, because of the diversity of exposure temperatures and times (Table 3), recovery metric and recovery temperatures, we recommend caution when comparing CCRT among species, studies, and laboratories.

## 6. Detecting variation in low temperature performance among individuals, populations, seasons and treatments

Phenotypic plasticity is the ability of an organism with a given genotype to express different phenotypes. Phenotypic plasticity in thermal biology includes hardening, acclimation, which occurs in the laboratory, and acclimatization, which happens in nature, although the mechanisms of acclimation and acclimatization are assumed to be similar (Kingsolver, 2009; Tattersall et al., 2012), but different to the mechanisms underlying hardening (Sinclair and Roberts, 2005; Teets and Denlinger, 2013). Developmental

**Table 3**

Examples of time and temperature treatments for measuring chill coma recovery. Time and temperature exposures often vary widely between studies and labs, and will depend on the low-temperature tolerance of the species of interest.

Study	Study species	Time (h)	Temperature
Andersen et al. (2015b)	<i>Drosophila</i> spp.	2	– 2 °C
Findsen et al. (2013)	<i>Locusta migratoria</i>	2	– 4 °C
Ransberry et al. (2011)	<i>Drosophila melanogaster</i>	6	0 °C
Coello Alvarado et al. (2015)	<i>Gryllus veletis</i> and <i>Gryllus pennsylvanicus</i>	12	0 °C
David et al. (2003)	<i>Drosophila subobscura</i> (four populations)	16	– 7 °C to + 2 °C

plasticity is the response to conditions experienced during development (e.g. Sisodia and Singh (2010)), while maternal (or trans-generational) effects refer to epigenetic signals passed from the mother to offspring that determine phenotype (e.g. Magiafoglou and Hoffmann (2003)). The time scale of phenotypic plasticity responses can range from minutes or hours (often termed ‘hardening’ responses) to long-term shifts, such as seasonal changes in cold tolerance (Fischer and Karl, 2010; Sinclair and Roberts, 2005). Genetic adaptation is plasticity on an evolutionary scale, and may play out among populations or species (Gibert and Huey, 2001; Kellermann et al., 2009; Sinclair et al., 2012).

All of these levels of variation in physiology can dramatically alter cold tolerance; thus, one way to account for this variation is to attempt to induce as much plasticity in cold tolerance as possible. Although this approach may not reveal the absolute limits of cold tolerance (and cannot determine the adaptive significance of the plasticity), it can at least indicate the presence of plasticity, and put a study into the context of a species’ plasticity. Here we define and explain the major sources of plasticity in insect low temperature performance, and discuss approaches that can be used to detect this plasticity.

### 6.1. Phenotypic plasticity of low temperature biology in nature

Phenotypic plasticity of cold tolerance is readily observed in populations of insects in nature at multiple timescales, most commonly as seasonal changes in cold tolerance. For example, field-collected larvae of the freeze-avoidant codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) are more cold-tolerant during the winter than in the summer (Khani and Moharramipour, 2010). Similarly, emerald ash borer have depressed supercooling points in winter (Crosthwaite et al., 2011), and freeze-tolerant alpine cockroaches have a lower  $LT_{50}$  in winter (Sinclair, 1997), both of which enhance overwintering survival.

In addition to seasonal variation in cold tolerance, there is also evidence of phenotypic plasticity over shorter timescales in nature. For example, survival of cold exposure increases at night and decreases during the day in field-caged *Drosophila melanogaster* (Overgaard and Sørensen, 2008). Similarly, the supercooling point distribution of field-collected Antarctic springtails *Desoria klovstadi* (Collembola: Isotomidae) decreases markedly between mid-day and midnight, reflecting changes in ambient temperature (Sinclair et al., 2003c). Thus, it is important to consider these natural variations in cold tolerance when determining the overwintering potential of an insect.

