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Deep supercooling, vitrification and limited survival to –100°C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae

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SUMMARY

Larvae of the freeze-avoiding beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) in Alaska have mean supercooling points in winter of –35 to –42°C, with the lowest supercooling point recorded for an individual of –58°C. We previously noted that some larvae did not freeze when cooled to –80°C, and we speculated that these larvae vitrified. Here we present evidence through differential scanning calorimetry that *C. c. puniceus* larvae transition into a glass-like state at temperatures <–58°C and can avoid freezing to at least –150°C. This novel finding adds vitrification to the list of insect overwintering strategies. While overwintering beneath the bark of fallen trees, *C. c. puniceus* larvae may experience low ambient temperatures of around –40°C (and lower) when microhabitat is un-insulated because of low snow cover. Decreasing temperatures in winter are correlated with loss of body water from summer high levels near 2.0 to winter lows near 0.4 mg mg^{-1} dry mass and concomitant increases in glycerol concentrations (4–6 mol l^{-1}) and thermal hysteresis. Finally, we provide direct evidence that *Cucujus* from Wiseman, Alaska, survive temperatures to –100°C.

Key words: SCP, supercooling point, AFP, antifreeze protein, DSC, differential scanning calorimetry, TH, thermal hysteresis.

INTRODUCTION

The red flat bark beetle, *Cucujus clavipes*, has a broad latitudinal range in North America that extends from above the Arctic Circle (~68°N) to North Carolina (~35°N), and exists as a western subspecies (*C. c. puniceus* Mannerheim) that occurs from Alaska to the Pacific coast and as an eastern subspecies (*C. c. clavipes* Fabricius) that extends east from the Great Plains (Thomas, 2002). Consequently, investigations of this species present an opportunity to study insect overwintering physiology over a large latitudinal expanse including Interior Alaska, one of the coldest environments in North America.

Insects that overwinter in freezing regions survive either by being freeze tolerant (able to survive the freezing of their extra-cellular water) or freeze avoiding (Zachariassen, 1985; Bale, 1987; Storey and Storey, 1988; Block, 1990; Danks, 1991; Duman et al., 1991; Lee and Denlinger, 1991), and, in one case, simultaneously freeze tolerant and avoiding (Sformo et al., 2009). Insects, and certain other terrestrial arthropods such as collembola, avoid freezing by use of combinations of molar concentrations of cryoprotectant polyols such as glycerol, antifreeze proteins (Duman, 2001), removal and/or masking of ice nucleators (Neven et al., 1986; Duman, 2001), and/or dehydration (Rickards et al., 1987; Lundheim and Zachariassen, 1993; Worland, 1996; Holmstrup and Sömme, 1998; Worland et al., 1998; Danks, 2000; Block, 2003; Worland and Block, 2003). Some collembola and earthworm cocoons (Holmstrup et al., 2002) and the Antarctic midge *Belgica antarctica* (Elnitsky et al., 2008) cryoprotectively undergo extreme water loss by dehydrating until they are in vapor pressure equilibrium with surrounding ice and therefore do not freeze.

Overwintering adaptations are exaggerated in insects from arctic and subarctic regions, where temperatures can reach below –60°C (Danks, 1981; Miller, 1982; Ring, 1982; Sömme and Block, 1991). Alaska populations of *C. c. puniceus* larvae are known to be freeze avoiding and routinely supercool to group means near –40°C, with individuals supercooling to as low as –58°C (Bennett et al., 2005); in some cases larvae did not freeze when cooled to –80°C in that study. This level of supercooling appeared irregularly among larvae collected near Fairbanks, AK (64°72′N) but appeared more often in larvae collected further north near Wiseman, AK (67°37′N) in late November to March. In that study, factors identified as contributing to the ability of *C. c. puniceus* to supercool to low temperatures were production of antifreeze proteins (AFPs), accumulation of glycerol, diapause (reduced metabolism in winter) and extensive dehydration. In contrast to the Alaskan *C. c. puniceus*, *C. c. clavipes* larvae from northern Indiana (41°45′N) show mean winter supercooling points (SCPs) of –23°C and do not dehydrate or diapause in winter (Bennett et al., 2005).

Dehydration contributes to the ability to supercool to low temperatures in Alaskan *C. c. puniceus*, in part, by causing the concentrations of AFPs and glycerol to increase (by as much as fivefold) over the levels synthesized prior to dehydration, and by decreasing the amount of water in the insect that is available for freezing (Bennett et al., 2005). Antifreeze proteins can mask ice nucleators and also inhibit inoculative freezing initiated by external ice in contact with the cuticle. AFPs enhance supercooling by both these mechanisms in larvae of the beetle *Dendroides canadensis* (Olsen et al., 1998; Olsen and Duman, 1997a; Olsen and Duman, 1997b; Duman, 2002). *C. c. puniceus* produce a family of AFPs that are similar but not identical to those of *D. canadensis* (Duman et al., 1998; Duman et al., 2004; Andorfer and Duman, 2000). The extreme desiccation of *C. c. puniceus* makes it impossible to sample hemolymph from animals in mid-winter. However, when hemolymph was collected in the autumn (after AFPs and glycerol had been produced, but prior to dehydration) and then concentrated

