Western University
Scholarship@Western

Biology Publications | Biology Department

10-1-2015

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

Brent J Sinclair
bsincla7@uwo.ca

Litza E Coello Alvarado

Laura V Ferguson

Follow this and additional works at: https://ir.lib.uwo.ca/biologypub

Part of the Biology Commons

Citation of this paper:

Sinclair, Brent J; Coello Alvarado, Litza E; and Ferguson, Laura V, "An invitation to measure insect cold tolerance: Methods, approaches, and workflow." (2015). *Biology Publications*. 68.
https://ir.lib.uwo.ca/biologypub/68

REVIEW

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

Brent J. Sinclair*, Litza E. Coello Alvarado & Laura V. Ferguson

Department of Biology, University of Western Ontario, London, ON, Canada

Address for correspondence: Dept. Biology, University of Western Ontario, London, ON, N6A 5B7, Canada

Email: bsincla7@uwo.ca; tel: 519-661-2111 x83138; fax: 519-661-3935

13 ## Abstract

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

31

32 **Keywords:** chill coma, critical thermal minimum, supercooling point, cold tolerance
33 strategy, lower lethal temperature, rapid cold-hardening, acclimation, deacclimation,
34 phenotypic plasticity, experimental design

35 ## 1. Introduction

36 Temperature constrains the geographic distribution and seasonal activity of insects
37 (Chown and Nicolson, 2004), and therefore can directly or indirectly affect the spread and
38 impact of invasive pests, the success of species introduced for biological control, and the
39 dynamics of native insect populations (Bale and Hayward, 2010). In temperate, polar and
40 montane habitats, the majority of insects spend a large proportion of their life in an
41 overwintering stage, and must survive the low temperatures and accompanying
42 environmental stressors that are associated with winter (Leather et al., 1993; Williams et
43 al., 2015). Similarly, insects in deserts and tropical high mountains can also be regularly
44 exposed to potentially-lethal freezing conditions (Sømme, 1995; Sømme et al., 1996;
45 Sømme and Zachariassen, 1981). Thus, low temperature biology is a key component of
46 insect fitness, and one of the best determinants of insect distribution (Andersen et al.,
47 2015b).

48

49 Most insects are ectotherms, and as such, their body temperatures are generally
50 similar to the ambient microclimate temperature, and changes in ambient temperature can
51 thus have drastic effects on the physiology of an insect. Thus, measuring low temperature
52 performance (which we refer to loosely here as 'cold tolerance') is an excellent way to
53 incorporate the pervasive effects of temperature in studies ranging from ecological (e.g. van
54 Dooremalen et al., 2013) to molecular (e.g. Reis et al., 2011). In the cold, many insects
55 enter a reversible state of paralysis, called chill coma, at the critical thermal minimum
56 (CT_{min}) (MacMillan and Sinclair, 2011a). At sub-zero temperatures, insects risk freezing of

57 the body fluids, as well as a host of other low temperature injuries (Denlinger and Lee,
58 2010). The ability of an insect to survive at low temperatures is referred to as its cold
59 hardiness, and their responses to low temperature have generally been categorized as chill-
60 susceptible, freeze-avoidant, and freeze-tolerant (Fig. 1, see Section 3) (Bale, 1993).

61

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

explanations of why we favour 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.

102

103 More commonly, insects are cooled using specialised cooling equipment, such as rate-
104 controlled incubators (e.g. Ju et al., 2011) or refrigerated circulators (e.g. Marshall and
105 Sinclair, 2011). These are often programmable, allowing precise cooling, hold ('soak' in
106 engineering terminology), and warming programmes. Refrigerated circulators will require
107 a bath liquid (e.g. glycol, alcohol, or a synthetic or mineral oil) for sub-freezing use. In our
108 laboratory we use ethylene glycol (usually mixed 1:1 with water) for moderate low
109 temperatures (c. -20 °C) because it has a lower viscosity than the less-toxic propylene
110 glycol and can be heated. We use methanol (either undiluted or mixed 1:1 with water) for
111 lower temperatures. Samples (in containers) can either be placed directly in the bath, or the
112 batch liquid can be circulated to a cooling stage; in our laboratory we circulate liquid to an
113 insulated aluminium block that is milled with holes to accommodate various sample
114 container sizes, from 1.5 mL microcentrifuge tubes to 35 mL standard *Drosophila* vials.

115

116 Thermoelectric cooling via Peltier devices can be used to construct efficient cooling
117 devices (especially if the cooling modules are stacked and liquid-cooled), and we have used
118 these in the past (e.g. Sinclair et al., 2003a; Sinclair and Sjursen, 2001); unfortunately,
119 neither of these devices (nor designs for them) are commercially available, so they must be
120 custom-designed and –built (Wharton and Rowland, 1984). However, they are light and
121 robust and use little power, so are ideal for field situations. Another field alternative is to
122 use an endothermic reaction to provide cooling. For example, Sømme et al. (1993) used a
123 $CaCl_2 \cdot 6H_2O$/crushed ice mixture to determine SCPs of mites at a field camp in Antarctica.
124 This has the disadvantage of producing chemical waste, but greatly reduces the electricity

125 requirements for cooling in the field. Another approach to control cooling rate is to use a
126 set-temperature incubator or freezer, and to moderate the cooling rate (if not the final
127 temperature) using insulation, such as a Styrofoam box (e.g. Hao and Kang, 2004).

128

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

142

143 Investigations of insect cold tolerance initially adopted a uniform cooling rate of
144 approximately 1 °C/min to standardize results (Salt, 1966). Although this rate is
145 convenient, it is significantly faster than the cooling rates experienced in nature [e.g.
146 Sinclair (2001) measured a maximum cooling rate of 0.01 °C/min in alpine microhabitats

147 in New Zealand –100-fold slower than used in these laboratory studies] and several studies

148 have shown that fast cooling rates lead to additional cold shock (e.g. Bale et al., 1989;

149 Cloudsley-Thompson, 1973; Miller, 1978). In particular, directly plunging an insect into an

150 extreme temperature can significantly decrease survivorship, compared to that achieved

151 during a ramping regime (Nguyen et al., 2014). Conversely, slow cooling rates can make

152 experiments impractically long, and may allow insects to mount a physiological response to

153 cold, such as that observed during rapid cold-hardening (Kelty and Lee, 1999; Nguyen et

154 al., 2014). Most authors currently choose cooling rates that compromise between

155 ecological relevance and time investment; generally 0.1 to 0.5 °C/min (e.g. Boardman et

156 al., 2012; Crosthwaite et al., 2011; Renault et al., 2004; Sformo et al., 2011). It is clear that

157 the rate of temperature change does affect the response of the insect (this has also been

158 vigorously discussed in the context of high temperature tolerances, e.g. Santos et al., 2011;

159 Terblanche et al., 2007; Terblanche et al., 2011). Thus, it is important to maintain

160 consistent cooling rates among sites or species for comparisons, and to be cognizant of the

161 assumptions inherent in comparing thermal limits among studies that used different

162 exposure conditions.

163

164 *2.2 Temperature measurement*

165 Thermocouples are the most common way to measure insect temperatures during

166 cooling. Thermocouples are thin wires that measure temperature via the changes in the

167 voltage output of a bimetallic junction. Usually one thermocouple is used per insect,

168 although it is also possible to increase throughput by carefully placing more than one

169 (small) insect on a thermocouple (e.g. springtails; Sinclair et al., 2006a), or by connecting

170 multiple thermocouples in series (Nedvěd et al., 1995). Thermocouples differ in the types
171 of metal used, and in the diameter of the wire; any thermocouple type is acceptable if it
172 returns valid measurements over an appropriate range of sub-zero temperatures and is
173 compatible with the recording device. Types K (chromel-alumel) and T (copper-
174 constantan) are most common in insect cold tolerance studies. Smaller-diameter
175 thermocouples will yield a faster response (but are more fragile); we have found 36 AWG
176 thermocouples to be a suitable compromise. Pre-made thermocouples can be purchased in
177 various lengths, or thermocouple wire can be purchased and used to make thermocouples
178 by stripping the wires and twisting them together to create a thermocouple junction
179 (damaged pre-made thermocouples can also be repaired this way). Thermistors can be used
180 in an identical manner to thermocouples for SCP measurements (provided they are small
181 enough), but require specific data acquisition apparatus.

182

183 To detect freezing, thermocouples must be close enough to (or in contact with) the insect
184 during cooling so they can detect the latent heat of crystallization that indicates ice
185 formation. The lowest temperature that precedes this exotherm is termed the supercooling
186 point (the SCP; Fig. 1). Small insects and eggs can be attached to thermocouples by coating
187 the tip of the thermocouple with a thin film of petroleum jelly, heat sink compound, or
188 vacuum grease, which will adhere to the insect with a light touch (Fig. 2A). This has been
189 particularly successful with springtails and mites (Block, 1982b; Block and Young, 1979).
190 For larger insects (e.g. crickets and beetles), thermocouples may be maintained in contact
191 with the cuticle by placing (or chasing) the insect into a microcentrifuge tube or pipette tip,
192 and packing cotton wool in to hold the animal and a thermocouple in place (Fig. 2B).

193 Removable adhesive putty (such as the sticky tack used to attach pictures to walls) may
194 also be used to hold the tip of the thermocouple in contact with an insect such as a phasmid
195 (Fig. 2C). Exotherms can often be detected from large organisms even if the thermocouple
196 is not in direct contact with the animal. This approach – placing the thermocouple on the
197 outside of a sealed respirometry chamber containing the animal – has worked for large
198 caterpillars (e.g. Sinclair et al., 2004) and frogs (Sinclair et al., 2013).

199

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

206

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

215

216 Differential scanning calorimetry measures heat flow into or out of a sample,
217 usually for chemical analysis. However, the sensitivity and precision of DSC means it can
218 be used to measure freezing in small insects placed in the cooling chamber. Many DSC
219 instruments are cooled by liquid nitrogen, and can therefore be used to measure very low
220 SCPs (e.g. below -40 °C). Differential Scanning Calorimetry likely avoids artifacts from
221 thermocouple contact, and can be used for multiple small insects at once (e.g. Worland and
222 Convey, 2001). Infrared thermography is another approach to detecting and measuring
223 SCPs without physical contact. This approach has been used successfully to detect SCPs
224 (Palmer et al., 2004), to analyze the ice formation process (Sformo et al., 2009), and to
225 separate freezing of insects from ice formation in their substrate (Koštál et al., 2012). It is
226 now possible to purchase infrared cameras that interface with a smartphone, so the
227 availability of this technology may increase rapidly. Infrared 'thermocouples' are also
228 commercially available, but have not, to our knowledge, been used for measuring insect
229 cold tolerance in the published literature.

230

231 *2.3 Measuring Supercooling Points*

232 The supercooling point is the temperature at which freezing begins, and ice
233 formation will usually then proceed from the site of nucleation to other parts of the insect's
234 body (Lee and Lewis, 1985). Many investigations of insect cold tolerance begin with
235 preliminary measurements of the SCP because this provides an anchor point about which
236 the cold tolerance strategy can be determined: an initial step is to determine whether insects
237 survive freezing or not (see Section 3.1). Additionally, changes in the SCP with treatment

238 or season can indicate biochemical or physiological changes, even if the SCP is not a good
239 measure of survival of low temperatures (Coleman et al., 2014). Because the SCP does not
240 necessarily equate to cold-hardiness, it must be interpreted with caution, and in many cases
241 has limited ecological relevance (Bale, 1987; Baust and Rojas, 1985; Renault et al., 2002).
242 However, for freeze tolerant and freeze-avoidant insects (see below), the SCP does
243 represent an important physiological threshold or lower lethal limit, respectively, and can
244 indicate ecologically-relevant variation in cold tolerance in the latter (e.g. Crosthwaite et
245 al., 2011; van der Merwe et al., 1997; Worland et al., 2006).

246

247 Measuring the SCP is as simple as placing an insect in contact with a thermocouple,
248 and cooling it at a reproducible rate to a temperature at which all of the individuals have
249 frozen. The exotherm released by the latent heat of crystallisation is easily detected (Fig.
250 3). The SCP is defined as the lowest temperature recorded prior to the initiation of the
251 exotherm, the duration and shape of which will depend on the size of the insect. Larger
252 insects (e.g. crickets and caterpillars) have more water, and the freezing process takes
253 longer than in small insects (*Drosophila* and springtails) yielding distinctly different shapes
254 (Fig. 3). In terms of data acquisition, higher sampling rates may be required to capture the
255 SCP of small insects (e.g. springtails) with exotherms that occur rapidly releasing a small
256 amount of heat. In general, a sampling rate of <1 Hz is suitable for larger insects, and (from
257 experience) 10 Hz should be sufficient for even small insects, insect eggs, Collembola, and
258 mites. If the cooling method results in fluctuations in temperature, or there is a large
259 amount of condensation present, it is sometimes possible to detect artifactual freezing

260 events that are not the result of the insect freezing (Fig. 3). A simple solution to this is to
261 include a thermocouple without an insect attached, to allow interpretation of any artifacts.
262
263 Supercooling points are usually measured on 20-30 individuals, which provides a
264 robust sample size with which to assess the shape of the distribution of SCPs. Although
265 cold-hardy populations of insects often have unimodal SCP distributions, some species
266 (and also microarthropods like springtails and mites) have bimodal SCP distributions
267 (Cannon and Block, 1988; Sinclair et al., 2006a; Figure 4). Bimodal SCP distributions can
268 arise due to variation in the presence of ice nucleators or differences in body composition.
269 For example, springtails that have recently fed (introducing ice nucleators into the gut
270 leading to a high group of SCPs; Sømme, 1982), and/or moulted (removing nucleators;
271 Worland et al., 2006) have very different SCPs. Starved adult *Alphitobius diaperinus*
272 beetles also had a bimodal SCP distribution, but this was due to s starvation × sex
273 interaction such that the SCPs of males decreased with starvation, but the SCps of females
274 did not (Salin et al., 2000). Although techniques do exist to determine breakpoints in
275 bimodal distributions (e.g. Aldrich, 1987), many authors simply decide on an *a priori* high
276 group-low group divider by collating all SCPs and visually assessing a histogram for an
277 obvious break and then compare the frequency, means or medians of the high and low
278 groups among treatments using standard methods (e.g. Sinclair et al., 2006a). Because
279 supercooling point distributions are seldom normal, and abnormally high or low values
280 from one or two individuals can skew the entire distribution, care must be taken in using
281 parametric statistics when comparing SCPs among samples or treatments. In addition,
282 because larger insects contain more water, they have a higher probability of freezing, and

283 therefore higher SCPs. One approach to account for this potential confounding factor is to
284 incorporate body size (e.g. as mass) into statistical model (or conduct analyses on residuals
285 of a regression of body size on SCP), demonstrated by Hahn et al. (2008). This may be
286 necessary if (e.g.) dietary treatments yield individuals of markedly different body sizes. An
287 alternate approach (when possible) is to use broadly size-matched individuals for
288 comparison.

289

290 Ice formation can take only a few seconds in small animals, particularly at low
291 SCPs. By contrast, freezing can be a slow process in larger insects. For example, the peak
292 of the exotherm in 3-5 g alpine weta, *Hemideina maori* (Orthoptera: Anostostomatidae)
293 occurs approximately 1.5 min after the SCP, but at that point only 5 % of the body water is
294 converted into ice. Ice content reached an equilibrium 70-80 % after c. 8 h (Figure 5;
295 Ramløv and Westh, 1993). Because the amount of heat released is proportional to the
296 quantity of water converted to ice, it is tempting to use the exotherm area to estimate ice
297 content. However, a thermocouple does not capture the heat release accurately enough to
298 quantify ice formation from its measurements (or to usefully interpret the highest
299 temperature the thermocouple records), so the amount of ice formed must be measured
300 calorimetrically (e.g. Block et al., 1998; Layne and Blakeley, 2002; Ramløv and Westh,
301 1993).

302

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 °C and -22 °C but are killed by brief exposures between -8 °C and -12 °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 °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

327 rod gall fly *Eurosta solidaginis* (Diptera: Tephritidae) freeze around -8 °C but can survive
328 with more than 60 % of their body converted to ice, with mortality beginning below -25 °C
329 (Morrissey and Baust, 1976). Many temperate species switch from one strategy to another
330 at the onset of winter (Salt, 1961), and the presence or absence of external ice can also
331 determine cold tolerance strategy (e.g. Shimada and Riihimaa, 1988).

332

333 Defining a species' cold tolerance strategy is essential for interpreting the SCP, and
334 for determining the subsequent approach to measuring lethal temperatures (see Section 4).
335 However, while the cold tolerance strategy helps explain how insects respond to and
336 survive exposure to low temperatures, it does not predict or quantify survival in a particular
337 winter or overwintering site, nor does it designate absolute levels of cold toelrance (Bale,
338 1993; Sinclair, 1999). For example, in temperate southwestern Ontario, Canada, winter
339 temperatures regularly drop below -25 °C, yet freeze avoidant (e.g. Crosthwaite et al.,
340 2011), freeze-tolerant (e.g. Marshall and Sinclair, 2011) and chill-susceptible (Udaka and
341 Sinclair, 2014) species are all abundant. Thus, cold tolerance strategy is not a substitute for
342 understanding lethal limits, and cannot be used on its own to predict survival. Despite its
343 inability to predict cold tolerance, the cold tolerance strategy can indicate potential
344 prediction and control methods (see Leather et al., 1993, for a comprehensive review). For
345 example, freeze-avoidant species may be susceptible to conditions that promote ice
346 formation, such as the application of ice nucleators (e.g. Fields, 1993; Lee et al., 1994).