### 6.2. Rapid cold-hardening

Rapid cold-hardening (RCH) is the shortest time-scale of plastic responses, and describes enhanced survival of temperature extremes after a brief (minutes to c. 3 h) pre-exposure to sub-lethal

temperatures (reviewed by Teets and Denlinger, 2013). Rapid cold hardening can affect a range of cold tolerance parameters; for example, by decreasing LLT (Lee et al., 1987),  $CT_{min}$  and CCRT (Ransberry et al., 2011), and even modifying the SCP in some cases (e.g. Worland and Convey (2001)). Thus, RCH rapidly shifts insects from a non-cold-hardy to a cold-hardy state in life stages that are not normally cold-tolerant, or at times of year when seasonal cold tolerance has not yet been acquired. Because of this rapid shift in cold tolerance, parameters like LLT may be substantially underestimated if RCH is not taken into account. For example, non-diapausing adults of the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae), have only 15% survival after 1 h at  $-7^{\circ}C$ , but survival increases to 90% if the  $-7^{\circ}C$  exposure is preceded by 4 h at  $0^{\circ}C$  (Lee et al., 1987). Rapid cold-hardening is mainly expressed in chill-susceptible species, but has been reported in freeze-tolerant and freeze-avoidant species as well (Lee and Denlinger, 2010).

RCH may be induced by slow cooling, diurnal cycles and mild low temperatures (usually  $-2$  to  $+4^{\circ}C$ ) (Teets and Denlinger, 2013). Unlike the heat shock response (see Harrison et al. (2012)), RCH does not normally require a recovery period after the pre-exposure to express the enhanced cold tolerance. Nevertheless, including a recovery period is commonly incorporated in laboratory assessments of RCH (e.g. Jakobs et al. (2015), Nunamaker (1993), Sinclair and Chown (2003)). Because the mechanisms of RCH are still poorly understood (Teets and Denlinger, 2013), it is not entirely clear whether these different treatments eliciting 'RCH' are actually triggering the same physiological response, so it is important to report the methods fully, and potentially include several different treatments in a study (e.g. Nunamaker, 1993).

### 6.3. Acclimation, deacclimation and acclimatization

Cold acclimation and acclimatization are processes that improve cold-hardiness over a span of days-to-weeks, within a single life stage. Acclimation can have a marked effect on cold tolerance; for example, a six-week cold acclimation increased survival of the rusty grain beetle, *Cryptolestes ferrugineus*, from 1.4 days to 24 days at  $-10^{\circ}C$ . Cold-acclimation regimes may be based on measured field conditions (e.g. Jakobs et al. (2015)), or based on convenience (e.g. the  $4^{\circ}C$  of many domestic and laboratory refrigerators). Notably, acclimation under fluctuating temperatures usually leads to greater cold tolerance (Colinet et al., 2015). Although laboratory acclimation can never properly replicate field conditions, the aim of incorporating laboratory acclimation is to provide some indication of the extent of plasticity, and therefore the extent to which potential cold tolerance is being underestimated.

Both acclimation and acclimatization are reversible; in the *C. ferrugineus* example above, the enhanced cold tolerance was lost within a week of returning the insects to the rearing temperature of  $30^{\circ}C$  (Fields et al., 1998), a process termed deacclimation. In the field, deacclimation could occur during seasonal temperature variation (e.g. mid-winter thaws). For example, Sobek-Swant et al. (2012) found that winter-acclimatized freeze-avoidant emerald ash borer prepupae had increased SCPs and decreased glycerol concentrations after exposure to mid-winter warm spells ( $+10$  and  $+15^{\circ}C$ ), and that this deacclimation was not reversed when the prepupae were returned to  $-10^{\circ}C$ . Deacclimation is rarely included in cold tolerance studies, but is clearly relevant when attempting to understand the plasticity of cold tolerance, particularly in relation to variable environments.

### 6.4. Developmental and cross-generational plasticity

Developmental plasticity is the non-reversible change in phenotype of one life stage that is decided by the experience of an

earlier life stage (for review and examples, see Davidowitz and Nijhout (2004), Kingsolver and Huey (2008), Nylin and Gotthard (1998)). For example, *Bicyclus* butterflies reared at a high temperature have slower chill coma recovery than those reared at a lower temperature, regardless of their adult experience (Franke et al., 2012); by contrast, flour beetles raised under high temperatures have faster chill coma recovery than their low temperature counterparts (Scharf et al., 2015). Developmental plasticity is thus important when considering overwintering, because entry into appropriate physiological state for overwintering may be initiated during an earlier life stage.