3.2-fold to reflect a level of dehydration near that of winter larvae, the concentrated hemolymph exhibited nearly 13°C of thermal hysteresis (Bennett et al., 2005). This is the highest thermal hysteresis ever measured in association with any organism. Thermal hysteresis activity (THA) is the difference between the freezing and melting points of the sample and reflects the amount of depression of the freezing point caused by the AFP, in the presence of ice, below the melting point (DeVries, 1986). However, the level of protection afforded to whole insects by AFPs generally greatly exceeds the magnitude of THA that can be measured in the hemolymph (Zachariassen and Husby, 1982; Olsen et al., 1998; Duman, 2001; Duman, 2002; Duman et al., 2004). Consequently, the protection provided by AFPs in *C. c. puniceus* larvae is probably much greater than even the level of THA demonstrated in the concentrated hemolymph.

While a few northern or alpine insects have been shown to have supercooling points of –40 to –60°C, not much is known about the mechanisms that contribute to this. Three freeze-avoiding Alaskan and Canadian Rocky Mountain species that overwinter in willow galls in exposed branches were found to have mean SCPs of –56 to –58°C, with individual SCPs to –63°C (Miller and Warner, 1987; Miller, 1982; Ring and Tesar, 1980). Mean SCPs of –54°C have been reported in larvae of the beetle *Pytho deplanatus* that overwinter under the bark of fallen spruce trees in the Canadian Rockies (Ring, 1982). These levels of freeze avoidance have been attributed to high concentrations of polyols and removal of ice nucleators and are described as extreme or deep supercooling. This paper examines the deep supercooling capacity, defined as cooling to below –58°C without freezing, of *C. c. puniceus* larvae in Alaska. We examine correlations among body water, glycerol concentration, thermal hysteresis and ambient temperature to identify physiological and abiotic factors associated with deep supercooling. We demonstrate that *C. c. puniceus* larvae can avoid freezing at temperatures down to –150°C, their body water vitrifies (turns to glass) at a mean temperature of approximately –76°C, and larvae can survive exposure to –100°C.

MATERIALS AND METHODS

Insect collection and microhabitat characteristics

We collected *C. c. puniceus* larvae from under the bark of standing dead *Poplar* spp. trees near Wiseman and Fairbanks, AK, each September in 2005 to 2007. Larvae were placed in plastic food storage containers (20 cm×15 cm×10 cm; 20–150 per box) perforated for gas exchange, along with moist bark from their host trees. Insects were left to acclimatize to local winter conditions by placing the containers either on the ground or suspended (so as not to be covered by snow) for 1–4 months either in undisturbed wooded areas on the University of Alaska Fairbanks campus or near Wiseman, Alaska. Air and microhabitat temperatures at these overwintering locations were monitored using Hobo Pro Series data loggers and downloaded with BoxCar Pro 4 software (Onset Computer Corporation, Bourne, MA, USA).

Supercooling points

Insects were recovered from containers at different times in winter and brought into the laboratory at the Institute of Arctic Biology in Fairbanks to determine the supercooling point (SCP) and water content. Insects retrieved from Wiseman were kept at –18 to –20°C during transport to Fairbanks and tested within 9 h. Larvae were tested for individual SCP by placing a thermocouple junction (copper–constantan, 36 gauge) against their body in a 0.6 ml plastic tube. Thermocouple leads, monitoring up to 16 larvae at a time, were attached to a computer-controlled multi-channel thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, OH, USA) that recorded temperature every 5 s. Closed tubes were placed inside a 500 ml glass beaker that was covered and mostly submerged in an alcohol–water cooling bath. Once the temperature of the insects equilibrated to 0°C, bath temperature was reduced at 0.2°C min^{-1}, typically to –60 to –70°C. Before and after supercooling runs were performed, each thermocouple-attachment was visually inspected to ensure that the thermocouple junction was in direct contact with the insect. The lowest body temperature recorded at the release of the latent heat of fusion, as evidenced by an exotherm in the temperature recording, was recorded as the SCP, and since *C. c. puniceus* is a freeze-sensitive insect (Bennett et al., 2005), the SCP is also the lower lethal temperature. Larvae that did not exhibit an exotherm when cooled to –60°C or lower were recorded as deep supercooling.

Water content

Individual larvae were weighed to the nearest 0.1 mg and then dried at 60°C (48 h) to constant mass. Absolute body water content (WC) was calculated as mg water mg^{-1} dry mass (Rojas et al., 1986; Hadley, 1994).

Glycerol

Individuals were randomly chosen from those sampled during the 2005 and 2006 field seasons and, based on supercooling point determinations, categorized as freezing (exotherm present) or deep supercooling. Fresh and dry mass (nearest 0.1 mg) were recorded, and individuals were homogenized in distilled water at a 1:1000 dilution. Samples were centrifuged and the supernatant removed. Sub-samples of 20 µl were removed and further diluted by a factor of ten and analyzed for glycerol content using the Boehringer–Mannheim (Marshall, MI, USA) Glycerol Kit. This method measures the amount of NADH oxidized to NAD^+ that is stoichiometrically proportional to the initial glycerol concentration of the sample. The glycerol concentration of each individual was calculated based on the glycerol determination and the body water content, assuming equal distribution of glycerol. Glycerol standards were run to check the accuracy of the procedure.