347

348 *3.1 Determining cold tolerance strategy*

349 Cold tolerance strategy is best inferred from two pieces of information gathered
350 *from the same individual* during a cold exposure: 1) Whether or not the insect froze, and 2)
351 whether or not it survived (Table 1; Salt, 1961). Cold tolerance strategy can be determined
352 with a small number of individuals cooled, in contact with thermocouples, to a temperature
353 close to the SCP. By rewarming all of the individuals once half of them have frozen
354 (evident from exotherms), it is possible to generate a population of insects that includes
355 individuals that did and did not freeze, but that were all exposed to the same low
356 temperature. The cold tolerance strategy can then be determined from the survival of these
357 individuals (Tables 1 and 2; see section 4.1 for a discussion of survival metrics).

358

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

367

368 Ice formation is a slow process, particularly in larger insects (Fig. 5). Thus,
369 survival of brief exposures below the SCP may not reflect survival of ‘complete’ freezing
370 (i.e. freezing to an equilibrium ice content). While it is possible to measure the ice content

371 of insects (e.g. Block et al., 1998; Layne and Blakeley, 2002; Ramløv and Westh, 1993),
372 holding insects below the SCP for a longer period (e.g. overnight) can indicate whether the
373 animal is tolerant of equilibrium ice content (Dennis et al., 2015; Sinclair et al., 1999).
374 Insects that do not survive complete ice formation [termed 'partially freeze tolerant' by
375 Sinclair (1999)] may nevertheless use freeze tolerance as a component of their cold
376 tolerance strategy, depending on the environmental conditions, particularly if sub-freezing
377 conditions are brief (e.g. Sømme et al., 1996; Sømme and Zachariassen, 1981), although
378 this has not been well-explored. Voituron et al. (2002) hypothesised that this partial freeze
379 tolerance may be an important intermediate step in the evolution of freeze tolerance.
380 Similarly, although some insects may survive short periods of acute cold exposure if they
381 remain unfrozen, freeze-avoidant insects must remain unfrozen when supercooled for long
382 periods (Sømme, 1996). The ability to remain supercooled is easily tested by cooling
383 insects to close to the SCP, and holding them at that temperature, for example, overnight
384 (e.g. Crosthwaite et al., 2011).

385

386 Some species of insect tolerate freezing only if ice formation is initiated
387 (inoculated) at relatively high subzero temperatures (Lee, 2010). For example, larvae of
388 *Chymomyza costata*,(Diptera: Drosophilidae) were initially classified as freeze-avoidant,
389 but if freezing is inoculated by contact with ice crystals, then diapausing larvae will survive
390 below -80 °C (Shimada and Riihimaa, 1988). This is most relevant to species expected to
391 overwinter in moist conditions, and ice formation can be inoculated by placing an animal in
392 contact with a moist substrate (e.g. Boardman et al., 2012; Koštál and Havelka, 2000),
393 applying ice (e.g. Layne et al., 1990), or applying a known ice nucleator, such as silver

394 iodide (e.g. Strachan et al., 2011). Cold tolerance strategy can then be assessed as
395 described above.
396

397 To identify cryoprotective dehydration, insects need to be exposed to low
398 temperatures in the presence of (but not actually in contact with) ice. In the field, this
399 occurs in frozen soil or moss (Holmstrup, 2014), and the most common laboratory method
400 is to confine insects in a mesh or perforated container within a larger sealed container that
401 contains crushed ice (e.g. Elnitsky et al., 2008; Sørensen and Holmstrup, 2011; Udaka and
402 Sinclair, 2014), which is exposed to cold, usually in an incubator or other large chamber.
403 The hallmarks of cryoprotective dehydration are a decrease in water content (usually
404 measured gravimetrically), such that the concentration of solutes in the body fluids
405 increases to yield a melting point approximately equivalent to the ambient temperature,
406 rendering the insect essentially unfreezable [Fig. 6; see Holmstrup (2014) for an extensive
407 review]. Simultaneously, the insect may also accumulate low molecular weight
408 cryo/anydroprotectants, such as trehalose. Thus, insects using cryoprotective dehydration
409 will have both reduced water content and depressed supercooling points, and these should
410 decrease in concert with decreasing temperature.
411

412 Cold tolerance strategy is not necessarily fixed. First, many species only express
413 their winter-relevant cold-tolerance strategy after appropriate seasonal cues. For example,
414 *Eurosta solidaginis* is chill-susceptible in the summer, but freeze-tolerant in the winter (Lee
415 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 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; Nedvěd, 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.

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

450

451 *4.1 Estimating Lower lethal limits*

452 Lower lethal limits are usually determined by exposing individual insects (or more
453 commonly, groups of individuals) to a pre-determined range of test temperatures, each for
454 the same fixed period of time. Approximately five temperatures spanning the range of 0 to
455 100 % mortality are usually sufficient to determine the LT_{50}, although additional
456 temperatures are sometimes added to obtain a finer-scale estimate of LT_{50}. After cold
457 exposure, insects are returned to recovery conditions (e.g. room temperature or the rearing
458 temperature), and allowed to recover before survival is assessed.

459

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

477

478 Determining LLT is, by necessity, an iterative process. Ideally, survival metrics,
479 SCP, and cold tolerance strategy have already been determined, and can help to determine a
480 starting point, with the goal of anchoring the analysis with exposures to temperatures that
481 yield 0 % and 100 % survival, with additional replicates to fill the intervening space (e.g.
482 Udaka and Sinclair, 2014). An alternative approach is to expose groups of insects to a pre-

483 determined set of temperatures (e.g. at 5 °C intervals; Bouchard et al., 2006; Kleynhans et
484 al., 2014). This approach has advantages in design and execution; in particular, it can allow
485 random assignment of insects to treatments, allow all of the exposures to be made on a
486 single day, and is more suitable for using longer-term measures of survival.

487

488 Assessing mortality after cold exposures requires a consistent (and ecologically-
489 relevant) estimate of whether or not an insect is alive, and an appropriate time period to
490 capture mortality caused by cold, but exclude mortality from other causes. Ideally, survival
491 to reproduction indicates that an insect has retained fitness after cold exposure (Baust and
492 Rojas, 1985), but this is surprisingly rare as a metric. Survival to the reproductive stage for
493 immature insects (i.e. successful eclosion as an adult) is an ecologically-relevant estimate
494 of mortality that is more common (e.g. Strachan et al., 2011; Williams et al., 2014).
495 However, for species that do poorly in captivity, those with long life cycles, or because of
496 pressure to obtain data, most researchers choose simple metrics of survival that can be
497 rapidly assessed. These metrics are often a coordinated movement (standing or a righting
498 response, e.g. MacMillan and Sinclair, 2011b; Nyamukondiwa et al., 2011; Tomcala et al.,
499 2006), although resumption of feeding, or a more ‘athletic’ ability (running, jumping, or
500 flying) would also be appropriate. More simple measures can be problematic, as they can
501 lack an assessment of biologically-relevant performance. For example, cold-exposed
502 insects can be injured and able to move in an uncoordinated fashion, but unable to perform
503 more complex functions. Such individuals have been characterized as ‘injured’ (Koštál et
504 al., 2006; MacMillan and Sinclair, 2011b) or ‘moribund’ (Sinclair, 1997). These

505 individuals are usually considered 'dead' in analyses on the basis that since they could not
506 reproduce, their fitness is zero.
507
508 Mortality is usually assessed within the first 24 hours to a few days after cold exposure.
509 Thus, lethal temperature estimates may ignore mortality that occurs in the longer term, and
510 also disregard sub-lethal effects on behavior or reproductive capacity (Baust and Rojas,
511 1985). For example, adults of the freeze-tolerant carabid *Pterostichus brevicornis* will
512 survive for a few days when frozen and thawed under sub-optimal conditions, but die after
513 a week (Miller, 1969). By contrast, it is sometimes possible to significantly reduce the
514 recovery period needed for a survival assessment. For example, Udaka & Sinclair (2014)
515 found no difference in survival estimates of cold-exposed *Curculio glandium* (Coleoptera:
516 Curculionidae) larvae made 15 min and 24 h after rewarming. Choosing a timeframe and
517 metric for assessment of mortality will thus depend on the goal of the study; when
518 assessing potential for overwintering survival, longer-term survival and reproductive-
519 capacity measures may be most appropriate.
520
521 Lower lethal temperature experiments are usually conducted with sequential
522 exposure of different groups of individuals to a range of temperatures (e.g. Bouchard et al.,
523 2006; Figure 7). The response variable is percent or proportion mortality (or survival, for
524 the optimistic). Thus, the unit of replication in an LLT study is a group of individuals, not
525 the individuals themselves; five groups of ten individuals gives n=5, whereas one group of
526 fifty (the same number of animals) gives n=1. Because survival is expressed as a

527 proportion, groups of fewer than five are less useful than larger groups, but the balance
528 between resolution and replication usually leads most researchers to use groups of 5-10.
529 There are statistical power advantages to treating each individual's survival separately
530 (using, for example, survival or failure time analyses, e.g. Jakobs et al., 2015), however,
531 researchers rarely take this approach, perhaps because most exposures are conducted in
532 groups. Even if individuals are individually housed, a single exposure treatment should
533 probably be considered a group, to avoid pseudoreplication (McArdle, 1996). When using
534 multiple experimental groups of individuals (e.g. 3-5) at each temperature, 3-5 independent
535 LLT curves do not need to be generated; instead, the order of exposure temperatures can be
536 randomized to yield replication, and all the data considered in the same model. Because
537 lethal limits are estimated using a regression approach, it is not necessary to expose insects
538 to precisely determined temperatures; it is sufficient to have an accurate measurement of
539 the temperature to which each group was exposed.

540

541 Lethal temperatures can be estimated graphically by plotting the survival
542 proportions by temperature and 'eyeballing' the lethal limits (Fig. 7A). However, a formal
543 statistical analysis of survival is relatively simple, and can yield values for LT_{50} and LLT
544 (and any other level of mortality), and also variance or confidence intervals around those
545 estimates. Because survival has a binomial error distribution (individuals are either alive or
546 dead), linear models, such as ANOVA, are inappropriate for determining LLT (although
547 ANOVA may be suitable for comparing among models, particularly in R, where this is a
548 common approach – e.g. Crawley, 2005). A logistic regression (Fig. 7B) with logit link and
549 binomial error distribution (i.e. a probit analysis) is, *a priori*, most appropriate (e.g. Bürgi

550 and Mills, 2010; Sinclair, 1997). Alternative forms of survival analysis (e.g. Ransberry et
551 al., 2011) are inappropriate for LLT analyses, because they assume that an individual is
552 followed throughout an exposure until it dies – clearly this is not the case when the
553 individual has to be rewarmed to ascertain survival.

554

555 **5. Chill Coma**

556 When cooled, a majority of insects will slow down and eventually lose the ability to
557 move. At a specific temperature, an insect will cross a threshold (the critical thermal
558 minimum, CT_{min}), into a reversible state of paralysis, known as chill coma. (Hazell and
559 Bale, 2011). Because insects cannot move, feed, reproduce, or evade predators while in
560 chill coma, the CT_{min} provides a useful lower limit to insect function (Andersen et al.,
561 2015b; David et al., 1998; Gibert et al., 2001; MacMillan and Sinclair, 2011a), and is often
562 used to approximate insect cold tolerance because it is broadly correlated to lethal limits
563 (Andersen et al., 2015b). Resumption of movement after rewarming (chill coma recovery,
564 CCR) is usually measured as the amount of time it takes insects to recover from a
565 standardised period in chill coma (David et al., 1998; Gibert et al., 2001; MacMillan and
566 Sinclair, 2011a). Additionally, because chill coma is usually non-lethal, it is possible to use
567 insects for further experiments, or even breed from them, allowing for selection
568 experiments (e.g. Bertoli et al., 2010; Franke et al., 2012; Mori and Kimura, 2008; Telonis-
569 Scott et al., 2009).

570

571 Although insects may accumulate injuries (or die) while in chill coma (MacMillan

572 and Sinclair, 2011a), CT_{min} and CCR are not measures of cold tolerance, but rather of
573 resistance to the effects of cold (although it provides a useful estimate of thermal biology
574 for comparative purposes; Andersen et al., 2015b). CT_{min} is also limited as a measurement
575 of resistance to cold, because it can only be measured in mobile life stages; specifically,
576 monitoring when an insect enters chill coma requires identifying and timing the loss or
577 recovery of some form of movement. Some insects become immobile when disturbed (e.g.
578 some species of stick insects and beetles), making it difficult to determine the CT_{min}.
579 Finally, not all species have a CT_{min}. For example, freezing occurs before chill coma in the
580 freeze-avoidant Antarctic springtail, *Isotoma klovstadi* (Sinclair et al., 2006a) and in some
581 freeze-tolerant cockroaches (Sinclair and Chown, 2005).
582

583 *5.1 Chill coma Onset (CT_{min})*

584 The CT_{min} is the most common measure of the threshold at which chill coma onset
585 (CCO) occurs. A similar measure of maintenance of performance at low temperatures,
586 knockdown time, could be used to determine the amount of time at a predetermined
587 temperature after which coordination is lost (cf. Hoffmann et al., 1997). However, it is
588 unclear whether these two approaches measure the same physiological phenomenon
589 (MacMillan and Sinclair, 2011a), so CT_{min} and knockdown time should not be used
590 interchangeably. Because most literature currently uses CT_{min} we suggest that this provides
591 the measure of chill coma onset that is most readily comparable among studies (and is the
592 approach we will discuss further below), assuming assessments have been made under
593 comparable conditions.
594

595 To measure the CT_{min}, the point at which an insect loses its ability to move needs to
596 be identified. This could be through direct observation during cooling (e.g. Klok and
597 Chown, 1997), or through identification of failure to remain in a chamber, or on a perch
598 (e.g. Huey et al., 1992). These observations need to be coupled to a measurement of
599 temperature in the chamber (or even at the surface of the insect). When direct observations
600 are being made, an ideal response will be repeatable among researchers and individual
601 animals, involuntary, and easily discerned, although stimulus is often necessary at low
602 temperatures because insects move more slowly (MacMillan and Sinclair, 2011a). Motor
603 responses include the righting response (standing after being placed on back or knocked
604 over; e.g. David et al., 1998), response to a stimulus such as prodding with a probe (e.g.
605 Klok and Chown, 1997), or coordinated standing (e.g. Koštál et al., 2004); an appropriate
606 species-specific response can be identified via a pilot study. We note (from experience) that
607 some species are simply not tractable for CT_{min} studies; for example, stick insects are
608 immobile when threatened – this is it hard to tell if a stick insect is not moving because it
609 cannot move, or because it will not move.
610
611 The cooling rate used for CT_{min} measurement is a balance between being slow enough to
612 ensure that the insect's body temperature does not lag behind the chamber (leading to
613 inaccurate estimation of CT_{min}), and being fast enough to avoid substantial physiological
614 (e.g. acclimation) responses during cooling (Huey et al., 1992). Thus, most CT_{min} protocols
615 require less than 2 h to complete the entire assay. Most studies use cooling rates between
616 0.1 and 0.25 °C/min for determining CT_{min} (e.g. Kleynhans et al., 2014; MacMillan and
617 Sinclair, 2011b; Terblanche et al., 2006). Note that there has been considerable recent

618 debate on the ‘correct’ rate for measuring high temperature tolerances (Rezende et al.,
619 2011; Santos et al., 2011; Terblanche et al., 2007; Terblanche et al., 2011), and it is
620 therefore imperative to clearly report the conditions when describing CT_{min} experiments. To
621 expedite measurements, it should be possible to rapidly reduce the temperature from the
622 rearing temperature to an intermediate temperature (e.g. 10 °C), allowing equilibration
623 before cooling at the pre-determined rate. To our knowledge, the consequences of different
624 equilibration times in such protocols have not been explored.
625

626 A variety of methods can be used to cool individual insects for manual CT_{min}
627 determination, including controlled-temperature incubators (e.g. Hu and Appel, 2004),
628 immersing insects in tubes or containers in the bath of a refrigerated circulator (e.g. Klok
629 and Chown, 1997), or using purpose-built chambers cooled by fluid circulated from
630 refrigerated circulators (e.g. MacMillan and Sinclair, 2011b; Sinclair et al., 2006b). Higher
631 throughput has been achieved by placing a population of insects into a column with
632 baffles/perches (Huey et al., 1992; Ransberry et al., 2011; Renault and Lalouette, 2012).
633 The insects lose coordination at the CT_{min} as the column is cooled, and can be collected at
634 intervals (or counted electronically; e.g. Shuman et al., 1996) as they fall out the bottom of
635 the column, with each interval representing individuals that entered chill coma over a
636 specific time period.
637

638 Other methods to observe the CT_{min} have also been explored. Renault et al. (1999)
639 used a microbalance to detect activity during declining temperature to identify the CT_{min} in
640 a beetle. Similarly, Hazell et al. (2008) describe making a video recording as an insect is

641 cooled, and then extracting from the video the point at which the last voluntary movement
642 was made [Everatt et al. (2013) describe a means to automate this measurement].
643 Alternately, physiological measurements may be plausibly developed that allows the CT_{min}
644 to be identified from a physiological threshold. Such a measure could use heart function
645 (Andersen et al., 2015a), metabolic rate (Lighton and Turner, 2004; Sinclair et al., 2004), or
646 automated activity monitoring (MacMillan et al., 2012a), although most of these
647 approaches are low-throughput.
648

649 Individual- or group-based studies of insect CT_{min} usually use 10-20 individuals per
650 treatment (e.g. Clarke et al., 2013; Jumbam et al., 2008; Klok and Chown, 1997; Renault
651 and Lalouette, 2012), but the knockdown column approach allows for larger sample sizes
652 (as many as 263 flies in Ransberry et al., 2011). Critical thermal minima can be compared
653 among treatments by treating each individual as a replicate, and using conventional
654 statistics (e.g. ANOVA; Klok and Chown, 1997), or non-linear approaches such as
655 Generalized Linear Models, if not normally distributed. Critical thermal minima may also
656 satisfy the assumptions of survival analyses such as Accelerated Failure Time models (e.g.
657 Ransberry et al., 2011).
658

659 *5.2 Chill Coma Recovery*

660 Because chill coma is reversible and movement is easy to detect in mobile insects,
661 recovery from chill coma is also used as a measure of cold tolerance. Although some
662 authors have measured this as a temperature at which movement reinitiates (e.g. Sinclair et
663 al., 2006a), current research suggests that chill coma recovery is a complex interplay

664 between the rate of loss of homeostasis, the low temperature equilibrium attained, and the
665 rate and threshold for recovery (Fig. 8). Thus, the most-used metric tends to be time until
666 recovery under standardised conditions (Chill Coma Recovery time; CCRT), since this
667 minimises confounding variables (e.g. confounding the rate of recovery versus a
668 temperature threshold for recovery). Here we will therefore discuss only CCRT, although
669 we note that although it is simple to measure under standardised conditions, it is difficult to
670 interpret in ecological context.