In some species, the overwintering phenotype is determined not by an individual's experience, but by the experience of the previous generation. For example, diapause by *Aedes* eggs is determined by the photoperiod experienced by the mother (Denlinger and Armbruster, 2014). Both developmental plasticity and trans-generational effects are easily missed in laboratory studies, and could therefore mean that laboratory studies underestimate low temperature performance in the field, but, to our knowledge, have been rarely explored.

### 6.5. Detecting phenotypic plasticity

The complexity of phenotypic plasticity means that it cannot be completely encompassed in simple laboratory experiments. Thus, we emphasize that the goal of laboratory studies of phenotypic plasticity is to provide an indication of the extent to which the full cold tolerance potential is being captured by the laboratory experiments. A valuable alternative or complement to these experiments is to conduct seasonal measurements of the cold tolerance of field-collected (or at least field-caged) individuals (e.g. Baust and Miller (1970), Crosthwaite et al. (2011), Košťál et al. (2014), Udaka and Sinclair (2014)). These field studies may provide the best, and most ecologically-relevant, estimate of the cold tolerance changes driven by the combined effects of temperature, photoperiod and trans-generational and developmental plasticity.

The general approach to detecting plasticity of cold tolerance in the laboratory is to apply pre-treatments or acclimation treatments, and measure some (preferably ecologically-relevant) response variable or phenotype, such as  $CT_{min}$ , SCP or LLT. The treatments could include both brief pre-treatments (to elicit rapid cold-hardening) and longer treatments (to elicit acclimation responses). One convenient approach to detecting plasticity phenotypes is the 'discriminating temperature' approach (Powell and Bale, 2004). In this approach, a discriminating temperature (one that causes  $\sim 80\%$  mortality) is chosen from preliminary investigations. Insects are then exposed to various pre-treatments and acclimations, and their survival is measured only at that temperature. While the discriminating temperature approach does not estimate the change in absolute limits of survival, increased survival does provide evidence of plasticity, which can be explored in greater detail as required. Discriminating temperatures are most often used in studies documenting the RCH response (e.g. Sinclair and Chown (2006)), but can also be used to explore other sources of plasticity, including heat shock and acclimation (e.g. Rajamohan and Sinclair (2008, 2009)). However, SCP is a relevant metric for freeze-avoidant species (e.g. Worland and Convey (2001)), and  $CT_{min}$  or CCRT may also be used to identify plasticity in a relatively high-throughput fashion (e.g. Everatt et al. (2013), Fischer et al. (2010), Hoffmann et al. (2005), Sisodia and Singh (2010)).

There is an almost infinite variety of combinations of potential cues for phenotypic plasticity (Table 4), so if the goal is simply to detect plasticity, it is most convenient to choose a small combination of treatments that encompass a variety of cues and time frames. Because it is the plasticity itself, not the precise cues, that is important, it can be useful to deliberately conflate photoperiod