Thermal hysteresis

In winter, hemolymph cannot be sampled because of the dehydrated state of insects. Consequently, to compare thermal hysteresis activities homogenates of larvae were prepared. Dry larvae were homogenized in a volume of water equal to 100 times the body water. This solution was further diluted 1:7 with 40 mmol l^{-1} phosphate buffer (pH 7.5) and sub-samples were tested for thermal hysteresis using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) (Chakrabartty and Hew, 1991). A micrometer syringe delivered 25–100 nl of sample into heavy mineral oil located in the sample well of the osmometer. The sample was initially frozen by cooling to –40°C and then warmed until a single small ice crystal remained. Temperatures were increased by 0.01–0.02°C until the crystal disappeared, as assessed visually, thus determining the melting point temperature. This routine was repeated at temperatures below the melting point with a single small crystal present and the temperature was slowly lowered until the crystal grew; this is the freezing point temperature.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a technique used to measure the change in heat capacity that is indicative of transitions

to or from a vitrified or glassy state. The phase transition of a liquid to a vitreous state occurs at the glass transition temperature. To determine the glass transition temperature, an insect is sealed in an aluminum sample pan within a chamber that also contains a reference pan with no insect present. The two pans are heated and cooled to maintain equivalent temperatures. This requires differential heating and cooling due to the presence of the insect. By monitoring heat flow and change in temperature over time, the heat capacity is measured. When a liquid transitions to a vitreous condition, there is solidification with a concomitant change in heat capacity but without release of the latent heat of crystallization (Fahy, 1995).

When supercooling tests demonstrated that larvae retrieved from the field could deep supercool to –70°C, additional individuals from a second collection container were shipped on dry ice to 21st Century Medicine in Fontana, CA, USA. There, a Perkin-Elmer DSC 7 with Pyris software tested for vitrification of insects through differential scanning calorimetry. The temperature scale of the instrument was calibrated by measuring the onset of the crystal transition of cyclohexane at –87.06°C and the temperature when ice water melts while warming at 1°C min^{-1}. Heat flow was calibrated by measuring the area under the melting curve of a known mass of water ice. Four larvae of approximately 10 mg mass each were folded and sealed in individual aluminum sample pans (Perkin-Elmer part no. 0219-0062). Then each larva was cooled from +10 to –150°C at a rate of 1°C min^{-1} and then warmed to +10°C at a rate of 40°C min^{-1}. Thermograms obtained at this high warming rate provided high sensitivity to detect small phase transitions. Additionally, each sample was cooled to –150°C at a rate of 100°C min^{-1}, and thermograms were obtained during warming to +10°C at 5°C or 10°C min^{-1}. Three smaller larvae (<2 mg) that deep supercooled were tested separately by cooling from +10°C to –150°C at a rate of 10°C min^{-1} and then warming back to +10°C at 10°C min^{-1}.

Survival

In winter 2005–2006, if a majority of insects taken from the outdoor enclosure on a specific date did not freeze when cooled to below –60°C, additional insects from the same group were cooled in plastic tubes at 1°C min^{-1} to –80 or –100°C. Insects in this second trial that did not display an exotherm were warmed at the same rate to –16°C and placed back into the container onto cold (–18°C) bark either in a plastic bag (N=12) or not (N=33). Containers were then returned to the outdoor enclosure and replaced under snow. On 1st May, containers were warmed to 20°C, and insects were assessed for survival and activity over a 1-week period. In winter 2007–2008, after larvae from the field containers exhibited deep supercooling, whole containers with larvae on bark and within plastic bags were cooled at 1°C min^{-1} to –80 or –100°C before being returned to the field, and larvae were checked for survival in April.

Statistical analysis

Normally distributed data (Shapiro–Wilk test, P>0.05) were compared by t-test or Tukey–Kramer for multiple comparison; non-normally distributed data were compared with a Mann–Whitney and χ^2-tests (SAS 9.1, SAS Institute, Inc). Unless otherwise noted, values are presented as mean ± s.e.m. To assess association, the Spearman Rank Correlation test was used with averaged rank used for tied observations. Since winter values for mean SCPs and body water content of larvae collected in Wiseman and held either in Wiseman or Fairbanks did not differ (Mann–Whitney, P>0.2), these data were combined and are collectively referred to as the Wiseman population.

RESULTS

Microhabitat characteristics

Below freezing temperatures began in mid-August in Wiseman and in mid-September in Fairbanks and extended to mid-April in Fairbanks and mid-May in Wiseman [see Bennett et al. (Bennett et al., 2005) for more detail of conditions at these two sites]. Although air temperatures regularly decreased to lower than –40°C, insulating snow cover resulted in minimum ground temperatures typically near –20°C in both locations. In Wiseman during 2006–2007, however, low snow cover resulted in similar conditions below and above the snow for much of the winter (Fig. 1). On 7th January 2007, for example, above-snow temperature was –43°C and below-snow temperature was –40°C. In summer, there were several transient cold snaps in Wiseman including on 4th June 2006 when ambient temperatures reached near –5°C. in Fig. 2 above- and below-snow temperatures are presented for Fairbanks from October to April 2005–2006 and 2007–2008.