671

672 To measure CCRT, insects are exposed to a temperature-time combination that
673 induces chill coma, and returned to a recovery temperature (often the rather vague 'room
674 temperature' – we recommend at least reporting and measuring that temperature). The
675 insects are observed, and the time taken to resume a pre-determined behavior or activity is
676 recorded. Movements and behaviors include coordinated leg movement (Halle et al.,
677 2015), standing or walking (e.g. Macdonald et al., 2004), righting response, or abdominal
678 contractions (e.g. Macmillan et al., 2012b). It should be possible to use video analysis to
679 automate CCRT (e.g. by modifying the method described by Hazell et al., 2008 for CT_{min}).
680 Insects can be cold-exposed via refrigerated baths or incubators (see Section 2.1); however,
681 because many insects enter chill coma around 0 °C, an ice-water slurry is often sufficient
682 (e.g. Gibert et al., 2001; Nilson et al., 2006; Ransberry et al., 2011). Recovery can be
683 observed in any clear container; a convenient approach for smaller insects, such as
684 *Drosophila* is to transfer the insect to 6- or 12-well cell culture plates, which allows
685 multiple individuals to be observed simultaneously. The recovery temperature will likely
686 influence the CCRT, however, this has not been well-explored (Macmillan et al., 2012b).

687

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

703

704 Because of its simplicity, sample sizes for CCRT can be larger than those for CT_{min},
705 typically 10-20 individuals/treatment (e.g. Andersen et al., 2015b; David et al., 2003;
706 Findsen et al., 2013; Gibert et al., 2001; Macdonald et al., 2004; Ransberry et al., 2011).
707 Because only a limited number of individuals can be observed at a time (typically <20, if
708 the observation interval is 20-30 s), we recommend that the treatments in each observation
709 set be randomised. Analysis approaches mirror those of CT_{min}, although because the CCRT

710 has a lower bound (an insect can't take a negative time to recover), survival analyses or
711 generalized linear models might be more appropriate than linear parametric statistics such
712 as ANOVA. Chill coma recovery time is often compared among treatments measured
713 together as a way to examine relative differences; however, because of the diversity of
714 exposure temperatures and times (Table 3), recovery metric and recovery temperatures, we
715 recommend caution when comparing CCRT among species, studies, and laboratories.

716

717 6. Detecting variation in low temperature performance among 718 individuals, populations, seasons and treatments

719 Phenotypic plasticity is the ability of an organism with a given genotype to express
720 different phenotypes. Phenotypic plasticity in thermal biology includes hardening,
721 acclimation, which occurs in the laboratory, and acclimatization, which happens in nature,
722 although the mechanisms of acclimation and acclimatization are assumed to be similar
723 (Kingsolver, 2009; Tattersall et al., 2012), but different to the mechanisms underlying
724 hardening (Sinclair and Roberts, 2005; Teets and Denlinger, 2013). Developmental
725 plasticity is the response to conditions experienced during development (e.g. Sisodia and
726 Singh, 2010), while maternal (or trans-generational) effects refer to epigenetic signals
727 passed from the mother to offspring that determine phenotype (e.g. Magiafoglou and
728 Hoffmann, 2003). The time scale of phenotypic plasticity responses can range from
729 minutes or hours (often termed ‘hardening’ responses) to long-term shifts, such as seasonal
730 changes in cold tolerance (Fischer and Karl, 2010; Sinclair and Roberts, 2005). Genetic
731 adaptation is plasticity on an evolutionary scale, and may play out among populations or
732 species (Gibert and Huey, 2001; Kellermann et al., 2009; Sinclair et al., 2012).

733

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

741

742 *6.1 Phenotypic plasticity of low temperature biology in nature*

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

751 In addition to seasonal variation in cold tolerance, there is also evidence of
752 phenotypic plasticity over shorter timescales in nature. For example, survival of cold
753 exposure increases at night and decreases during the day in field-caged *Drosophila*
754 *melanogaster* (Overgaard and Sørensen, 2008). Similarly, the supercooling point
755 distribution of field-collected Antarctic springtails *Desoria klovstadi* (Collembola:

756 Isotomidae) decreases markedly between midday and midnight, reflecting changes in
757 ambient temperature (Sinclair et al., 2003c). Thus, it is important to consider these natural
758 variations in cold tolerance when determining the overwintering potential of an insect.

759

760 *6.2 Rapid cold-hardening*

761 Rapid cold-hardening (RCH) is the shortest time-scale of plastic responses, and
762 describes enhanced survival of temperature extremes after a brief (minutes to c. 3 h) pre-
763 exposure to sub-lethal temperatures (reviewed by Teets and Denlinger, 2013). Rapid cold
764 hardening can affect a range of cold tolerance parameters; for example, by decreasing LLT
765 (Lee et al., 1987), CT_{min} and CCRT (Ransberry et al., 2011), and even modifying the SCP in
766 some cases (e.g. Worland and Convey, 2001). Thus, RCH rapidly shifts insects from a non-
767 cold-hardy to a cold-hardy state in life stages that are not normally cold-tolerant, or at times
768 of year when seasonal cold tolerance has not yet been acquired. Because of this rapid shift
769 in cold tolerance, parameters like LLT may be substantially underestimated if RCH is not
770 taken into account. For example, non-diapausing adults of the elm leaf beetle
771 *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae), have only 15 % survival after 1 h at
772 -7 °C, but survival increases to 90 % if the -7 °C exposure is preceded by 4 h at 0 °C (Lee
773 et al., 1987). Rapid cold-hardening is mainly expressed in chill-susceptible species, but has
774 been reported in freeze-tolerant and freeze-avoidant species as well (Lee and Denlinger,
775 2010).

776

777 RCH may be induced by slow cooling, diurnal cycles and mild low temperatures
778 (usually -2 to +4 °C) (Teets and Denlinger, 2013). Unlike the heat shock response (see

779 Harrison et al., 2012), RCH does not normally require a recovery period after the pre-
780 exposure to express the enhanced cold tolerance. Nevertheless, including a recovery period
781 is commonly incorporated in laboratory assessments of RCH (e.g. Jakobs et al., 2015;
782 Nunamaker, 1993; Sinclair and Chown, 2003). Because the mechanisms of RCH are still
783 poorly understood (Teets and Denlinger, 2013), it is not entirely clear whether these
784 different treatments eliciting 'RCH' are actually triggering the same physiological
785 response, so it is important to report the methods fully, and potentially include several
786 different treatments in a study (e.g. Nunamaker, 1993).
787

788 *6.3 Acclimation, deacclimation and acclimatization*

789 Cold acclimation and acclimatization are processes that improve cold-hardiness
790 over a span of days-to-weeks, within a single life stage. Acclimation can have a marked
791 effect on cold tolerance; for example, a six-week cold acclimation increased survival of the
792 rusty grain beetle, *Cryptolestes ferrugineus*, from 1.4 days to 24 days at -10 °C. Cold-
793 acclimation regimes may be based on measured field conditions (e.g. Jakobs et al., 2015),
794 or based on convenience (e.g. the 4 °C of many domestic and laboratory refrigerators).
795 Notably, acclimation under fluctuating temperatures usually leads to greater cold tolerance
796 (Colinet et al., 2015). Although laboratory acclimation can never properly replicate field
797 conditions, the aim of incorporating laboratory acclimation is to provide some indication of
798 the extent of plasticity, and therefore the extent to which potential cold tolerance is being
799 underestimated.
800

801 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 °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 °C), and that this deacclimation was not reversed when the prepupae were returned to -10 °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

825 experience, but by the experience of the previous generation. For example, diapause by
826 *Aedes* eggs is determined by the photoperiod experienced by the mother (Denlinger and
827 Armbruster, 2014). Both developmental plasticity and trans-generational effects are easily
828 missed in laboratory studies, and could therefore mean that laboratory studies
829 underestimate low temperature performance in the field, but, to our knowledge, have been
830 rarely explored.
831

832 *6.5 Detecting phenotypic plasticity*

833 The complexity of phenotypic plasticity means that it cannot be completely
834 encompassed in simple laboratory experiments. Thus, we emphasise that the goal of
835 laboratory studies of phenotypic plasticity is to provide an indication of the extent to which
836 the full cold tolerance potential is being captured by the laboratory experiments. A
837 valuable alternative or complement to these experiments is to conduct seasonal
838 measurements of the cold tolerance of field-collected (or at least field-caged) individuals
839 (e.g. Baust and Miller, 1970; Crosthwaite et al., 2011; Koštál et al., 2014; Udaka and
840 Sinclair, 2014). These field studies may provide the best, and most ecologically-relevant,
841 estimate of the cold tolerance changes driven by the combined effects of temperature,
842 photoperiod and trans-generational and developmental plasticity.
843

844 The general approach to detecting plasticity of cold tolerance in the laboratory is to
845 apply pre-treatments or acclimation treatments, and measure some (preferably ecologically-
846 relevant) response variable or phenotype, such as CT_{min}, SCP or LLT. The treatments
847 could include both brief pre-treatments (to elicit rapid cold-hardening) and longer

848 treatments (to elicit acclimation responses). One convenient approach to detecting
849 plasticity phenotypes is the 'discriminating temperature' approach (Powell and Bale, 2004).
850 In this approach, a discriminating temperature (one that causes ~80 % mortality) is chosen
851 from preliminary investigations. Insects are then exposed to various pre-treatments and
852 acclimations, and their survival is measured only at that temperature. While the
853 discriminating temperature approach does not estimate the change in absolute limits of
854 survival, increased survival does provide evidence of plasticity, which can be explored in
855 greater detail as required. Discriminating temperatures are most often used in studies
856 documenting the RCH response (e.g. Sinclair and Chown, 2006), but it can also be used to
857 explore other sources of plasticity, including heat shock and acclimation (e.g. Rajamohan
858 and Sinclair, 2008, 2009). However, SCP is a relevant metric for freeze-avoidant species
859 (e.g. Worland and Convey, 2001), and CT_{min} or CCRT may also be used to identify
860 plasticity in a relatively high-throughput fashion (e.g. Everatt et al., 2013; Fischer et al.,
861 2010; Hoffmann et al., 2005; Sisodia and Singh, 2010).

862

863 There is an almost infinite variety of combinations of potential cues for phenotypic
864 plasticity (Table 4), so if the goal is simply to detect plasticity, it is most convenient to
865 choose a small combination of treatments that encompass a variety of cues and time
866 frames. Because it is the plasticity itself, not the precise cues, that is important, it can be
867 useful to deliberately conflate photoperiod and temperature cues to maximize the likelihood
868 of inducing plasticity. For example, acclimation treatments often combine short day length
869 with low temperatures, without controlling for each factor independently (e.g. Jakobs et al.,
870 2015).

871

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

885

886 Using the discriminating temperature approach means that a simple comparison of
887 low temperature survival by each treatment to that of the control can reveal significant
888 effects of the plasticity treatments. In this approach, the unit of replication is the group (see
889 also section 4.1), and 5-10 groups per treatment should provide adequate power. As with
890 LLT measurements, it is best if replicates are randomized, and controls run alongside
891 treatment groups. Some care may need to be taken to control for aging if this is a potential
892 factor in lengthy acclimation treatments, as aging can decrease (e.g. Halle et al., 2015) or
893 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 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 an invasion 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-

916 relevant and easy to measure, while in the former case, the emphasis may be on
917 determining thresholds that can be explored in the context of the habitat. No single
918 measure will be appropriate for all species or situations, and (like all work on living
919 organisms), it is best to begin with some basic observations. In this final section, we
920 provide two suggested approaches, one for a general exploration of low temperature
921 biology, the other for choosing a measure of low temperature performance.

922

923 To begin a comprehensive description of insect cold tolerance, we recommend
924 beginning with two simple observations: SCP (Section 2.3) and CT_{min} (Section 5.1). These
925 two parameters provide anchor points for designing experiments to further investigate
926 CCRT, cold tolerance strategy, and lethal limits (Fig. 10). In turn, these measures help to
927 determine appropriate response variables for examining phenotypic plasticity (Section 6).
928 Examples of this approach can be found in many of the papers cited in this review (e.g.
929 Koštál et al., 2014; Sinclair and Chown, 2005; Slabber and Chown, 2004). Subsequent
930 investigations of the biochemistry underlying plasticity and cold tolerance, for example,
931 changes in hemolymph composition, can then be incorporated into suitably-informed
932 sampling and analyses, that are beyond the scope of this review (see, e.g., Bale and
933 Hayward, 2010; Duman et al., 1991; Lee, 2010; Storey and Storey, 1991, 2013;
934 Zachariassen, 1991; Zachariassen and Kristiansen, 2000; Zachariassen et al., 2004). As we
935 have discussed, the biology of the organism will determine the utility of metrics used –
936 overwintering and diapausing insects, for example, often do not merit measurement of
937 CT_{min}, so CT_{min} is consequently absent from studies on many overwintering insects (e.g.
938 Koštál et al., 2014). A reasonable description of cold tolerance can be made with small

939 sample size, by carefully combining individual measures of SCP, survival, and lower lethal
940 temperature (e.g. Sinclair and Chown, 2005).
941

942 Choosing a single metric as a proxy for cold tolerance in a wider study can be more
943 difficult. If a more-or-less complete description of cold tolerance is available, it is a simple
944 matter to compare (or correlate) the possible variables, and choose a metric that is broadly
945 representative of thermal tolerances and logistically convenient for the organism at hand.
946 In *Drosophila*, for example, the CT_{min} provides a reasonably good proxy for cold tolerance
947 (including LT_{50}), and is thus a parsimonious choice for comparison among treatments
948 (Andersen et al., 2015b). If this information is not available, then choosing an appropriate
949 metric is going to be based partially on the life history of the organism, and striking a
950 useful measure may be somewhat dependent on luck. In Table 5, we present the
951 advantages and disadvantages of using each metric to facilitate choosing a particular one to
952 suit the aim of the study.
953

954 **8. Final remarks**

955 Low temperatures are one of the key limiting factors for the distribution and
956 performance of many terrestrial insects, and cold is a key component of overwintering
957 stress in temperate, polar, and alpine habitats. Because of this, the signal-to-noise ratio for
958 measures of cold tolerance is large, providing useful information in comparing insects at
959 multiple scales in time and space. In particular, to fully understand the implications of
960 climate change, there is increasing need to expand beyond growing-season studies to

biology in the fall and winter (Gallinat et al., 2015; Williams et al., 2015), for which low temperature biology is a critical component. 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 favourite and we therefore end by encouraging others to join us.

9. Acknowledgements

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 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).

10. References

Aldrich, J.C., 1987. Graphical analyses of bimodal frequency distributions with an example using *Cancer pagurus*. J Zool, Lond 211, 307-319.

Andersen, J.L., Macmillan, H.A., Overgaard, J., 2015a. Temperate *Drosophila* preserve cardiac function at low temperature. J Insect Physiol 77, 26-32.

Andersen, J.L., Manenti, T., Sorensen, J.G., MacMillan, H.A., Loeschcke, V., Overgaard, J., 2015b. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Funct Ecol 29, 55-65.

Asahina, E., 1969. Frost resistance in insects. Adv Insect Physiol 6, 1-49.

Bale, J.S., 1987. Insect cold hardiness: Freezing and supercooling - an ecophysiological perspective. J Insect Physiol 33, 899-908.

Bale, J.S., 1993. Classes of insect cold hardiness. Funct Ecol 7, 751-753.

Bale, J.S., 2002. Insects and low temperatures: from molecular biology to distributions and abundance. Phil Trans R Soc Lond B 357, 849-861.

Bale, J.S., Hansen, T.N., Nishino, M., Baust, J.G., 1989. Effect of cooling rate on the survival of larvae, pupariation, and adult emergence of the gallfly *Eurosta solidaginis*. Cryobiol 26, 285-289.

Bale, J.S., Hayward, S.A.L., 2010. Insect overwintering in a changing climate. J Exp Biol 213, 980-994.

Baust, J.G., Miller, L.K., 1970. Seasonal variations in glycerol content and its effect on cold-hardiness in the Alaskan carabid beetle, *Pterostichus brevicornis*. J Insect Physiol 16, 979-990.

Baust, J.G., Rojas, R.R., 1985. Insect cold hardiness: Facts and fancy. J Insect Physiol 31, 755-759.

Beaudry, R.M., Moggia, C.E., Retamales, J.B., Hancock, J.F., 1998. Quality of 'Ivanhoe' and 'Bluecrop' blueberry fruit transported by air and sea from Chile to North America. Hortscience 33, 313-317.

Bemani, M., Izadi, H., Mandian, K., Khani, A., Samih, M.A., 2012. Study on the physiology of diapause, cold hardiness and supercooling point of overwintering pupae of the pistachio fruit hull borer, *Arimania comaroffi*. J Insect Physiol 58, 897-902.