**Table 4**  
Examples of cues and conditions for inducing phenotypic plasticity in insect cold tolerance.

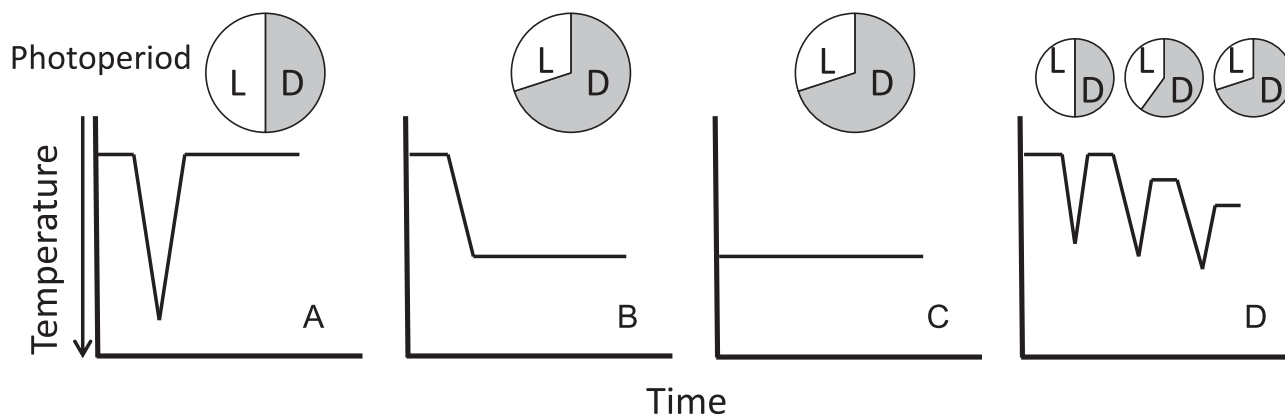
Cue/condition	Example	Reference
<b>Shortened photoperiod</b>	Shortened photoperiod (12 h of light, 12 h of dark) triggers increased cold-hardiness in <i>Aulacophora nigripennis</i> (Coleoptera: Chrysomelidae)	Watanabe and Tanaka (1998)
<b>Changes in diet</b>	<i>Drosophila ananassae</i> larvae reared on carbohydrate-rich food are more cold-tolerant. <i>Chymomyza costata</i> larvae reared on a proline-rich diet are more freeze-tolerant	Sisodia and Singh (2012) Košťál et al. (2011b)
<b>Maternal effects</b>	The LT <sub>50</sub> of <i>Sitobion avenae</i> offspring is lower if adults are reared at 10 °C, compared to 20 °C.	Powell and Bale (2008)
<b>Acute decrease in temperature</b>	<i>Rapid cold hardening</i> : Exposure to 5 °C for 30 min increases survival of <i>Drosophila melanogaster</i> at –5 °C 10 min to 1 hr exposure to 0 °C increases the freezing tolerance of <i>Sarcophaga crassipalpis</i> <i>Slow cooling</i> : Cooling at 0.05 °C/min or 0.1 °C/min increased survival of <i>Drosophila melanogaster</i> held at –7 °C for one hour	Czajka and Lee (1990) Chen et al. (1987) Kelty and Lee (1999)
<b>Heat shock</b>	Exposure to 34 °C for 40–80 min increases survival of <i>Drosophila melanogaster</i> larvae at 0 °C Heat pre-treatment (36.5 °C, 1 hr) increased cold tolerance in larvae of <i>Drosophila melanogaster</i>	Burton et al. (1988) Rajamohan and Sinclair (2008)
<b>Acclimation</b>	<i>Short (5–10 d)</i> : <i>Halmaeus atriceps</i> were held at 5 °C for 7 d <i>Slow decrease</i> : Acclimation to 15 °C for 1 wk, followed by 6 °C for 1 wk, extends survival of <i>Drosophila melanogaster</i> at 0 °C <i>Ecologically relevant</i> : Lepidopteran pupae were exposed to fluctuating temperatures of 14:10 °C day: night for 2 wk, followed by 10:6 °C day: night for 2 wk, followed by a constant 6 °C	Slabber and Chown (2005) Košťál et al. (2011a) Williams et al. (2012)
<b>Rearing temperature</b>	Three generations of <i>Sitobion avenae</i> were reared at 10 °C	Powell and Bale (2005)

and temperature cues to maximize the likelihood of inducing plasticity. For example, acclimation treatments often combine short day length with low temperatures, without controlling for each factor independently (e.g. Jakobs et al. (2015)).

We suggest four basic treatment approaches that encompass the main components of plasticity (Fig. 9): (A) some form of pre-treatment intended to elicit rapid cold-hardening (Lee et al., 1987); (B) a 5–10 day low temperature-short day length treatment to elicit an acclimation response (e.g. Slabber and Chown (2005)); (C) development at low temperature with a short day length (e.g. Colinet and Hoffmann (2012)); and (D) Fluctuating temperatures and decreasing photoperiod, for example based upon climate (e.g. a nearby weather station) or microclimate (e.g. Jakobs et al. (2015)). The precise temperature and photoperiod details, as well as the duration of the treatments, will depend on the natural history and timing of the life cycle of the organism, its lifespan, and its propensity for laboratory culture. A developmental acclimation treatment will not necessarily be possible for a univoltine species, or one that is not readily reared in the laboratory. Deacclimation has been poorly explored, but is usually achieved through exposures to warmer-than-usual winter temperatures for a period of days (see Section 6.2).