Supercooling points and water content

Changes in SCP, WC and ambient temperature over three overwintering seasons showed generally the same trends (Fig. 2 and positive correlations Table 1; note that these data do not include larvae that deep supercooled and did not freeze). As ambient temperatures decreased between summer and autumn, mean supercooling points determined in *Cucujus* larvae collected from Fairbanks and Wiseman populations declined by 20°C from values as high as –7.0°C and by a further 6–7°C between autumn and winter to average seasonal minima of approximately –37°C (Table 1). The lowest individual SCP was –54.3°C. Body water content of larvae also decreased between summer and winter from 2.1 to 0.8 mg mg dry mass^{-1} (averages combined for both locations; Table 1). Associations between SCPs and WCs by season and location are reported as correlation coefficients (Table 1), and, except for the summer Wiseman value, all are significant (P<0.0001). Results restricted to dates when both freezing and deep supercooling occurred in the same experimental run indicate that variation in water content of Fairbanks larvae explained approximately 24% of the variation in SCP (P<0.001, ρ=0.4976, N=175). For Wiseman larvae, water content explained approximately 41% of the variation in SCP (P<0.001, ρ=0.6393, N=202). Overall, mean SCPs decreased with

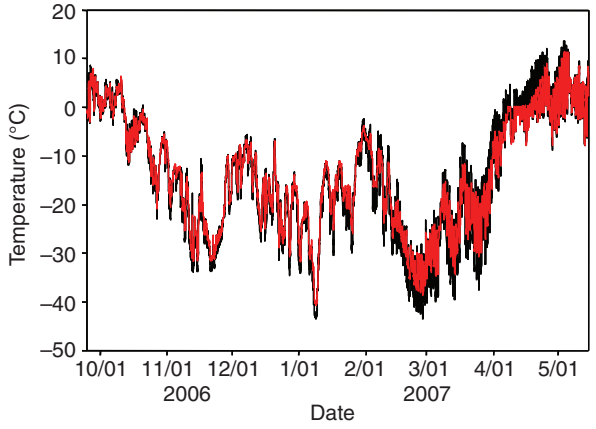

Fig. 1. Above- and below-snow temperatures (black and red lines, respectively) at Wiseman in October 2006 to May 2007. These data show the similarity between above- and below-snow temperatures as a result of the lack of snow accumulation.

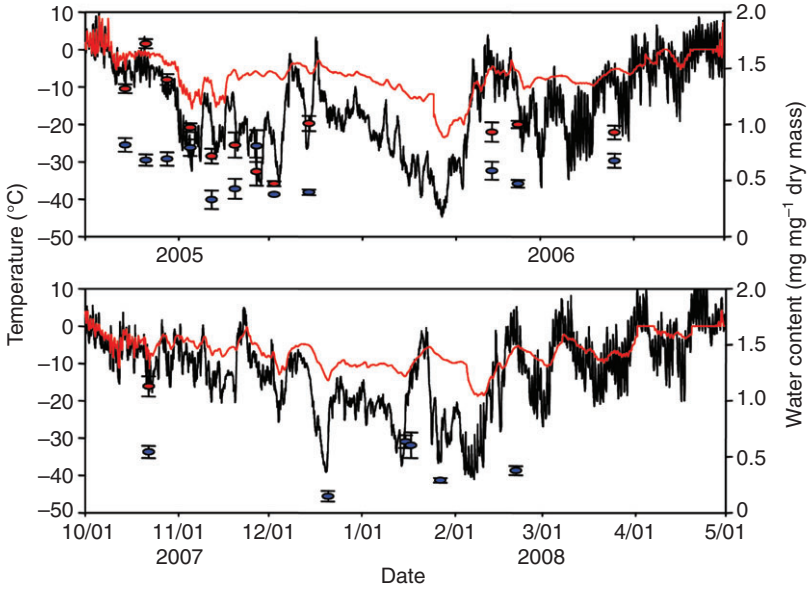

Fig. 2. Above- and below-snow temperatures (black and red lines, respectively) at Fairbanks, and supercooling points (blue ovals) and water content (red ovals) of Wiseman larvae held in Fairbanks from October 2005 to May 2006 and October 2007 to May 2008. On some dates, larvae deep supercooled (did not freeze), and these are not displayed. Supercooling point and water content values are means ± s.e.m.

mean WC, when values from insects collected at both sites throughout the study are compared (Fig. 3).

Deep supercooling and vitrification

Over half (111 of 210; 52.8%) of the *Cucujus* larvae from the Wiseman population did not exhibit an exotherm when cooled to between –60 and –70°C during November to February in 2005–2008 (Table 2). Over half (84 of 165; 50.9%) of the larvae from the Fairbanks population also did not exhibit an exotherm when cooled to between –60 to –70°C during December to March in 2005–2008 (Table 2). We refer to this result as deep supercooling, and we believe these individuals entered a stable, non-crystalline, glass-like state. When individuals from Fairbanks and Wiseman were tested on the same day during December to February 2007, 18 of 26 (69.2%) Fairbanks individuals and 11 of 34 (32.3%) Wiseman insects deep supercooled (χ^2=2.74; d.f.=1, P=0.09). There was no significant difference (P>0.05, Tukey–Kramer adjustment for multiple comparisons test) between WC of individuals that deep supercooled between populations: Fairbanks WC was 0.4±0.02, N=52 (winter), and Wiseman WC was 0.3±0.02, N=32 (autumn) and 0.4±0.02, N=59 (winter).