1022Bertoli, C.I., Scannapieco, A.C., Sambucetti, P., Norry, F.M., 2010. Direct and correlated
1023 responses to chill-coma recovery selection in *Drosophila buzzatii*. Entomol Exp Appl 134,
1024 154-159.
1025Block, W., 1982a. Cold hardiness in invertebrate poikilotherms. Comp Biochem Physiol 73A,
1026 581-593.
1027Block, W., 1982b. Supercooling points of insects and mites on the Antarctic Peninsula. Ecol
1028 Entomol 7, 1-8.
1029Block, W., Wharton, D.A., Sinclair, B.J., 1998. Cold tolerance of a New Zealand alpine
1030 cockroach, *Celatoblatta quinquemaculata* (Dictyoptera, Blattidae). Physiol Entomol 23, 1-
1031 6.
1032Block, W., Young, S.R., 1979. Measurement of supercooling in small arthropods and water
1033 droplets. Cryo-Lett 1, 85-91.
1034Boardman, L., Grout, T.G., Terblanche, J.S., 2012. False codling moth *Thaumatotibia leucotreta*
1035 (Lepidoptera, Tortricidae) larvae are chill-susceptible. Insect Sci 19, 315-328.
1036Bouchard, R.W., Carrillo, M.A., Kells, S.A., Ferrington, L.C., 2006. Freeze tolerance in larvae of
1037 the winter-active *Diamesa mendotae* Muttkowski (Diptera : Chironomidae): a contrast to
1038 adult strategy for survival at low temperatures. Hydrobiologia 568, 403-416.
1039Bürgi, L.P., Mills, N.J., 2010. Cold tolerance of the overwintering larval instars of light brown
1040 apple moth *Epiphyas postvittana*. J Insect Physiol 56, 1645-1650.
1041Burton, V., Mitchell, H.K., Young, P., Petersen, N.S., 1988. Heat-shock protection against cold
1042 stress of *Drosophila melanogaster*. Mol Cell Biol 8, 3550-3552.
1043Cannon, R.J.C., Block, W., 1988. Cold tolerance of microarthropods. Biol Rev 63, 23-77.
1044Chen, C.-P., Denlinger, D.L., Lee, R.E., Jr, 1987. Cold-shock injury and rapid cold hardening in
1045 the flesh fly *Sarcophaga crassipalpis*. Physiol Zool 60, 297-304.
1046Chown, S.L., Nicolson, S.W., 2004. Insect Physiological Ecology. Mechanisms and Patterns.
1047 Oxford University Press, Oxford.
1048Clarke, M.W., Thompson, G.J., Sinclair, B.J., 2013. Cold Tolerance of the Eastern Subterranean
1049 Termite, Reticulitermes flavipes (Isoptera: Rhinotermitidae), in Ontario. Environ Entomol
1050 42, 805-810.
1051Cloudsley-Thompson, J.L., 1973. Factors influencing the supercooling of tropical arthropoda,
1052 especially locusts. J Nat Hist 7, 471-480.

1053 Coello Alvarado, L.E., MacMillan, H.A., Sinclair, B.J., 2015. Chill-tolerant *Gryllus* crickets
1054 maintain ion balance at low temperatures. J Insect Physiol 77, 15-25.
1055 Coleman, P.C., Bale, J.S., Hayward, S.A.L., 2014. Cross-generation plasticity in cold hardiness is
1056 associated with diapause, but not the non-diapause developmental pathway, in the blow fly
1057 *Calliphora vicina*. J Exp Biol 217, 1454-1461.
1058 Colinet, H., Hoffmann, A.A., 2012. Comparing phenotypic effects and molecular correlates of
1059 developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*.
1060 Funct Ecol 26, 84-93.
1061 Colinet, H., Sinclair, B.J., Vernon, P., Renault, D., 2015. Insects in fluctuating thermal
1062 environments. Annu Rev Entomol 60, 123-140.
1063 Crawley, M.J., 2005. Statistics: an introduction using R. Wiley, Chichester.
1064 Crosthwaite, J.C., Sobek, S., Lyons, D.B., Bernards, M.A., Sinclair, B.J., 2011. The
1065 overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire
1066 (Coleoptera: Buprestidae). J Insect Physiol 57, 166-173.
1067 Czajka, M.C., Lee, R.E., Jr., 1990. A rapid cold-hardening response protecting against cold shock
1068 injury in *Drosophila melanogaster*. J Exp Biol 148, 245-254.
1069 David, J.R., Gibert, P., Moreteau, B., Gilchrist, G.W., Huey, R.B., 2003. The fly that came in
1070 from the cold: geographic variation of recovery time from low-temperature exposure in
1071 *Drosophila subobscura*. Funct Ecol 17, 425-430.
1072 David, R.J., Gibert, P., Pla, E., Petavy, G., Karan, D., Moreteau, B., 1998. Cold stress tolerance
1073 in *Drosophila*: Analysis of chill coma recovery in *D. melanogaster.* J Therm Biol 23, 291-
1074 299.
1075 Davidowitz, G., Nijhout, H.F., 2004. The physiological basis of reaction norms: The interaction
1076 among growth rate, the duration of growth and body size. Integr Comp Biol 44, 443-449.
1077 Denlinger, D.L., Armbruster, P.A., 2014. Mosquito Diapause. Annu Rev Entomol 59, 73-93.
1078 Denlinger, D.L., Lee, R.E., 2010. Insect Low Temperature Biology. Cambridge University Press,
1079 Cambridge.
1080 Dennis, A.B., Dunning, L.T., Sinclair, B.J., Buckley, T.R., 2015. Parallel molecular routes to cold
1081 adaptation in eight genera of New Zealand stick insects. Sci Reports 5, 13965.
1082 Duman, J.G., Wu, D.W., Xu, L., Tursman, D., Olsen, T.M., 1991. Adaptations of insects to
1083 subzero temperatures. Q Rev Biol 66, 387-410.

1084 Elnitsky, M.A., Hayward, S.A.L., Rinehart, J.P., Denlinger, D.L., Lee, R.E., 2008.
1085 Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic
1086 midge, *Belgica antarctica*. J Exp Biol 211, 524-530.
1087 Everatt, M.J., Bale, J.S., Convey, P., Worland, M.R., Hayward, S.A.L., 2013. The effect of
1088 acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates. J
1089 Insect Physiol 59, 1057-1064.
1090 Fields, P.G., 1993. Reduction of cold tolerance of stored-product insects by ice-nucleating-active
1091 bacteria. Environ Entomol 22, 470-476.
1092 Fields, P.G., Fleurat-Lessard, F., Lavenseau, L., Febvay, G., Peypelut, L., Bonnot, G., 1998. The
1093 effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino
1094 acid levels in *Sitophilus granarius* and *Cryptolestes ferrugineus* (Coleoptera). J Insect
1095 Physiol 44, 955-965.
1096 Findsen, A., Andersen, J.L., Calderon, S., Overgaard, J., 2013. Rapid cold hardening improves
1097 recovery of ion homeostasis and chill coma recovery time in the migratory locust, *Locusta*
1098 *migratoria*. J Exp Biol 216, 1630-1637.
1099 Fischer, K., Dierks, A., Franke, K., Geister, T.L., Liszka, M., Winter, S., Pflicke, C., 2010.
1100 Environmental Effects on Temperature Stress Resistance in the Tropical Butterfly *Bicyclus*
1101 *anynana*. PLoS ONE 5, e15284.
1102 Fischer, K., Karl, I., 2010. Exploring plastic and genetic responses to temperature variation using
1103 copper butterflies. Clim Res 43, 17-30.
1104 Franke, K., Dierks, A., Fischer, K., 2012. Directional selection on cold tolerance does not
1105 constrain plastic capacity in a butterfly. BMC Evol Biol 12, 14.
1106 Gallinat, A.S., Primack, R.B., Wagner, D.L., 2015. Autumn, the neglected season in climate
1107 change research. Trends Ecol Evol 30, 169-176.
1108 Gibert, P., Huey, R.B., 2001. Chill-coma temperature in *Drosophila*: Effects of developmental
1109 temperature, latitude, and phylogeny. Physiol Biochem Zool 74, 429-434.
1110 Gibert, P., Moreteau, B., Petavy, G., Karan, D., David, J.R., 2001. Chill-coma tolerance, a major
1111 climatic adaptation among *Drosophila* species. Evolution 55, 1063-1068.
1112 Hahn, D.A., Martin, A.R., Porter, S.D., 2008. Body Size, but Not Cooling Rate, Affects
1113 Supercooling Points in the Red Imported Fire Ant, *Solenopsis invicta*. Environ Entomol
1114 37, 1074-1080.

1115 Halle, S., Nowizki, A., Scharf, I., 2015. The consequences of parental age for development, body
1116 mass and resistance to stress in the red flour beetle. Biol J Linn Soc 115, 305-314.
1117 Hao, S.G., Kang, L., 2004. Supercooling capacity and cold hardiness of the eggs of the
1118 grasshopper *Chorthippus fallax* (Orthoptera : Acrididae). Eur J Entomol 101, 231-236.
1119 Harrison, J.F., Woods, H.A., Roberts, S.P., 2012. Ecological and Environmental Physiology of
1120 Insects. Oxford, New York.
1121 Hatherly, I.S., Bale, J.S., Walters, K.F.A., 2005. UK winter egg survival in the field and
1122 laboratory diapause of *Typhlodromips montdorensis*. Physiol Entomol 30, 87-91.
1123 Hazell, S.P., Bale, J.S., 2011. Low temperature thresholds: Are chill coma and CT(min)
1124 synonymous? J Insect Physiol 57, 1085-1089.
1125 Hazell, S.P., Pedersen, B.P., Worland, M.R., Blackburn, T.M., Bale, J.S., 2008. A method for the
1126 rapid measurement of thermal tolerance traits in studies of small insects. Physiol Entomol
1127 33, 389-394.
1128 Hoffmann, A.A., Dagher, H., Hercus, M., Berrigan, D., 1997. Comparing different measures of
1129 heat resistance in selected lines of *Drosophila melanogaster*. J Insect Physiol 43, 393-405.
1130 Hoffmann, A.A., Shirriffs, J., Scott, M., 2005. Relative importance of plastic vs genetic factors in
1131 adaptive differentiation: geographical variation for stress resistance in *Drosophila*
1132 *melanogaster* from eastern Australia. Funct Ecol 19, 222-227.
1133 Holmstrup, M., 2014. The ins and outs of water dynamics in cold tolerant soil invertebrates. J
1134 Therm Biol 45, 117-123.
1135 Holmstrup, M., Bayley, M., Ramløv, H., 2002. Supercool or dehydrate? An experimental
1136 analysis of overwintering strategies in small permeable Arctic invertebrates. Proc Natl
1137 Acad Sci USA 99, 5716-5720.
1138 Horwath, K.L., Duman, J.G., 1984. Yearly variations in the overwintering mechanisms of the
1139 cold-hardy beetle *Dendroides canadensis.* Physiol Zool 57, 40-45.
1140 Hu, X.P., Appel, A.G., 2004. Seasonal variation of critical thermal limits and temperature
1141 tolerance in Formosan and eastern subterranean termites (Isoptera : Rhinotermitidae). Env
1142 Entomol 33, 197-205.
1143 Huey, R.B., Crill, W.D., Kingsolver, J.G., Weber, K.E., 1992. A Method for Rapid Measurement
1144 of Heat or Cold Resistance of Small Insects. Funct Ecol 6, 489-494.

1145Jakobs, R., Gariepy, T.D., Sinclair, B.J., 2015. Adult plasticity of cold tolerance in a continental-
1146 temperate population of *Drosophila suzukii*. J Insect Physiol 79, 1-9.
1147Ju, R.T., Xiao, Y.Y., Li, B., 2011. Rapid cold hardening increases cold and chilling tolerances
1148 more than acclimation in the adults of the sycamore lace bug, *Corythucha ciliata* (Say)
1149 (Hemiptera: Tingidae). J Insect Physiol 57, 1577-1582.
1150Jumbam, K.R., Jackson, S., Terblanche, J.S., McGeoch, M.A., Chown, S.L., 2008. Acclimation
1151 effects on critical and lethal thermal limits of workers of the Argentine ant, *Linepithema*
1152 *humile*. J Insect Physiol 54, 1008-1014.
1153Kellermann, V., van Heerwaarden, B., Sgro, C.M., Hoffmann, A.A., 2009. Fundamental
1154 Evolutionary Limits in Ecological Traits Drive *Drosophila* Species Distributions. Science
1155 325, 1244-1246.
1156Kelty, J.D., Lee, R.E., Jr., 1999. Induction of rapid cold hardening by ecologically relevant
1157 cooling rates in *Drosophila melanogaster*. J Insect Physiol 45, 719-726.
1158Khani, A., Moharramipour, S., 2010. Cold hardiness and supercooling capacity in the
1159 overwintering larvae of the codling moth, *Cydia pomonella*. J Insect Sci 10, 12.
1160Kingsolver, J.G., 2009. The Well-Temperatured Biologist. Amer Nat 174, 755-768.
1161Kingsolver, J.G., Huey, R.B., 2008. Size, temperature, and fitness: three rules. Evol Ecol Res 10,
1162 251-268.
1163Kleynhans, E., Conlong, D.E., Terblanche, J.S., 2014. Host plant-related variation in thermal
1164 tolerance of *Eldana saccharina*. Entomol Exp Appl 150, 113-122.
1165Klok, C.J., Chown, S.L., 1997. Critical thermal limits, temperature tolerance and water balance
1166 of a sub-Antarctic caterpillar, *Pringleophaga marioni* (Lepidoptera: Tineidae). J Insect
1167 Physiol 43, 685-694.
1168Koštál, V., Havelka, J., 2000. Diapausing larvae of the midge *Aphidoletes aphidimyza* (Diptera:
1169 Cecidomyiidae) survive at subzero temperatures in a supercooled state but tolerate
1170 freezing if inoculated by external ice. Eur J Entomol 96, 433-436.
1171Koštál, V., Korbelova, J., Rozsypal, J., Zahradnickova, H., Cimlova, J., Tomcala, A., Simek, P.,
1172 2011a. Long-Term Cold Acclimation Extends Survival Time at 0 degrees C and Modifies
1173 the Metabolomic Profiles of the Larvae of the Fruit Fly *Drosophila melanogaster*. PLoS
1174 One 6, 10.

1175 Košťál, V., Miklas, B., Dolezal, P., Rozsypal, J., Zahradnickova, H., 2014. Physiology of cold
1176 tolerance in the bark beetle, *Pityogenes chalcographus* and its overwintering in spruce
1177 stands. J Insect Physiol 63, 62-70.
1178 Košťál, V., Simek, P., 2000. Overwintering strategy in *Pyrrhocoris apterus* (Heteroptera): the
1179 relations between life-cycle, chill tolerance and physiological adjustments. J Insect
1180 Physiol 46, 1321-1329.
1181 Košťál, V., Simek, P., Zahradnickova, H., Cimlova, J., Stetina, T., 2012. Conversion of the chill
1182 susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. Proc
1183 Natl Acad Sci U S A 109, 3270-3274.
1184 Košťál, V., Vambera, J., Bastl, J., 2004. On the nature of pre-freeze mortality in insects: water
1185 balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. J Exp
1186 Biol 207, 1509-1521.
1187 Košťál, V., Yanagimoto, M., Bastl, J., 2006. Chilling-injury and disturbance of ion homeostasis in
1188 the coxal muscle of the tropical cockroach (*Nauphoeta cinerea*). Comp Biochem Physiol
1189 B 143, 171-179.
1190 Košťál, V., Zahradnickova, H., Simek, P., 2011b. Hyperprolinemic larvae of the drosophilid fly,
1191 *Chymomyza costata*, survive cryopreservation in liquid nitrogen. Proc Natl Acad Sci USA
1192 108, 13041-13046.
1193 Lalouette, L., Vernon, P., Amat, H., Renault, D., 2010. Ageing and thermal performance in the
1194 sub-Antarctic wingless fly *Anatalanta aptera* (Diptera: Sphaeroceridae): older is better.
1195 Biol Lett 6, 346-349.
1196 Layne, J.R., Blakeley, D.L., 2002. Effect of freeze temperature on ice formation and long- term
1197 survival of the wooly bear caterpillar (*Pyrrharctia isabella*). J Insect Physiol 48, 1133-
1198 1137.
1199 Layne, J.R., Lee, R.E., Huang, J.L., 1990. Inoculation triggers freezing at high subzero
1200 temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*).
1201 Can J Zool 68, 506-510.
1202 Leather, S.R., Walters, K.F.A., Bale, J.S., 1993. The Ecology of Insect Overwintering.
1203 Cambridge University Press, Cambridge.
1204 Lee, R.E., 1991. Principles of insect low temperature tolerance, in: Lee, R.E., Denlinger, D.L.
1205 (Eds.), Insects at Low Temperature. Chapman and Hall, New York, pp. 17-46.

1206Lee, R.E., 2010. A primer on insect cold tolerance, in: Denlinger, D.L., Lee, R.E. (Eds.), Low
1207 Temperature Biology of Insects. Cambridge University Press, Cambridge, pp. 3-34.
1208Lee, R.E., Chen, C.-P., Denlinger, D.L., 1987. A rapid cold-hardening process in insects. Science
1209 238, 1415-1417.
1210Lee, R.E., Costanzo, J.P., Kaufman, P.E., Lee, M.R., Wyman, J.A., 1994. Ice-nucleating active
1211 bacteria reduce the cold-hardiness of the freeze-intolerant Colorado Potato Beetle
1212 (Coleoptera: Chrysomelidae). J Econ Entomol 87, 377-381.
1213Lee, R.E., Denlinger, D.L., 2010. Rapid cold-hardening: Ecological significance and
1214 underpinning mechanisms, in: Denlinger, D.L., Lee, R.E. (Eds.), Low temperature biology
1215 of insects. Cambridge University Press, Cambridge, pp. 35-58.
1216Lee, R.E., Hankison, S.J., 2003. Acquisition of freezing tolerance in early autumn and seasonal
1217 changes in gall water content influence inoculative freezing of gall fly larvae, *Eurosta*
1218 *solidaginis* (Diptera, Tephritidae). J Insect Physiol 49, 385-393.
1219Lee, R.E., Lewis, E.A., 1985. Effect of temperature and duration of exposure on tissue ice
1220 formation in the gall fly, *Eurosta solidaginis* (Diptera, Tephritidae). Cryo-Lett 7, 25-34.
1221Li, N., Zachariassen, K., 2007. Cold hardiness of insects distributed in the area of Siberian Cold
1222 Pole. Comp Biochem Physiol A 146, S156-S157.
1223Lighton, J.R.B., Turner, R.J., 2004. Thermolimit respirometry: an objective assessment of critical
1224 thermal maxima in two sympatric desert harvester ants, *Pogonomyrmex rugosus* and *P.*
1225 *californicus*. J Exp Biol 207, 1903-1913.
1226Macdonald, S.S., Rako, L., Batterham, P., Hoffmann, A.A., 2004. Dissecting chill coma recovery
1227 as a measure of cold resistance: evidence for a biphasic response in *Drosophila*
1228 *melanogaster*. J Insect Physiol 50, 695-700.
1229MacMillan, H.A., Sinclair, B.J., 2011a. Mechanisms underlying insect chill-coma. J Insect
1230 Physiol 57, 12-20.
1231MacMillan, H.A., Sinclair, B.J., 2011b. The role of the gut in insect chilling injury: cold-induced
1232 disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. J Exp Biol
1233 214, 726-734.
1234MacMillan, H.A., Walsh, J.P., Sinclair, B.J., 2009. The effects of selection for cold tolerance on
1235 cross-tolerance to other environmental stressors in *Drosophila melanogaster*. Insect Sci
1236 16, 263-276.