Using the discriminating temperature approach means that a simple comparison of low temperature survival by each treatment to that of the control can reveal significant effects of the plasticity treatments. In this approach, the unit of replication is the group (see also Section 4.1), and 5–10 groups per treatment should provide adequate power. As with LLT measurements, it is best if replicates are randomized, and controls run alongside treatment groups. Some care may need to be taken to control for aging if this is a potential factor in lengthy acclimation treatments, as aging can decrease (e.g. Halle et al. (2015)) or increase (e.g. Lalouette et al. (2010)) cold tolerance, depending on the species. Survival of pre-treated groups can be directly compared to that of controls, using *t*-tests, ANOVA with planned comparisons, or GLZ (with binomial error distributions) and planned comparisons. If ANOVA or *t*-tests are used, note that the binomial error distribution inherent in survival data is inappropriate for these tests, but an arcsine-square-root transformation can normalize data somewhat prior to analysis.

Interpreting data from the discriminating temperature approach is difficult, because it allows the detection of plasticity, but does not quantify the magnitude of that plasticity. If survival increases markedly (e.g. from 20 to 100%), it makes it clear that LLT



**Fig. 9.** Examples of temperature & photoperiod manipulations used to induce phenotypic plasticity in the laboratory. (A) Acute exposure to low temperatures to induce a rapid cold-hardening response. (B) A 5–10 day exposure to low temperatures and short day-length. (C) Development under low temperatures and short day-length. (D) Exposure to gradually declining temperature and photoperiod. L=light, D=dark.

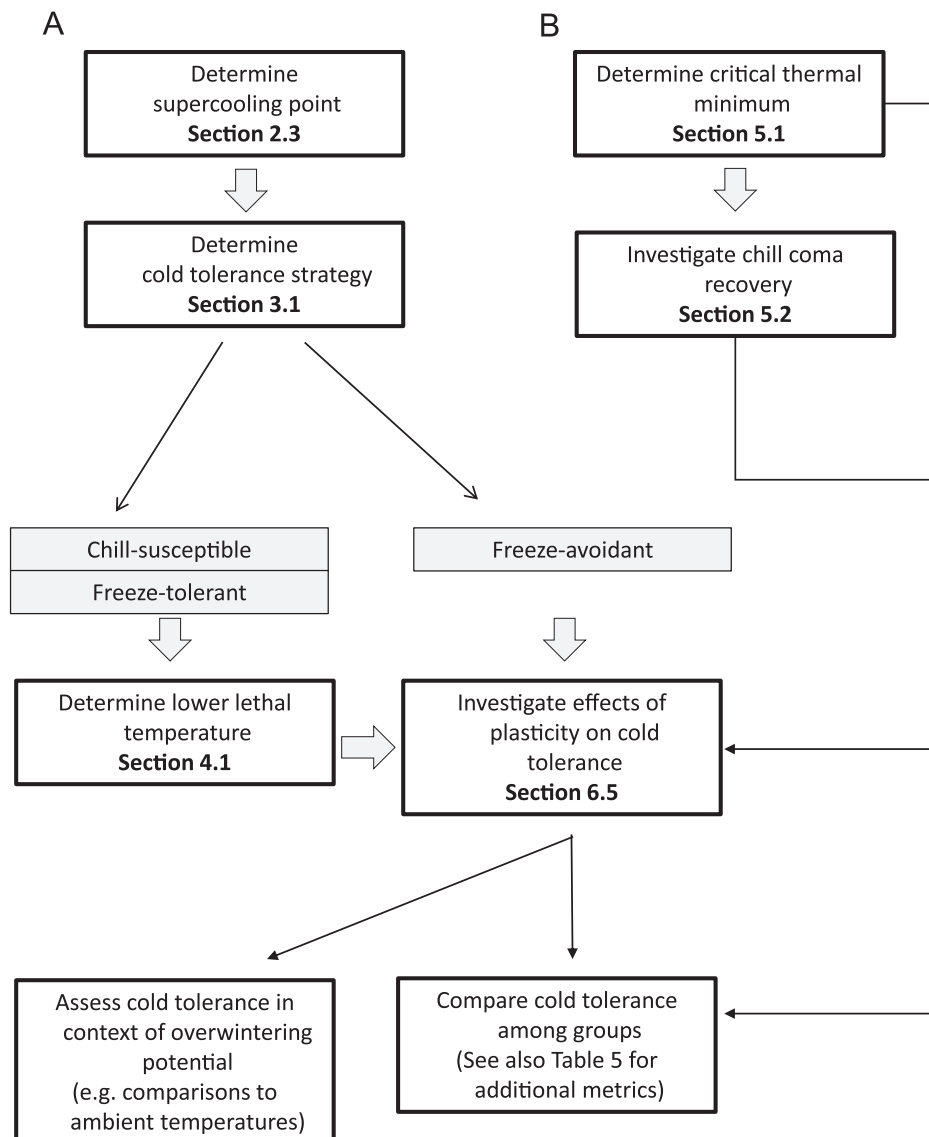
has been underestimated, but to obtain parametric predictions it will be best to construct new LLT curves with individuals in which plasticity has been detected (see [Section 4.1](#)). Note that it is possible that plasticity treatments may also modify the cold tolerance strategy (e.g. [Shimada and Riihimaa \(1988\)](#)), thereby modifying the approach to determining potential for overwintering survival.