Compared with larvae that froze, individuals that deep supercooled had half of the water content, a 1.57-fold greater concentration of glycerol, and a 1.16-fold greater level of thermal hysteresis (Table 3), all statistically significant differences.

Differential scanning calorimetry

No evidence of the formation of ice in deep supercooled *C. c. puniceus* larvae was observed by differential scanning calorimetry when larvae were cooled to –150°C at all cooling and warming rates studied. The most sensitive test used for detection of freezing events was cooling at 1°C min^{-1}, followed by warming at 40°C min^{-1}. The rapid warming rate increases detection sensitivity for melting peaks because a faster warming rate requires larger heat flow, helping raise small heat flow changes above the noise level (Saunders et al., 2004). In the experience of one of the authors (B.W.), the detection sensitivity of the instrument analysis software at this warming rate is approximately 0.05 J g^{-1}, corresponding to a mass of ice equal to 0.015% the sample mass.

Thermograms (change in heat capacity as a function of temperature) obtained during warming of four large larvae at 40°C min^{-1} show a large change in heat capacity within a narrow temperature range that indicates a transition to glass (Fig. 4). Glass transition or vitrification temperatures, indicated as the thermogram inflection point, while warming at 5°C min^{-1}, are presented in Table 4. Two large larvae showed a small glass transition near –97±1°C followed by a larger transition near –70°C. All four of the large larvae showed a consistent large glass transition at –76±1°C. The three small larvae (1.7–3.3 mg) showed higher and more variable glass transition temperatures. In addition, the glass transitions appeared at the same temperature (accounting for a few

Table 1. Seasonal changes in supercooling points and water content of insects that froze (exotherm)

Season	Months	Location	Supercooling point (°C)*	Water content† (exotherm)	Correlation coefficient
Summer	May–September	F	–8.2^{a}±0.3 (170)	2.0^{d}±0.04 (169)	0.36
	July	W	7.0^{a}±0.3 (21)	–2.2^{d}±0.2 (21)	NS
Autumn	October–December	F	–28.1^{b}±1.0 (65)	1.4^{e}±0.05 (65)	0.48
	October–November	W	–30.0^{b}±1.0 (91)	1.2^{f}±0.04 (91)	0.35
Winter	December–April	F	–36.4^{c}±0.7 (174)	0.8^{g}±0.03 (175)	0.49
	December–March	W	–38.7^{c}±0.8 (168)	0.8^{g}±0.02 (170)	0.62

F, Fairbanks; W, Wiseman.
*Values are means ± s.e.m. (N); †mg mg^{-1} dry mass.
Superscript letters indicate significant difference in column means (P<0.05) with the Tukey–Kramer adjustment for multiple comparisons test. Correlation coefficients were calculated within months and location categories with raw data.
Note that these data do not include deep supercooling individuals.

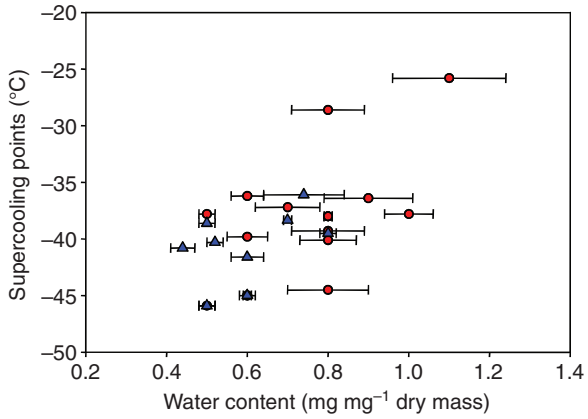

Fig. 3. Mean supercooling points and water content (mean ± s.e.m.) of larvae from Fairbanks (blue triangles) and Wiseman (red circles). Data are from November to February trials, when individuals were also capable of deep supercooling (no exotherm <–60 to –70°C); however, these deep supercooling larvae are not included in these data.

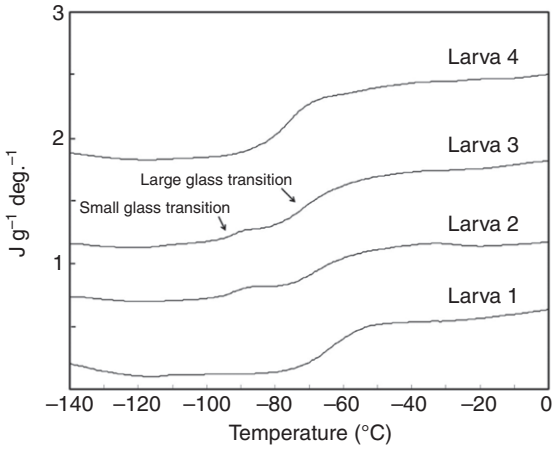

Fig. 4. Thermograms of larvae studied by DSC at a warming rate of 40°C min^{-1}. The thermogram heat flows are normalized to specific heat capacity units. The starting point on the vertical scale for each thermogram is arbitrary. Two of the four larvae exhibited a double glass transition.

degrees scanning lag) whether scanning up or down (warming or cooling) through the glass transition.