1237MacMillan, H.A., Williams, C.M., Staples, J.F., Sinclair, B.J., 2012a. Metabolism and energy
1238 supply below the critical thermal minimum of a chill-susceptible insect. J Exp Biol 215,
1239 1366-1372.
1240Macmillan, H.A., Williams, C.M., Staples, J.F., Sinclair, B.J., 2012b. Reestablishment of ion
1241 homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. Proc Natl
1242 Acad Sci USA 109, 20750-20755.
1243Magiafoglou, A., Hoffmann, A.A., 2003. Cross-generation effects due to cold exposure in
1244 *Drosophila serrata*. Funct Ecol 17, 664-672.
1245Marshall, K.E., Sinclair, B.J., 2011. The sub-lethal effects of repeated freezing in the woolly bear
1246 caterpillar *Pyrrharctia isabella*. J Exp Biol 214, 1205-1212.
1247McArdle, B.H., 1996. Levels of evidence in studies of competition, predation, and disease. N Z J
1248 Ecol 20, 7-15.
1249Miller, L.K., 1969. Freezing tolerance in an adult insect. Science 166, 105-106.
1250Miller, L.K., 1978. Freezing tolerance in relation to cooling rate in an adult insect. Cryobiol 15,
1251 345-349.
1252Mori, N., Kimura, M.T., 2008. Selection for rapid and slow recovery from chill- and heat-coma
1253 in *Drosophila melanogaster*. Biol J Linnean Soc 95, 72-80.
1254Morrissey, R., Baust, J.G., 1976. The ontogeny of cold tolerance in the gall fly, *Eurosta*
1255 *solidaginis*. J Insect Physiol 22, 431-438.
1256Nedvěd, O., 1998. Modelling the relationship between cold injury and accumulated degree days
1257 in terrestrial arthropods. Cryo-Lett 19, 267-274.
1258Nedvěd, O., Hodková, M., Brunnhofer, V., Hodek, I., 1995. Simultaneous measurement of low
1259 temperature survival and supercooling in a sample of insects. Cryo-Lett 16, 108-113.
1260Nedvěd, O., Lavy, D., Verhoef, H.A., 1998. Modelling the time-temperature relationship in cold
1261 injury and effect of high-temperature interruptions on survival in a chill-sensitive
1262 collembolan. Funct Ecol 12, 816-824.
1263Nguyen, C., Bahar, M.H., Baker, G., Andrew, N.R., 2014. Thermal Tolerance Limits of
1264 Diamondback Moth in Ramping and Plunging Assays. PLoS One 9, e87535.
1265Nilson, T.N., Sinclair, B.J., Roberts, S.P., 2006. The effects of carbon dioxide anesthesia and
1266 anoxia on rapid cold-hardening and chill coma recovery in *Drosophila melanogaster*. J
1267 Insect Physiol 52, 1027-1033.

1268 Nunamaker, R.A., 1993. Rapid Cold-Hardening in *Culicoides variipennis sonorensis* (Diptera,
1269 Ceratopogonidae). J Med Entomol 30, 913-917.
1270 Nyamukondiwa, C., Terblanche, J.S., Marshall, K.E., Sinclair, B.J., 2011. Basal cold but not heat
1271 tolerance constrains plasticity among *Drosophila* species (Diptera: Drosophilidae). J Evol
1272 Biol 24, 1927-1938.
1273 Nylin, S., Gotthard, K., 1998. Plasticity in life-history traits. Annu Rev Entomol 43, 63-83.
1274 Overgaard, J., Sørensen, J.G., 2008. Rapid thermal adaptation during field temperature variations
1275 in *Drosophila melanogaster*. Cryobiology 56, 159-162.
1276 Palmer, C.M., Siebke, K., Yeates, D.K., 2004. Infrared video thermography: a technique for
1277 assessing cold adaptation in insects. Biotechniques 37, 212-217.
1278 Pauli, J.N., Zuckerberg, B., Whiteman, J.P., Porter, W., 2013. The subnivium: a deteriorating
1279 seasonal refugium. Frontiers Ecol Env 11, 260-267.
1280 Powell, S.J., Bale, J.S., 2004. Cold shock injury and ecological costs of rapid cold hardening in
1281 the grain aphid Sitobion avenae (Hemiptera : Aphididae). J Insect Physiol 50, 277-284.
1282 Powell, S.J., Bale, J.S., 2005. Low temperature acclimated populations of the grain aphid
1283 *Sitobion avenae* retain ability to rapidly cold harden with enhanced fitness. J Exp Biol
1284 208, 2615-2620.
1285 Powell, S.J., Bale, J.S., 2008. Intergenerational acclimation in aphid overwintering. Ecol
1286 Entomol 33, 95-100.
1287 Rajamohan, A., Sinclair, B.J., 2008. Short-term hardening effects on survival of acute and
1288 chronic cold exposure by *Drosophila melanogaster* larvae. J Insect Physiol 54, 708-718.
1289 Rajamohan, A., Sinclair, B.J., 2009. Hardening trumps acclimation in improving cold tolerance
1290 of *Drosophila melanogaster* larvae. Physiol Entomol 34, 217-223.
1291 Ramløv, H., Westh, P., 1993. Ice formation in the freeze-tolerant alpine weta *Hemideina maori*
1292 Hutton (Orthoptera: Stenopelmatidae). Cryo-Lett 14, 169-176.
1293 Ransberry, V.E., Macmillan, H.A., Sinclair, B.J., 2011. The relationship between chill-coma
1294 onset and recovery at the extremes of the thermal window of *Drosophila melanogaster*.
1295 Physiol Biochem Zool 84, 553-559.
1296 Reis, M., Vieira, C.P., Morales-Hojas, R., Aguiar, B., Rocha, H., Schlotterer, C., Vieira, J., 2011.
1297 A Comparative Study of the Short Term Cold Resistance Response in Distantly Related
1298 *Drosophila* Species: The Role of *regucalcin* and *Frost*. PLoS One 6.

1299 Renault, D., Lalouette, L., 2012. Critical thermal minima of three sub-Antarctic insects from the
1300 French southern Indian Ocean islands. Antarct Sci 24, 43-44.
1301 Renault, D., Salin, C., Vannier, G., Vernon, P., 1999. Survival and chill-coma in the adult lesser
1302 mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), exposed to low
1303 temperatures. J Therm Biol 24, 229-236.
1304 Renault, D., Salin, C., Vannier, G., Vernon, P., 2002. Survival at low temperatures in insects:
1305 What is the ecological significance of the supercooling point? Cryolett 23, 217-228.
1306 Renault, D., Vernon, P., Vannier, G., 2004. Comparing the freezing susceptibility of third-instar
1307 larvae of *Gnorimus variabilis* (Cetoniidae : Trichiinae) from three distant geographical
1308 regions. Can J Zool 82, 873-879.
1309 Rezende, E.L., Tejedo, M., Santos, M., 2011. Estimating the adaptive potential of critical thermal
1310 limits: methodological problems and evolutionary implications. Funct Ecol 25, 111-121.
1311 Ring, R.A., Riegert, P.W., 1991. A tribute to R. W. Salt, in: Lee, R.E., Denlinger, D.L. (Eds.),
1312 Insects at Low Temperature. Chapman and Hall, New York, pp. 3-16.
1313 Salin, C., Renault, D., Vannier, G., Vernon, P., 2000. A sexually dimorphic response in
1314 supercooling temperature, enhanced by starvation, in the lesser mealworm *Alphitobius*
1315 *diaperinus* (Coleoptera : Tenebrionidae). J Therm Biol 25, 411-418.
1316 Salt, R.W., 1961. Principles of insect cold hardiness. Ann Rev Entomol 6, 55-74.
1317 Salt, R.W., 1966. Effect of cooling rate on the freezing temperatures of supercooled insects. Can
1318 J Zool 44, 655-659.
1319 Santos, M., Castaneda, L.E., Rezende, E.L., 2011. Making sense of heat tolerance estimates in
1320 ectotherms: lessons from *Drosophila*. Funct Ecol 25, 1169-1180.
1321 Scharf, I., Galkin, N., Halle, S., 2015. Disentangling the Consequences of Growth Temperature
1322 and Adult Acclimation Temperature on Starvation and Thermal Tolerance in the Red Flour
1323 Beetle. Evol Biol 42, 54-62.
1324 Sformo, T., Kohl, F., McIntyre, J., Kerr, P., Duman, J.G., Barnes, B.M., 2009. Simultaneous
1325 freeze tolerance and avoidance in individual fungus gnats, *Exechia nugatoria*. J Comp
1326 Physiol B 179, 897-902.
1327 Sformo, T., McIntyre, J., Walters, K.R., Barnes, B.M., Duman, J., 2011. Probability of freezing in
1328 the freeze-avoiding beetle larvae *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) from
1329 interior Alaska. J Insect Physiol 57, 1170-1177.

1330Shimada, K., Riihimaa, A., 1988. Cold acclimation, inoculative freezing and slow cooling:
1331 essential factors contributing to the freezing-tolerance in diapausing larvae of *Chymomyza*
1332 *costata* (Diptera: Drosophilidae). Cryo-Lett 9, 5-10.
1333Shuman, D., Coffelt, J.A., Weaver, D.K., 1996. A computer-based electronic fall-through probe
1334 insect counter for monitoring infestation in stored products. Trans ASAE 39, 1773-1780.
1335Sinclair, B., Klok, C., Scott, M., Terblanche, J., Chown, S., 2003a. Diurnal variation in
1336 supercooling points of three species of Collembola from Cape Hallett, Antarctica. J Insect
1337 Physiol 49, 1049-1061.
1338Sinclair, B., Vernon, P., Klok, C., Chown, S., 2003b. Insects at low temperatures: an ecological
1339 perspective. Trends Ecol Evol 18, 257-262.
1340Sinclair, B.J., 1997. Seasonal variation in freezing tolerance of the New Zealand alpine
1341 cockroach *Celatoblatta quinquemaculata.* Ecol Entomol 22, 462-467.
1342Sinclair, B.J., 1999. Insect cold tolerance: How many kinds of frozen? Eur J Entomol 96, 157-
1343 164.
1344Sinclair, B.J., 2001. Field ecology of freeze tolerance: interannual variation in cooling rates,
1345 freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta*
1346 *quinquemaculata*. Oikos 93, 286-293.
1347Sinclair, B.J., Chown, S.L., 2003. Rapid responses to high temperature and desiccation but not to
1348 low temperature in the freeze tolerant sub-Antarctic caterpillar *Pringleophaga marioni*
1349 (Lepidoptera, Tineidae). J Insect Physiol 49, 45-52.
1350Sinclair, B.J., Chown, S.L., 2005. Climatic variability and hemispheric differences in insect cold
1351 tolerance: support from southern Africa. Funct Ecol 19, 214-221.
1352Sinclair, B.J., Chown, S.L., 2006. Rapid cold-hardening in a Karoo beetle, *Afrinus* sp. Physiol
1353 Entomol 31, 98-101.
1354Sinclair, B.J., Gibbs, A.G., Lee, W.K., Rajamohan, A., Roberts, S.P., Socha, J.J., 2009.
1355 Synchrotron X-Ray Visualisation of Ice Formation in Insects during Lethal and Non-
1356 Lethal Freezing. Plos One 4, E8259.
1357Sinclair, B.J., Klok, C.J., Chown, S.L., 2004. Metabolism of the sub-Antarctic caterpillar
1358 *Pringleophaga marioni* during cooling, freezing and thawing. J Exp Biol 207, 1287-1294.

1359 Sinclair, B.J., Klok, C.J., Scott, M.B., Terblanche, J.S., Chown, S.L., 2003c. Diurnal variation in
1360 supercooling points of three species of Collembola from Cape Hallett, Antarctica. J Insect
1361 Physiol 49, 1049-1061.
1362 Sinclair, B.J., Roberts, S.P., 2005. Acclimation, shock and hardening in the cold. J Therm Biol
1363 30, 557-562.
1364 Sinclair, B.J., Sjursen, H., 2001. Cold tolerance of the Antarctic springtail *Gomphiocephalus*
1365 *hodgsoni* (Collembola, Hypogastruridae). Antarct Sci 13, 271-279.
1366 Sinclair, B.J., Stinziano, J.R., Williams, C.M., MacMillan, H.A., Marshall, K.E., Storey, K.B.,
1367 2013. Real-time measurement of metabolic rate during freezing and thawing of the wood
1368 frog, *Rana sylvatica*: implications for overwinter energy use. J Exp Biol 216, 292-302.
1369 Sinclair, B.J., Terblanche, J.S., Scott, M.B., Blatch, G., Klok, C.J., Chown, S.L., 2006a.
1370 Environmental physiology of three species of Collembola at Cape Hallett, North Victoria
1371 Land, Antarctica. J Insect Physiol 52, 29-50.
1372 Sinclair, B.J., Terblanche, J.S., Scott, M.B., Blatch, G.L., Klok, C.J., Chown, S.L., 2006b.
1373 Environmental physiology of three species of Collembola at Cape Hallett, North Victoria
1374 Land, Antarctica. J Insect Physiol 52, 29-50.
1375 Sinclair, B.J., Williams, C.M., Terblanche, J.S., 2012. Variation in thermal performance among
1376 insect populations. Physiol Biochem Zool 85, 594-606.
1377 Sinclair, B.J., Worland, M.R., Wharton, D.A., 1999. Ice nucleation and freezing tolerance in New
1378 Zealand alpine and lowland weta, *Hemideina* spp. (Orthoptera; Stenopelmatidae). Physiol
1379 Entomol 24, 56-63.
1380 Sisodia, S., Singh, B.N., 2010. Influence of developmental temperature on cold shock and chill
1381 coma recovery in *Drosophila ananassae*: Acclimation and latitudinal variations among
1382 Indian populations. J Therm Biol 35, 117-124.
1383 Sisodia, S., Singh, B.N., 2012. Experimental evidence for nutrition regulated stress resistance in
1384 Drosophila ananassae. PloS one 7, e46131.
1385 Slabber, S., Chown, S.L., 2004. Thermal tolerance and cold hardiness strategy of the sub-
1386 Antarctic psocid *Antarctopsocus jeanneli* Badonnel. Polar Biol 28, 56-61.
1387 Slabber, S., Chown, S.L., 2005. Differential responses of thermal tolerance to acclimation in the
1388 sub-Antarctic rove beetle *Halmaeusa atriceps*. Physiol Entomol 30, 195-204.

1389 Sobek-Swant, S., Crosthwaite, J.C., Lyons, D.B., Sinclair, B.J., 2012. Could phenotypic
1390 plasticity limit an invasive species? Incomplete reversibility of mid-winter deacclimation
1391 in emerald ash borer. Biol Invasions 14, 115-125.
1392 Sømme, L., 1982. Supercooling and winter survival in terrestrial arthropods. Comp Biochem
1393 Physiol 73A, 519-543.
1394 Sømme, L., 1995. Invertebrates in Hot and Cold Arid Environments. Springer-Verlag, Berlin.
1395 Sømme, L., 1996. The effect of prolonged exposures at low temperatures in insects. Cryo-Lett
1396 17, 341-346.
1397 Sømme, L., 2000. The history of cold hardiness research in terrestrial arthropods. CryoLett 21,
1398 289-296.
1399 Sømme, L., Davidson, R.L., Onore, G., 1996. Adaptations of insects at high altitudes of
1400 Chimborazo, Ecuador. Eur J Entomol 93, 313-318.
1401 Sømme, L., Strømme, A., Zachariassen, K.E., 1993. Notes on the ecology and physiology of the
1402 Antarctic oribatid mite *Maudheimia wilsoni*. Polar Res 12, 21-25.
1403 Sømme, L., Zachariassen, K.E., 1981. Adaptations to low temperature in high altitude insects
1404 from Mount Kenya. Ecol Entomol 6, 199-204.
1405 Sørensen, J.G., Holmstrup, M., 2011. Cryoprotective dehydration is widespread in Arctic
1406 springtails. J Insect Physiol 57, 1147-1153.
1407 Storey, K.B., Storey, J.M., 1991. Biochemistry of cryoprotectants, in: Lee, R.E., Denlinger, D.L.
1408 (Eds.). Chapman and Hall, New York, pp. 64-93.
1409 Storey, K.B., Storey, J.M., 2013. Molecular Biology of Freezing Tolerance. Compr Physiol 3,
1410 1283-1308.
1411 Strachan, L.A., Tarnowski-Garner, H.E., Marshall, K.E., Sinclair, B.J., 2011. The Evolution of
1412 Cold Tolerance in *Drosophila* Larvae. Physiol Biochem Zool 84, 43-53.
1413 Tattersall, G.J., Sinclair, B.J., Withers, P.C., Fields, P.A., Seebacher, F., Cooper, C.E., Maloney,
1414 S.K., 2012. Coping with Thermal Challenges: Physiological Adaptations to Environmental
1415 Temperatures. Compr Physiol 2, 2151-2202.
1416 Teets, N.M., Denlinger, D.L., 2013. Physiological mechanisms of seasonal and rapid cold-
1417 hardening in insects. Physiol Entomol 38, 105-116.