## 7. Suggested workflow

Leaving aside the thrill of basic discovery, there are two main reasons to measure low temperature performance in a previously-uninvestigated insect: (1) to provide a detailed description of its cold tolerance, perhaps in the context of a pest or interesting habitat or (2) to provide a measure of low temperature performance to facilitate comparisons among treatments or populations, or to round out a study incorporating a wider array of stressors. In the latter case, the priority is therefore to choose a metric that is both biologically-relevant and easy to measure, while in the former case, the emphasis may be on determining thresholds that can be explored in the context of the habitat. No single measure will be

appropriate for all species or situations, and (like all work on living organisms), it is best to begin with some basic observations. In this final section, we provide two suggested approaches, one for a general exploration of low temperature biology, the other for choosing a measure of low temperature performance.

To begin a comprehensive description of insect cold tolerance, we recommend beginning with two simple observations: SCP ([Section 2.3](#)) and  $CT_{min}$  ([Section 5.1](#)). These two parameters provide anchor points for designing experiments to further investigate CCRT, cold tolerance strategy, and lethal limits ([Fig. 10](#)). In turn, these measures help to determine appropriate response variables for examining phenotypic plasticity ([Section 6](#)). Examples of this approach can be found in many of the papers cited in this review (e.g. [Košťál et al. \(2014\)](#), [Sinclair and Chown \(2005\)](#), [Slabber and Chown \(2004\)](#)). Subsequent investigations of the biochemistry underlying plasticity and cold tolerance, for example, changes in hemolymph composition, can then be incorporated into suitably-informed sampling and analyses, that are beyond the scope of this review (see, e.g. [Bale and Hayward \(2010\)](#), [Duman et al. \(1991\)](#), [Lee \(2010\)](#), [Storey and Storey \(1991, 2013\)](#), [Zachariassen \(1991\)](#), [Zachariassen and Kristiansen \(2000\)](#), [Zachariassen](#)



**Fig. 10.** Recommended work flow to determine cold tolerance strategies and lower thermal limits for investigating overwintering potential and comparisons of cold tolerance among groups. (A) Work flow for determining cold tolerance strategy and overwintering potential. (B) Work flow for using lower limits as a physiological comparison among groups.

et al. (2004)). As we have discussed, the biology of the organism will determine the utility of metrics used – overwintering and diapausing insects, for example, often do not merit measurement of  $CT_{min}$ , so  $CT_{min}$  is consequently absent from studies on many overwintering insects (e.g. Košťál et al. (2014)). A reasonable description of cold tolerance can be made with small sample size, by carefully combining individual measures of SCP, survival, and lower lethal temperature (e.g. Sinclair and Chown (2005)).

Choosing a single metric as a proxy for cold tolerance in a wider study can be more difficult. If a more-or-less complete description of cold tolerance is available, it is a simple matter to compare (or correlate) the possible variables, and choose a metric that is broadly representative of thermal tolerances and logistically convenient for the organism at hand. In *Drosophila*, for example, the  $CT_{min}$  provides a reasonably good proxy for cold tolerance (including  $LT_{50}$ ), and is thus a parsimonious choice for comparison among treatments (Andersen et al., 2015b). If this information is not available, then choosing an appropriate metric is going to be based partially on the life history of the organism, and striking a useful measure may be somewhat dependent on luck. In Table 5, we present the advantages and disadvantages of using each metric to facilitate choosing one to suit the aim of the study.