The glass transition temperature of aqueous solutions in general increases with increasing solute concentration, and the higher glass transition temperature of the smaller larvae suggests the possibility of greater drying due to their greater surface-area-to-volume ratio; furthermore, the two separate glass transitions seen in some animals are indicative of two separate fluid compartments, one with a higher water concentration that undergoes a glass transition at a lower temperature, and one with a lower water concentration that transitions at a higher temperature.

Survival

Survivorship (in 2005–2006) of larvae from Fairbanks and Wiseman left below-snow to overwinter was 93% and 92%, respectively, when assessed in the spring of each year. In 2007–2008, Fairbanks and Wiseman individuals maintained above the snow in Fairbanks showed 57% and 80% survival, respectively, whereas 90% of below-snow individuals survived in both groups.

On 18th January 2006, larvae (N=16) from Wiseman held in Fairbanks were cooled in contact with individual thermocouples to –72°C at 1°C min^{-1}. No exotherms occurred. Twelve of these were placed on bark, sealed in a plastic bag, and returned to the outside enclosure. On 1st May 2006, 6 of twelve were alive. On 20th December 2007 half of Wiseman larvae retrieved from the outdoor enclosure (N=8) deep supercooled and half froze when cooled to –71°C. The next day, 41 additional larvae were retrieved from the same enclosure and cooled to –100°C at 1°C min^{-1}. These larvae were held below –58°C (the lowest exotherm temperature we have measured) for approximately 84 min. After reaching –100°C, they were warmed at 1°C min^{-1} to approximately –25°C and then returned to the enclosure, as above. On 7th April 2008, 3 of the 41 larvae were alive. No other insects (N=138) survived deep supercooling from –70°C to –100°C. Note that we have not yet directly shown that specimens found to vitrify, based on differential scanning calorimetry, are alive after rewarming, but our data appear to be compatible with the possibility of such a demonstration in the future.

Table 2. Proportion of individuals that deep supercooled compared with the total tested per supercooling run, by month and year in both populations, and the mean water content of individuals that deep supercooled

	Fairbanks			Wiseman	
Date	DS/total	DS WC	Date	DS/total	DS WC
December 05	1/12	0.6	November 05	5/13	0.6
January 06	10/10	0.2	November 05	3/15	0.4
March 06	1/14	0.6	November 05	9/16	0.4
December 06	5/11	0.4	November 06	19/24	0.3
December 06	7/7	0.5	December 05	12/13	0.4
January 07	1/11	0.4	December 05	2/12	0.9
February 07	1/15	0.4	January 07	4/13	0.4
February 07	4/6	0.4	February 07	2/16	0.5
March 07	14/16	0.4	February 07	10/12	0.4
December 07	10/14	0.2	February 07	7/8	0.3
December 07	1/12	0.2	December 07	11/29	NA
January 08	8/9	0.3	January 08	8/8	0.4
March 08	21/28	NA	January 08	12/16	NA
			January 08	7/15	NA
Grand mean±s.e.m. WC		0.38±0.04			0.45±0.05

DS, deep supercooled; WC, water content (mg mg^{-1} dry mass).

Table 3. Comparison of mean body water content, glycerol concentrations and thermal hysteresis levels in larvae that froze *versus* those that deep supercooled (did not freeze)

	Supercooling point (°C)	Water content[1] (mg mg^{-1} dry)	Glycerol[2] (Mol)	Thermal hysteresis[3] (°C)
Exotherm	–39.5±1.6 (39)	0.8±0.07[a] (39)	2.8±0.19[a] (37)	2.07±0.11[a] (21)
No exotherm	Did not freeze (36)	0.4±0.13[b] (36)	4.4±0.13[b] (36)	2.39±0.08[b] (21)

Individuals were sampled October to December 2005 and February to March 2007.
Superscript numbers indicate statistical test used; superscript letters indicate significant difference (P<0.05):
[1]Mann-Whitney, U=796.5, P<0.0001.
[2]t-test with unequal variance, t=6.73, d.f.=63.6, P<0.0001.
[3]t-test with equal variance, t=2.33, d.f.=40, P=0.02.
Values are means ± s.e.m. (N).

DISCUSSION

This study provides the first evidence of an insect avoiding freezing in extreme low temperatures by entering a stable, non-crystalline vitreous condition that is, at least in some individuals, survivable. Differential scanning calorimetry of rapidly cooled individuals reflected vitrification (glass transition) at temperatures as high as –58°C. This vitrified fluid is well-protected against freezing, showing no detectable tendency to form ice. The fact that these larvae can vitrify, however, does not mean that all survive these low temperatures. Half of the larvae cooled to between –70°C and –73°C survived, whereas only 7% of those cooled to –100°C survived. One possible reason for the lower survivorship between –70°C and –100°C may have to do with our method of transporting larvae from the enclosure to the low temperature bath and back to the enclosure. Larvae were deposited in an outside enclosure in September and were typically retrieved for testing when ambient conditions were less than –20°C (late November to March). Many of the containers that held the insects were frozen to the ground. To retrieve insects, the containers had to be forcefully removed. This jostling may cause already supercooled insects to nucleate or become mechanically damaged and thus be a source of mortality. Similar manipulations and rapid temperature changes in the laboratory also may be damaging.