1418 Telonis-Scott, M., Hallas, R., McKechnie, S.W., Wee, C.W., Hoffmann, A.A., 2009. Selection for
1419 cold resistance alters gene transcript levels in *Drosophila melanogaster*. J Insect Physiol
1420 55, 549-555.
1421 Terblanche, J.S., Deere, J.A., Clusella-Trullas, S., Janion, C., Chown, S.L., 2007. Critical thermal
1422 limits depend on methodological context. Proc R Soc B 274, 2935-2942.
1423 Terblanche, J.S., Hoffmann, A.A., Mitchell, K.A., Rako, L., le Roux, P.C., Chown, S.L., 2011.
1424 Ecologically relevant measures of tolerance to potentially lethal temperatures. J Exp Biol
1425 214, 3713-3725.
1426 Terblanche, J.S., Klok, C.J., Krafsur, E.S., Chown, S.L., 2006. Phenotypic plasticity and
1427 geographic variation in thermal tolerance and water loss of the tsetse *Glossina pallidipes*
1428 (Diptera : Glossinidae): Implications for distribution modelling. Amer J Trop Med Hyg 74,
1429 786-794.
1430 Tomcala, A., Tollarova, M., Overgaard, J., Simek, P., Koštál, V., 2006. Seasonal acquisition of
1431 chill tolerance and restructuring of membrane glycerophospholipids in an overwintering
1432 insect: triggering by low temperature, desiccation and diapause progression. J Exp Biol
1433 209, 4102-4114.
1434 Udaka, H., Sinclair, B.J., 2014. The overwintering biology of the acorn weevil, *Curculio*
1435 *glandium* in southwestern Ontario. J Therm Biol 44, 103-109.
1436 van der Merwe, M., Chown, S.L., Smith, V.R., 1997. Thermal tolerance limits in six weevil
1437 species (Coleoptera, Curculionidae) from sub-Antarctic Marion Island. Polar Biol 18, 331-
1438 336.
1439 van Dooremalen, C., Berg, M.P., Ellers, J., 2013. Acclimation responses to temperature vary with
1440 vertical stratification: implications for vulnerability of soil-dwelling species to extreme
1441 temperature events. Global Change Biol 19, 975-984.
1442 Voituron, Y., Mouquet, N., de Mazancourt, C., Clobert, J., 2002. To freeze or not to freeze? An
1443 evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms.
1444 Amer Nat 160, 255-270.
1445 Watanabe, M., Tanaka, K., 1998. Adult diapause and cold hardiness in Aulacophora nigripennis
1446 (Coleoptera : Chrysomelidae). J Insect Physiol 44, 1103-1110.
1447 Wharton, D.A., Rowland, J.J., 1984. A thermoelectric cooling stage for the measurement of the
1448 supercooling points of microscopic organisms. J Microsc 134, 299-305.

1449 Williams, C.M., Hellmann, J.J., Sinclair, B.J., 2012. Lepidopteran species differ in susceptibility
1450 to winter warming. Clim Res 53, 119-130.
1451 Williams, C.M., Henry, H.A.L., Sinclair, B.J., 2015. Cold truths: how winter drives responses of
1452 terrestrial organisms to climate change. Biol Rev 90, 214-235.
1453 Williams, C.M., Nicolai, A., Ferguson, L.V., Bernards, M.A., Hellmann, J.J., Sinclair, B.J., 2014.
1454 Cold hardiness and deacclimation of overwintering *Papilio zelicaon* pupae. Comp
1455 Biochem Physiol A 178, 51-58.
1456 Worland, M.R., Convey, P., 2001. Rapid cold hardening in Antarctic microarthropods. Funct Ecol
1457 15, 515-524.
1458 Worland, M.R., Leinaas, H.P., Chown, S.L., 2006. Supercooling point frequency distributions in
1459 Collembola are affected by moulting. Funct Ecol 20, 323-329.
1460 Zachariassen, K.E., 1985. Physiology of cold tolerance in insects. Physiol Rev 65, 799-832.
1461 Zachariassen, K.E., 1991. The water relations of overwintering insects, in: Lee, R.E., Denlinger,
1462 D.L. (Eds.). Chapman and Hall, New York, pp. 47-63.
1463 Zachariassen, K.E., Kristiansen, E., 2000. Ice nucleation and antinucleation in nature. Cryobiol
1464 41, 257-279.
1465 Zachariassen, K.E., Kristiansen, E., Pedersen, S.A., 2004. Inorganic ions in cold-hardiness.
1466 Cryobiol 48, 126-133.

1467

Figure Captions

Figure 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).

Figure 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.

Figure 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.

1489 **Figure 4. Supercooling point distributions from two different species of Antarctic**
1490 **Collembola. A.** A bimodal distribution of supercooling points of *Cryptopygus*
1491 *cisantarcticus* (Isotomidae). **B.** A unimodal distribution of supercooling points of *Friesea*
1492 *grisea* (Neanuridae). Redrawn from Sinclair et al., (2006a).

1493

1494 **Figure 5. Time course of ice accumulation in adult *Hemideina maori* held at -5 °C.**
1495 Redrawn after Ramløv and Westh (1993).

1496

1497 **Figure 6. Hallmarks of cryoprotective dehydration in insects. A.** Characteristically,
1498 insect body water content and the melting point of the insect will decrease as the ambient
1499 temperature decreases. Redrawn from Sinclair et al. (2003b). **B.** In the collembolan
1500 *Onychiurus arcticus,* the melting point of the body fluids does not change over time if the
1501 insect is held over water, whereas the melting point decreases over time and mimics that of
1502 the ambient temperature when the insect is held over ice. The dotted line represents the
1503 average melting point over ice and the solid line represents the ambient temperature.
1504 Redrawn from Holmstrup et al. (2002).

1505

1506 **Figure 7. Determining lethal temperatures. A.** Estimate of the lower lethal temperature of
1507 *Diamesa mendotae* based on survival of subzero temperatures. Flies were cooled to subzero
1508 temperatures and immediately re-warmed upon reaching the designated temperature.
1509 Survival was recorded 24h following exposure. The lower lethal temperature was estimated
1510 to be -21.5 °C. Redrawn from Bouchard et al. (2006). **B**. Survival of *Eldana saccharina*

1511 larvae exposed to low temperatures**.** Larvae were directly exposed to temperatures ranging

1512 from 10 °C to -10 °C for 2 h. Solid line represents the fitted logistic regression model. Re-

1513 drawn from Kleynhans et al. (2014).

1514

1515 **Figure 8. Variation in chill-coma recovery time depending on entry (A, B) and exit (C,**

1516 **D) from chill coma**. **A**. CCR of insects x, y, and z are equal, because equilibrium is reached

1517 during time spent in chill coma. **B.** CCR of x is less than y (and z), because the rate of entry

1518 into chill coma did not allow for equilibrium to be reached before recovery began. **C**. CCR

1519 of y is greater than x, despite the same time spent in chill coma, because the equilibrium of y

1520 is lower than that of x. **D**. The CCR of y is greater than x because recovery occurs at a

1521 slower rate. E_k = membrane equilibrium potential of K^+.

1522

1523 **Figure 9. Examples of temperature & photoperiod manipulations used induce**

1524 **phenotypic plasticity in the laboratory. A**. Acute exposure to low temperatures to induce a

1525 rapid cold-hardening response. **B**. A 5-10 day exposure to low temperatures and short day-

1526 length. **C.** Development under low temperatures and short day-length. **D.** Exposure to

1527 gradually declining temperature and photoperiod. **L** = light**, D =** dark.

1528

1529 **Figure 10.** Recommended work flow to determine cold tolerance strategies and lower

1530 thermal limits for investigating overwintering potential and comparisons of cold tolerance

1531 among groups. **A.** Work flow for determining cold tolerance strategy and overwintering

1532 potential. **B.** Work flow for using lower limits as a physiological comparison among groups.

1533 **Tables**

1534 **Table 1. Cold tolerance strategies of insects as determined by survival of internal ice**

1535 **formation.** Chill-susceptible insects die of injuries unrelated to freezing, freeze-avoidant

1536 insects die upon internal ice formation, and freeze-tolerant species are able to survive

1537 internal ice formation.

1538

1539

	Internal ice formed	**No internal ice formed**
Alive	Freeze-tolerant	Chill-susceptible or freeze-avoidant
Dead	Chill-susceptible or freeze-avoidant	Chill-susceptible

1540

1541

1542

1543

1544

1545 **Table 2. Cold tolerance strategies of insects as determined by survival following**
1546 **exposure to the supercooling point.** When half of the insects have reached their
1547 supercooling point during cooling, all insects are returned to warm conditions to monitor
1548 survival. This provides a group of insects that experienced freezing, and a group that did
1549 not, while maintaining exposure to approximately the same low temperature. Survival
1550 following freezing indicates freeze-tolerance, whereas survival until freezing is reached
1551 indicates freeze-avoidance. Mortality before the supercooling point is reached indicates
1552 chill-susceptibility. *Drosophila suzukii:* Jakobs et al. (2015); *Reticulitermes flavipes*: Clarke
1553 et al. (2013); *Perisphaeria* sp.: Sinclair and Chown (2005).

1554

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

1555

1556 **Table 3. Examples of time and temperature treatments for measuring chill coma**

1557 **recovery.** Time and temperature exposures often vary widely between studies and labs, and

1558 will depend on the low-temperature tolerance of the species of interest.

1559

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

1560

1561

1562 **Table 4. Cues and conditions for inducing phenotypic plasticity in insect cold tolerance.**

Cue/Condition		Example	Reference
Shortened Photoperiod		Shortened photoperiod (12 hours of light, 12 hours of dark) triggers increased cold-hardiness in *Aulacophoro nigripennis* (Coleoptera: Chrysomelidae)	(Watanabe and Tanaka, 1998)
Changes in diet		*Drosophila ananassae* larvae reared on carbohydrate-rich food are more cold- tolerant.	(Sisodia and Singh, 2012)
		Chymomyza costata larvae reared on a proline-rich diet are more freeze-tolerant	(Koštál et al., 2011b)
Maternal Effects		The LT_{50} of *Sitobion avenae* offspring is lower if adults are reared at 10°C, compared to 20°C.	(Powell and Bale, 2008)
Acute decrease in temperature	*Rapid cold hardening*	Exposure to 5 °C for 30 min increases survival of *Drosophila melanogaster* at -5 °C	(Czajka and Lee, 1990)
		10 min to 1 hr exposure to 0 °C increases the freezing tolerance of *Sarcophaga crassipalpis*	(Chen et al., 1987)
	Slow cooling	Cooling at 0.05 °C/min or 0.1 °C/min increased survival of *Drosophila melanogaster* held at -7 °C for one hour	(Kelty and Lee, 1999)
Heat shock		Exposure to 34 °C for 40-80 min increases survival of *Drosophila melanogaster* larvae at 0 °C	(Burton et al., 1988)
		Heat pre-treatment (36.5 °C, 1 hr) increased cold tolerance in larvae of *Drosophila melanogaster*	(Rajamohan and Sinclair, 2008)
Acclimation	*Short (5-10 d)*	*Halmaeusa atriceps* were held at 5 °C for 7 d	(Slabber and Chown, 2005)
	Slow decrease	Acclimation to 15 °C for 1 wk, followed by 6 °C for 1 wk, extends survival of *Drosophila melanogaster* at 0 °C	(Koštál et al., 2011a)
	Ecologically relevant	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	(Williams et al., 2012)

Rearing temperature	Three generations of *Sitobion avenae* were reared at 10 °C	(Powell and Bale, 2005)

1563

1564 **Table 5. Requirements and considerations for measurements of cold tolerance in insects**

1565

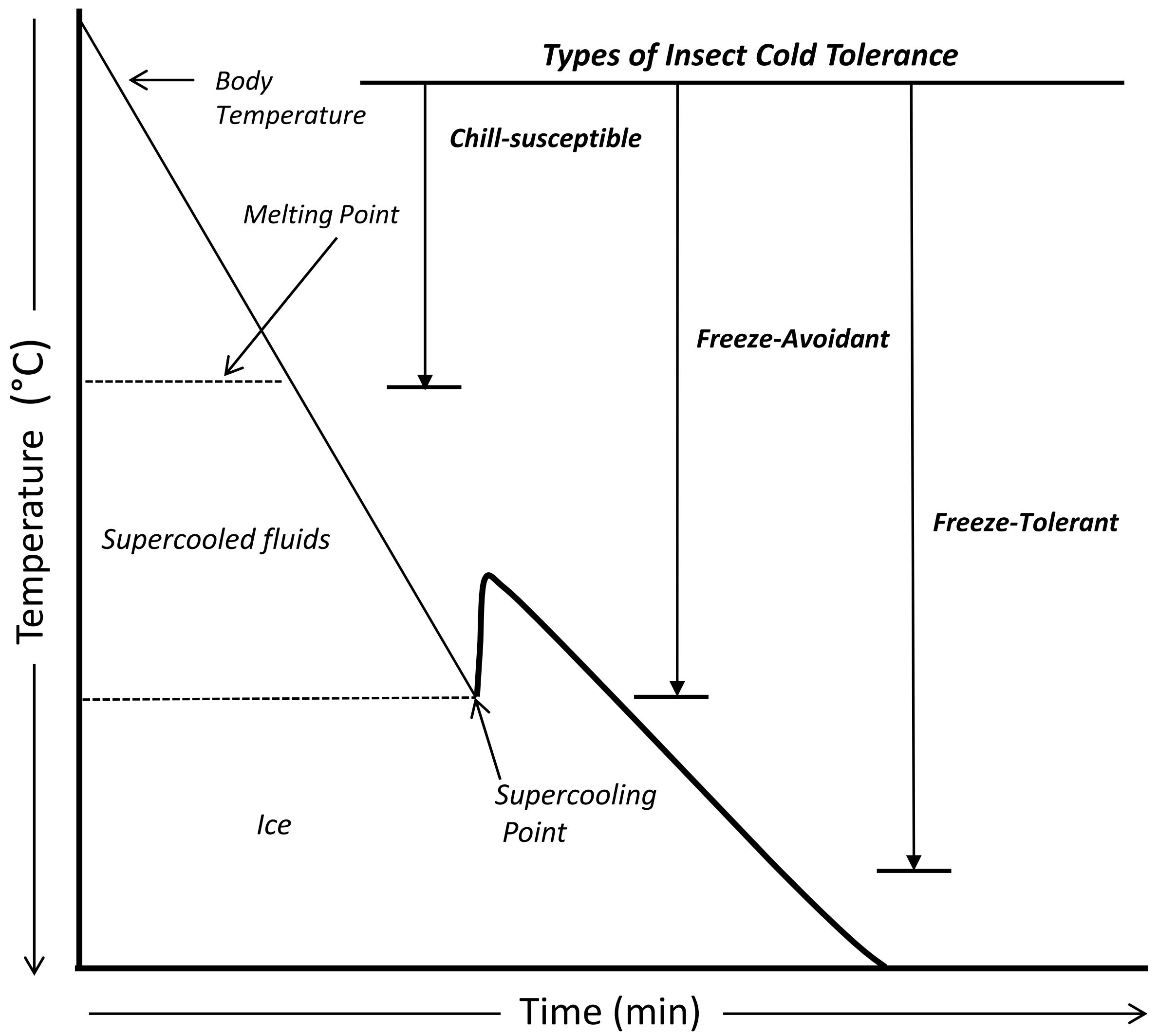
Measurement	Equipment requirements	Ecological relevance	Constraints	Advantages	Typical sample size
Chill coma recovery (CCR) Section 5.2	Simple cooling Simple constant temperature	Unclear	Active/mobile insects only Cannot compare among studies	Easy to measure Can be compared among treatment groups	10-20
Critical thermal minimum (CT_{min}) Section 5.1	Controlled cooling Temperature measurement	Moderate-high: sets the lower limit for activity	Continual observation Active/mobile insects only	Correlated to other measures of cold tolerance (e.g. in *Drosophila*) Can be compared among species and treatments Can be compared to ambient temperatures	10-20
Supercooling point (SCP) 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
Cold tolerance strategy Section 3.1	Controlled cooling Temperature measurement	None	Does not predict thermal limits	Informs interpretation of other measurements	8-10
Lower lethal temperature (LLT) Section 4.1	Controlled cooling Temperature measurement	High	Time-consuming	Directly relevant to the real world	5-10 groups of 5-10
Discriminating temperature	Simple, constant temperature	Moderate: predicts survival at this	Must determine appropriate	High throughput With careful choice of temperature, can	Groups of 5-10 with or