## 8. Final remarks

Low temperatures are one of the key limiting factors for the distribution and performance of many terrestrial insects, and cold is a key component of overwintering stress in temperate, polar, and alpine habitats. Because of this, the signal-to-noise ratio for measures of cold tolerance is large, providing useful information in comparing insects at multiple scales in time and space. In particular, to fully understand the implications of climate change, there is increasing need to expand beyond growing-season studies to biology in the autumn and winter (Gallinat et al., 2015; Williams et al., 2015), for which low temperature biology is critical. Thus, there is significant incentive for insect ecologists, both basic and applied, to measure some aspect of low temperature biology. It is our hope that this review will be of some value in demystifying what are, for the most part, relatively straightforward methods; our advice is, of course, our own, and we hope that we have also provided enough reference to the literature to allow a newcomer to the field to reflect on, and judge for themselves, our recommendations.

We end with the observation that the field of insect cold tolerance research has always been small and diverse. However, our experience has been that it is a welcoming community, and that for many of its members, cold tolerance is something of a side-interest. Nevertheless, for many of those researchers, the fascination of insect biology at low temperatures means that while insect cold tolerance is not necessarily the most substantial part of their research portfolio, it is almost always a favorite and we therefore end by encouraging others to join us.

## Acknowledgments

This document is derived from one originally commissioned by the Canadian Food Inspection Agency under Research Contract number 3872188. Thanks to Martin Damus and Ruth Jakobs for comments on earlier drafts, Stephanie Sobek-Swant for some initial discussions, and Eric Moise, Kennan Oyen, Inon Scharf, and two anonymous reviewers for feedback on a later draft. BJS thanks David Wharton, Bill Block, Roger Worland and Hans Ramløv for introducing him to the methods of insect cold tolerance. LVF was

**Table 5**  
Requirements and considerations for measurements of cold tolerance in insects.

Measurement	Equipment requirements	Ecological relevance	Constraints	Advantages	Typical sample size
<b>Chill coma recovery (CCR)</b> Section 5.2	Simple cooling; Simple constant temperature	Unclear	Active/mobile insects only; Cannot compare among studies	Easy to measure; Can be compared among treatment groups	10–20
<b>Critical thermal minimum (<math>CT_{min}</math>)</b> Section 5.1	Controlled cooling; Temperature measurement	Moderate–high; sets the lower limit for activity	Continuous observation; Active/mobile insects only	Correlated to other measures of cold tolerance (e.g. in <i>Drosophila</i> ); Can be compared among species and treatments; Can be compared to ambient temperatures	10–20
<b>Supercooling point (SCP)</b> Section 2.3	Controlled cooling; Temperature measurement	High if insect is freeze-avoidant; Important threshold for freeze-tolerant insects; No relevance if insect is chill-susceptible	Must consider sources of ice nucleation	High throughput; Can be compared among species and treatments; Can be compared to ambient temperatures	20–50
<b>Cold tolerance strategy</b> Section 3.1	Controlled cooling; Temperature measurement	None	Does not predict thermal limits	Informs interpretation of other measurements	8–10
<b>Lower lethal temperature (LLT)</b> Section 4.1	Controlled cooling; Temperature measurement	High	Time-consuming	Directly relevant to the real world	5–10 groups of 5–10
<b>Discriminating temperature</b> Section 6.1	Simple, constant temperature	Moderate; predicts survival at this temperature	Must determine appropriate temperature in advance	High throughput; With careful choice of temperature, can be ecologically relevant; Can be compared among groups/treatments	Groups of 5–10, with or without replicate groups

supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada. Work by BJS on insect cold tolerance in Canada has been supported by the Natural Sciences and Engineering Research Council of Canada, the Canadian Food Inspection Agency, The Ontario Ministry for Research and Innovation, the Canadian Foundation for Innovation, The University of Western Ontario, and the National Institutes of Health (USA).

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