Fairbanks and Wiseman, locations in interior Alaska, provide excellent low temperature settings to examine extreme overwintering physiology of insects. Both locations have some of the coldest environments in North America with official recordings of –52°C in Fairbanks in 1962 and –62°C in 1971 at Prospect Creek Camp (Schulski and Wendler, 2007) 87 km south of Wiseman and approximately 107 km south of the northern limit of *C. c. puniceus*. These minima are probably an under representation of temperatures that *C. c. puniceus* as a species have experienced, both historically and in recent years. Unofficial evidence suggests that temperatures a little below –60°C have recently been reached in the Wiseman area. This is low enough to expose *C. c. puniceus* to their highest glass transition temperature as recorded in the present study. Low ambient temperatures are usually buffered by an insulating layer of snow; however, there are years when snow cover is minimal, resulting in below-snow temperatures that are comparable to above-snow temperatures (Fig. 1).

Regardless of location, supercooled *C. c. punicius* larvae were regularly found in direct contact with ice crystals for months at a time and at low temperatures (Bennett et al., 2005). Since larvae were found not to be at increased risk of inoculative freezing (Bennett et al., 2005), direct ice contact and low temperature may enhance dehydration, which we assume is taking place through differential vapor pressure between the unfrozen insect and external ice. This process has been noted in other organisms such as earthworm cocoons (*Dendrobaena octaedra*) and collembola (*Onychiurus arcticus*) (Holmstrup et al., 2002), and the Antarctic midge, *Belgica antarctica* (Elnitsky et al., 2008). The direct contact between ice and the body of the larva reduces the diffusion distance, which is inversely proportional to water loss rate (Holmstrup and Zachariassen, 1996). Exposure to low temperatures increases differential vapor pressure between the frozen microhabitat and the supercooled body fluids; consequently, these organisms lose water to the environment until they come to a new equilibrium. It is problematic whether *C. c. puniceus* larvae reach vapor pressure equilibrium with the environment at the extremely low temperatures to which they are exposed, and consequently, they may not correctly be said to undergo ‘cryoprotective dehydration’ that requires vapor pressure equilibrium (Holmstrup et al., 2002). However, dehydration results in the low water contents that are found in larvae in winter, especially those that deep supercool. For Fairbanks larvae, the range of water contents associated with deep supercooling was 0.6–0.2 mg mg^{-1} dry mass (compared with 2.0 mg mg^{-1} dry mass in

Table 4. Temperatures of glass transition of *Cucujus clavipes puniceus* larvae

Individuals	Mass (mg)	Large glass transition temp. (°C)	Small glass transition temp.* (°C)	Magnitude (J g^{-1} deg.$^{-1}$) of large glass transition	Magnitude (J g^{-1} deg.$^{-1}$) of small glass transition
1	1.7	–66†	Not obs	0.6	Not obs
2	2.3	–58†	Not obs	0.6	Not obs
3	3.3	–71†	Not obs	0.6	Not obs
4	6.5	–76‡	Not obs	0.6	Not obs
5	10	–75‡	–96	0.45	0.15
6	10.6	–76‡	Not obs	0.6	Not obs
7	12	–76†	–98	0.45	0.15

*The small glass transition temperature, which could not be detected while warming at 5°C min^{-1}, was obtained from the 40°C min^{-1} thermogram and adjusted for thermal lag by subtracting the temperature difference between the large glass transition events on the 40°C min^{-1} and 5°C min^{-1} thermograms.
†Glass transition temperature was measured while warming at 10°C min^{-1}.
‡Glass transition temperature was measured while warming at 5°C min^{-1}.

summer) and 0.9-0.3 mg mg^{-1} dry mass for the Wiseman population. There is no significant difference in mean water content (approximately 0.4 mg mg^{-1} dry mass) between populations.

In addition to decreasing the amount of water available for freezing, dehydration also causes concentration of solutes. There was a 1.6-fold increase in mean glycerol concentration in individuals that did not freeze compared with insects that froze, with one individual having a glycerol concentration of 6.5 mol l^{-1} (Table 3). Although we did not directly measure antifreeze protein concentration, there was also a 1.2-fold increase in thermal hysteresis activity (a proxy for the presence and concentration of antifreeze proteins) in diluted homogenates of unfrozen individuals compared to those of individuals that froze. In general, the amount of dehydration measured could cause the concentration of antifreezes to increase by as much as fivefold; therefore, AFPs not only contribute to supercooling ability of non-deep supercooling individuals, but they also almost certainly contribute to the ability of individuals to deep supercool and vitrify. The inability to sample larval hemolymph, because of their extreme desiccation in winter, precludes the determination of hemolymph thermal hysteresis, therefore necessitating the measurement of thermal hysteresis in homogenates. As a result we do not know the thermal hysteresis activity of winter hemolymph; however, we reported earlier (Bennett et al., 2005) that hemolymph from autumn larvae prior to desiccation exhibited nearly 13°C of thermal hysteresis when concentrated 3.2× to reflect a level of dehydration experienced by winter larvae under a lesser level of dehydration. The known ability of AFPs nearly identical to those of *C.c. puniceus* to inhibit ice nucleators (Duman, 2001; Duman, 2002) indicates that the *Cucujus* AFPs assist deep supercooling and vitrification.