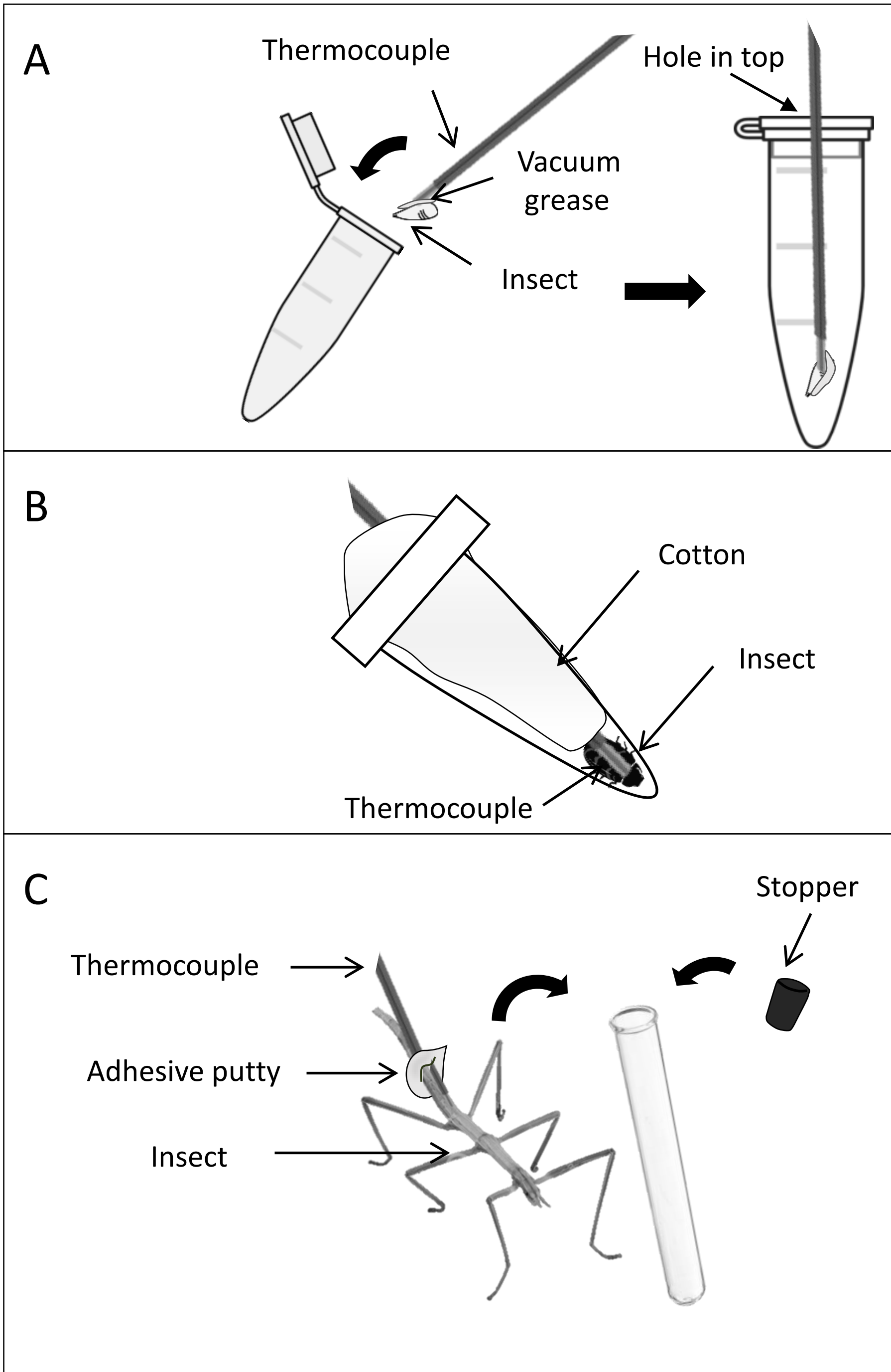
141

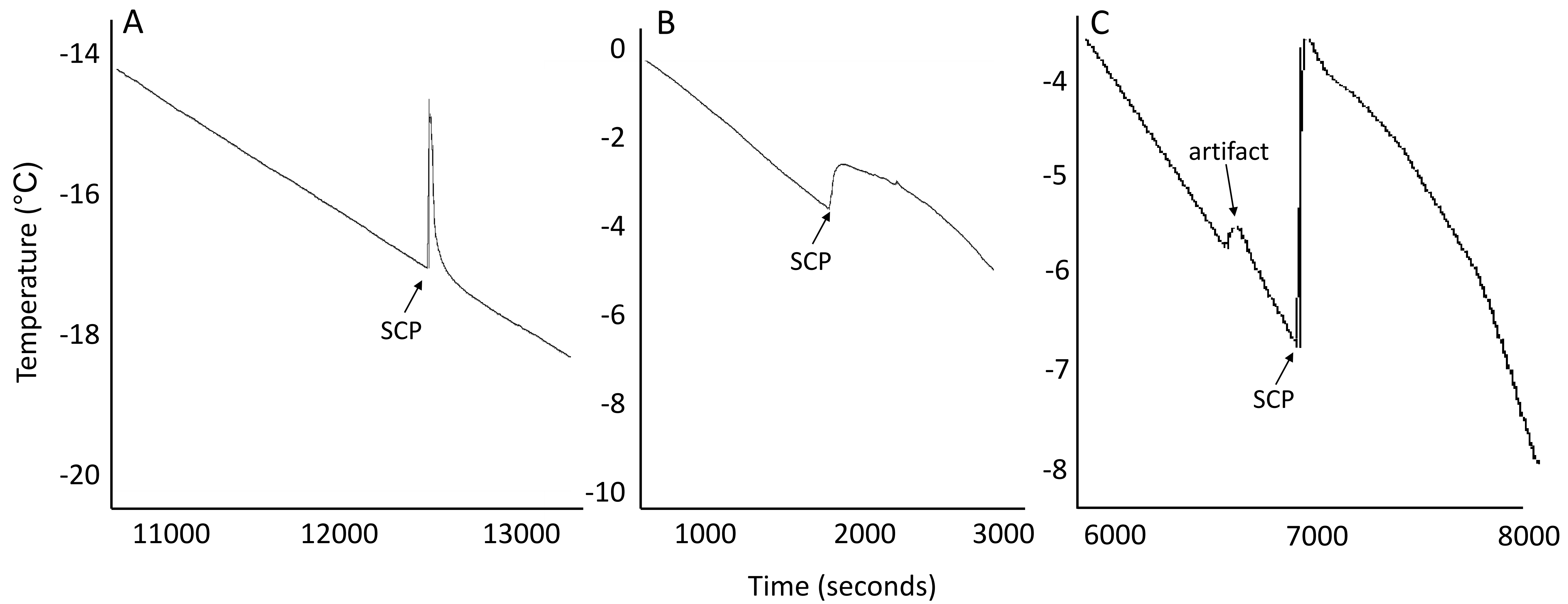
Section 6.1	temperature	temperature in advance	be ecologically relevant. Can be compared among groups/treatments	without replicate groups

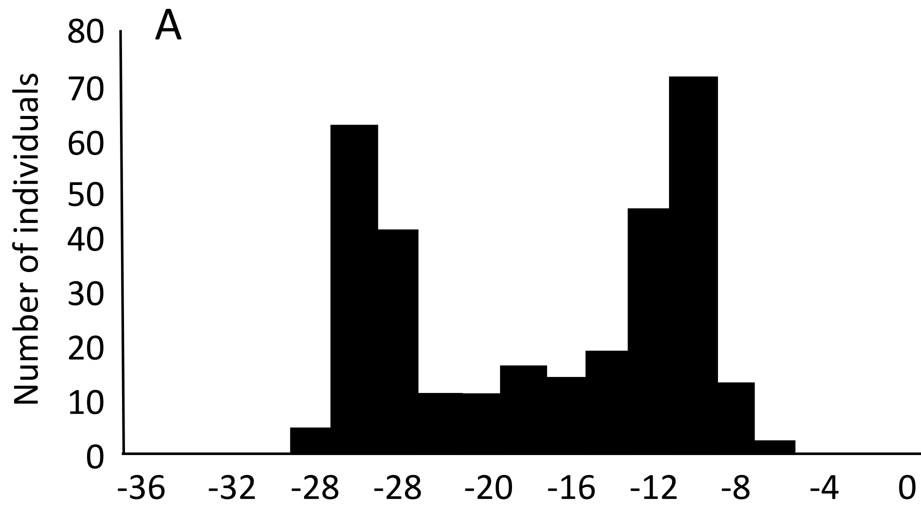
1566

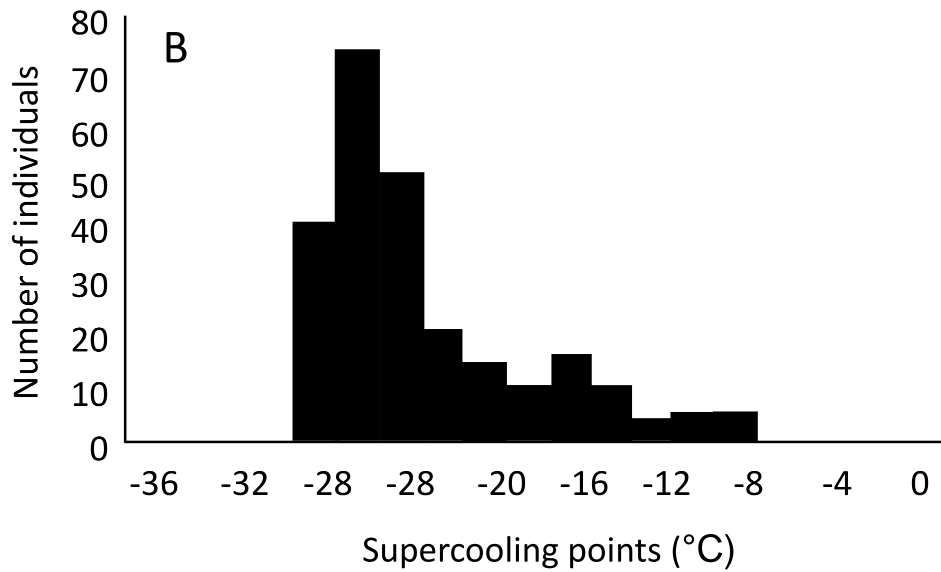
142
143

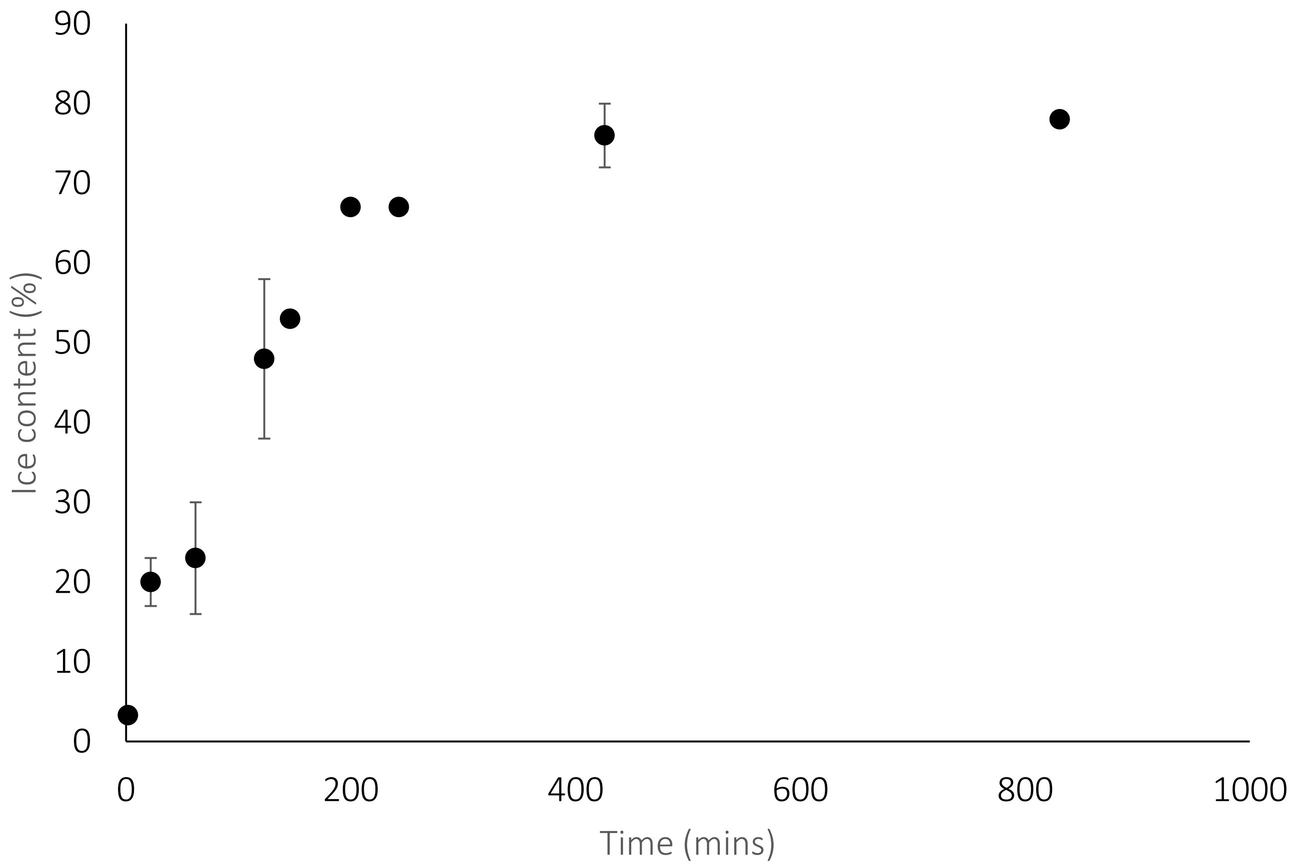

Types of Insect Cold Tolerance
Body
Temperature
Chill-susceptible
Melting Point
Freeze-Avoidant
Freeze-Tolerant
Supercooled fluids
Supercooling
Point
Ice
Temperature (°C)
Time (min)

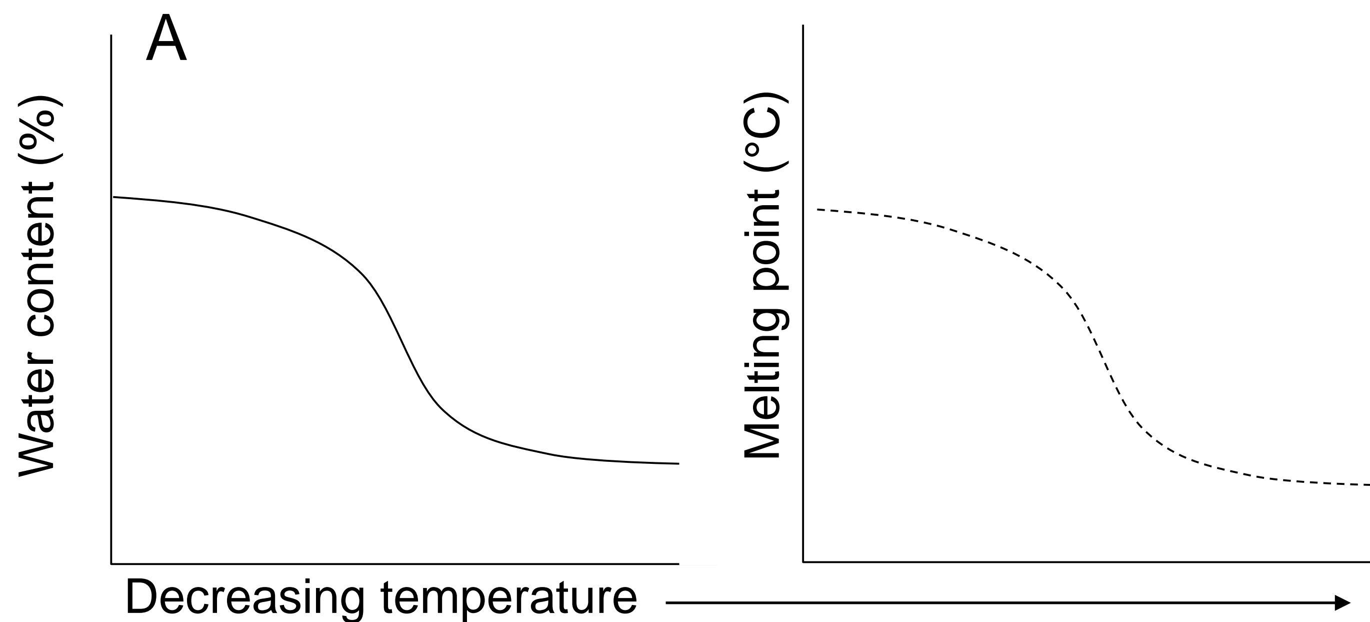

A
Thermocouple
Hole in top
Vacuum grease
Insect
B
Cotton
Insect
Thermocouple
C
Stopper
Thermocouple
Adhesive putty
Insect

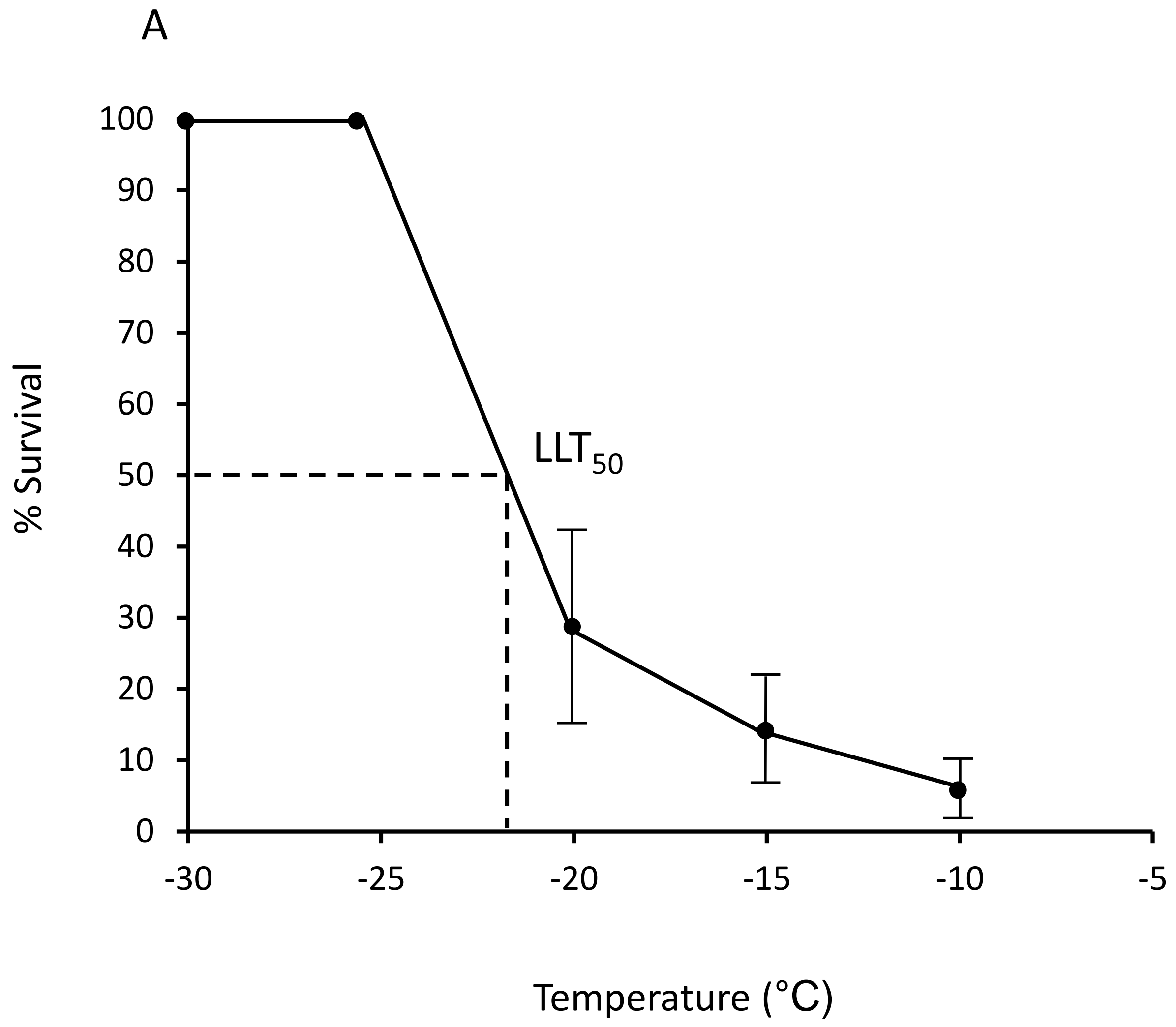

A
B
C
Temperature (°C)
-14
-16
-18
-20
SCP
11000
12000
13000
0
-2
-4
-6
-8
-10
SCP
1000
2000
3000
-4
-5
-6
-7
-8
artifact
SCP
6000
7000
8000
Time (seconds)

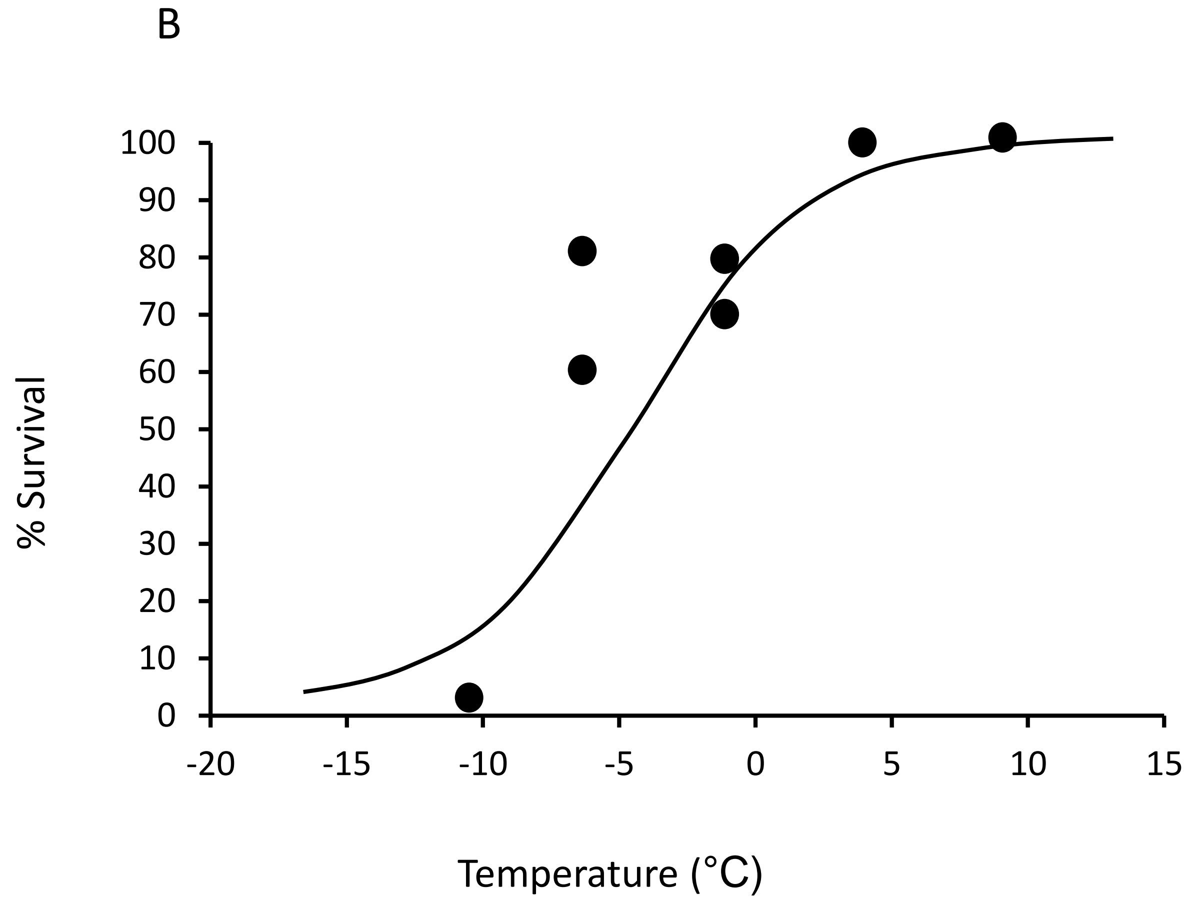

A
80
70
60
50
40
30
20
10
0
Number of individuals
-36
-32
-28
-28
-20
-16
-12
-8
-4
0

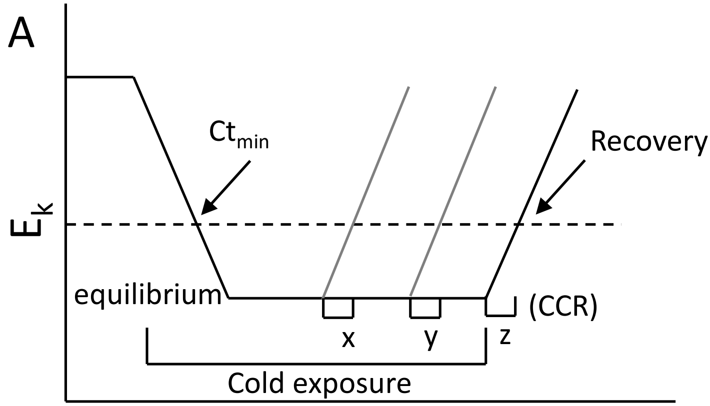

B
80
70
60
50
40
30
20
10
0
Number of individuals
-36
-32
-28
-28
-20
-16
-12
-8
-4
0
Supercooling points (°C)

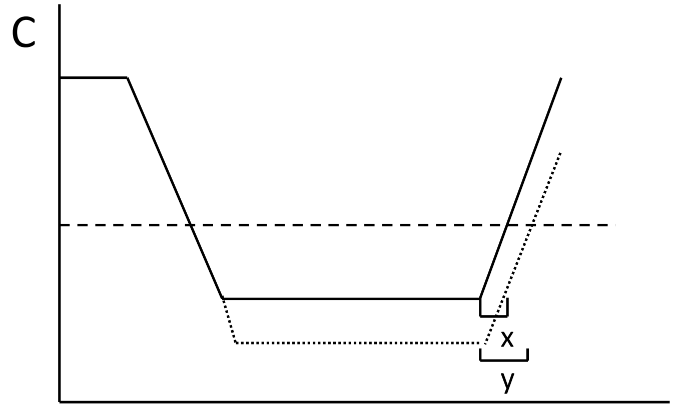

90
80
70
60
50
40
30
20
10
0
Ice content (%)
0
200
400
600
800
1000
Time (mins)

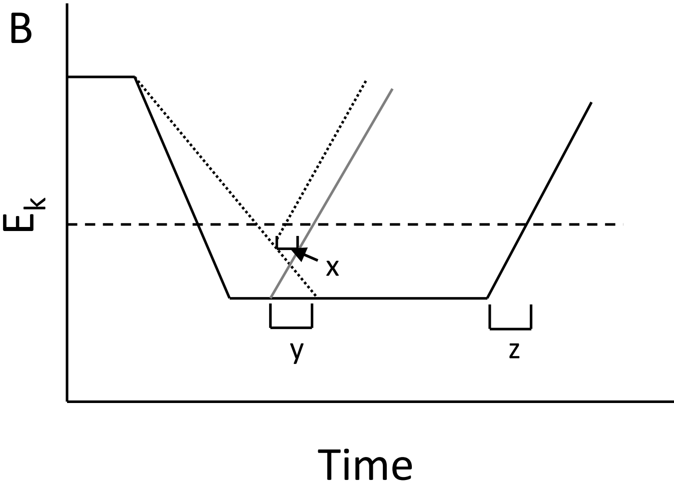

A
Water content (%)
Melting point (°C)
Decreasing temperature
B
Melting point over water
Melting point over ice
Ambient temperature (°C)
Melting point (°C)
0
-2
-4
-6
-8
0
2
4
6
8
10
12
14
16
Time (days)

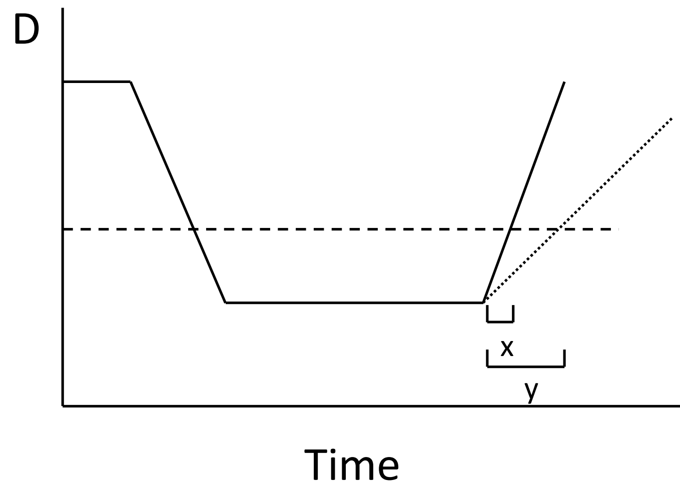

A
100
90
80
70
60
50
40
30
20
10
0
% Survival
LLT50
-30
-25
-20
-15
-10
-5
Temperature (°C)

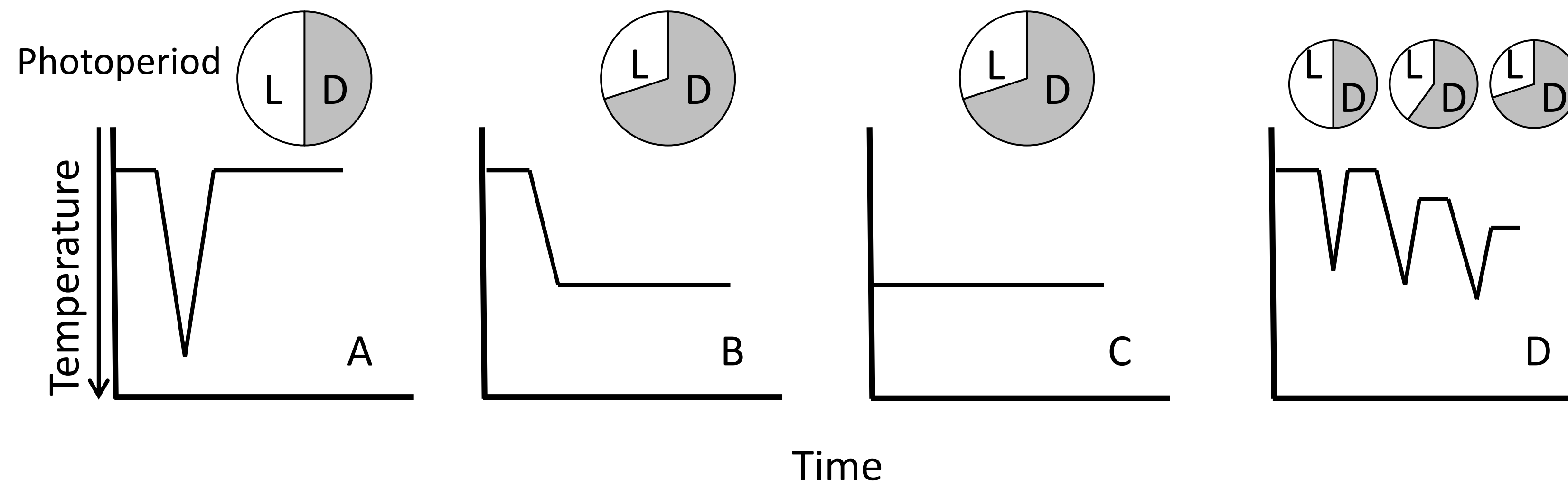

B
100
90
80
70
60
50
40
30
20
10
0
% Survival
-20
-15
-10
-5
0
5
10
15
Temperature (°C)


A
Ct_{min}
Recovery
E_k
equilibrium
x
y
z
(CCR)
Cold exposure

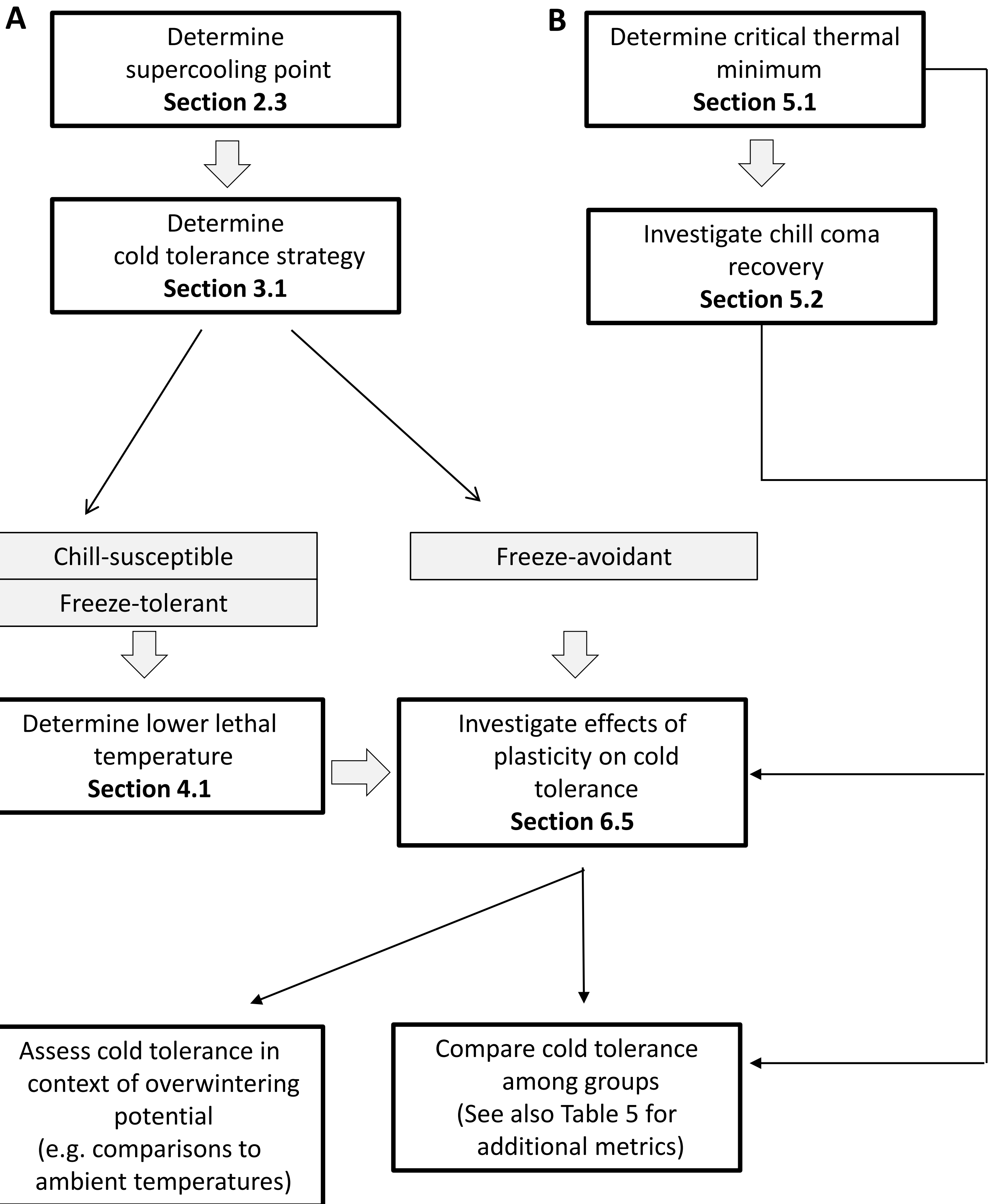

C
x
y


B
E_k
x
y
z
Time


D
x
y
Time



Photoperiod
L
D
Temperature
A
B
C
D
Time


A
Determine supercooling point
Section 2.3
Determine cold tolerance strategy
Section 3.1
Chill-susceptible
Freeze-tolerant
Freeze-avoidant
Determine lower lethal temperature
Section 4.1
Investigate effects of plasticity on cold tolerance
Section 6.5
Assess cold tolerance in context of overwintering potential
(e.g. comparisons to ambient temperatures)
Compare cold tolerance among groups
(See also Table 5 for additional metrics)
B
Determine critical thermal minimum
Section 5.1
Investigate chill coma recovery
Section 5.2