Although dehydration in Alaskan *C. clavipes* larvae contributes to cold hardiness, many insect species do not readily lose body water over the winter period yet still show a seasonal increase in supercooling capacity (Zachariassen, 1985; Duman et al., 1991; Bennett et al., 2005). Even the eastern subspecies of *Cucujus* from northern Indiana (~41°45′N) does not dehydrate in winter but supercools to approximately –23.0°C (Bennett et al., 2005). By contrast, the western subspecies investigated in this study loses body water and increases supercooling capacity during winter, with the annual minimum supercooling point for the eastern subspecies being achieved by the western subspecies in Alaska in October, prior to dehydration. For other insects, like the western subspecies of *C. clavipes*, increased supercooling capacity is correlated with a decrease in body water, although vapor pressure equilibrium, and therefore strict cryoprotective dehydration, may not be attained (Lundheim and Zachariassen, 1993; Gehrken, 1989; Rickards et al., 1987; Leather et al., 1993; Block, 2003; Worland and Block, 2003; Danks, 2000; Bennett et al., 2005). In fact, the association between water content and supercooling in this study indicates that Fairbanks populations maintain a moderate association (R^2=24%) between SCP and WC in autumn and winter. For the Wiseman population, the association increases from 24% in the autumn to near 40% in winter. Zachariassen et al. (Zachariassen et al., 2004a; Zachariassen et al., 2004b) explain the correlation between log body mass and supercooling point in freeze-avoiding insects by citing the work of Bigg (Bigg, 1953), who found a linear relationship between supercooling and the logarithm of water volume: as log mass (and volume of water) declines, supercooling capacity increases. As a consequence of low water content through dehydration and high solute concentration, the diffusion of water molecules should be inhibited at some low temperature, allowing the remaining water to turn to glass, a situation that has been described as a ‘viscous slowing down of supercooling liquid’ (Tarjus and Kivelson, 2000). We believe that this low temperature threshold in *C.c. puniceus* larvae is approximately –58°C. Bennett et al. (Bennett et al., 2005) and this study did not record any freezing events in *Cucujus* larvae at temperatures less than –58°C.

The present study provides direct evidence, through differential scanning calorimetry, that a glass transition temperature can occur at –58°C. By depressing the homogeneous nucleation temperature of body water to below the glass transition temperature (Fahy et al., 1984), tolerance of dehydration, and by a combination of noncolligative AFPs and colligative antifreeze (glycerol), *C. c. punicius* larvae should not be threatened with ice nucleation, even under extreme low ambient temperatures and in direct contact with ice crystals. Theoretically, vitrified larvae should be stable for long periods of time because of the unique properties of a vitrified substance. Larvae in a vitrified condition will not have the stress of volume expansion and grain growth of ice in tissues (Hochachka and Somero, 2002), and since the vitrified condition encompasses both intra-and extra-cellular fluids, vitrified larvae should not encounter the differential osmotic and ionic stress (Storey, 2004) that is associated with concentration of solutes when extracellular water freezes. It is interesting that Holmstrup et al. (Holmstrup et al., 2002) also demonstrated that ice formation did not occur in dehydrated collembola and earthworm cocoons cooled to –60°C, although vitrification was not reported.

We suggest that over a broad range of temperatures, *C. c. puniceus* larvae overwinter through a continuum of supercooling capacities by which they survive high latitude winters in a freeze-avoiding state. Production of AFPs in early autumn, followed by cessation of feeding and clearing of the gut, lower the SCPs into the –20°C range. Then, between autumn and winter glycerol accumulates, body water decreases, and there is a concomitant increase in solutes (including glycerol and antifreeze proteins) that affords individuals low temperature freezing resistance to approximately –40°C. As body water declines to near 0.4 mg mg^{-1} dry mass, the additional increase in solutes (which in one case reached approximately 6 mol l^{-1} glycerol) has the additional benefit of increasing viscosity, leading to a threshold for vitrification of approximately –58°C. We have measured hundreds of nucleation temperatures in winter *C. c. puniceus* larvae, and –58°C is the lowest SCP that we have recorded, a temperature which is consistent with the highest glass transition temperature measured in larvae. For temperatures lower than –58°C, the combination of low body water, increase in viscosity, and low temperatures, promote vitrification of body fluid that augments survival of individuals well beyond temperatures officially recorded in Alaska, putatively adding vitrification to the list of potential insect overwintering strategies. While most *C. c. puniceus* larvae overwintering *in situ* are covered by an insulating snow cover for much of the winter, this is not always the case (Fig. 1). Although our evidence of low temperature survival indicates that individuals can survive temperatures lower than the lowest glass transition range, we did not directly test survival and vitrification simultaneously. The next tasks are to examine whether larvae truly vitrify in nature and whether they are capable of surviving vitrification under laboratory conditions.

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