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Comparative overwintering physiology of Alaska and Indiana populations of the beetle *Cucujus clavipes* (Fabricius): roles of antifreeze proteins, polyols, dehydration and diapause

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Summary

The beetle *Cucujus clavipes* is found in North America over a broad latitudinal range from North Carolina (latitude ~35°N) to near tree line in the Brooks Range in Alaska (latitude, ~67°30′ N). The cold adaptations of populations from northern Indiana (~41°45′ N) and Alaska were compared and, as expected, the supercooling points (the temperatures at which they froze) of these freeze-avoiding insects were significantly lower in Alaska insects. Both populations produce glycerol, but the concentrations in Alaska larvae were much higher than in Indiana insects (~2.2 and 0.5 mol l^{-1}, respectively). In addition, both populations produce antifreeze proteins. Interestingly, in the autumn both populations have the same approximate level of hemolymph thermal hysteresis, indicative of antifreeze protein activity, suggesting that they synthesize similar amounts of antifreeze protein. A major difference is that the Alaska larvae undergo extreme dehydration in winter wherein water content decreases from 63–65% body water (1.70–1.85 g H_2O g^{-1} dry mass) in summer to 28–40% body water (0.40–0.68 g H_2O g^{-1} dry mass) in winter. These 2.5–4.6-fold reductions in body water greatly increase the concentrations of antifreeze in the Alaska insects. Glycerol concentrations would increase to 7–10 mol l^{-1} while thermal hysteresis increased to nearly 13°C (the highest ever measured in any organism) in concentrated hemolymph. By contrast, Indiana larvae do not desiccate in winter. The Alaska population also undergoes a diapause while insects from Indiana do not. The result of these, and likely additional, adaptations is that while the mean winter supercooling points of Indiana larvae were approximately –23°C, those of Alaska larvae were –35 to –42°C, and at certain times Alaska *C. clavipes* did not freeze when cooled to –80°C.

Key words: beetle, insect, cold tolerance, antifreeze protein, subzero adaptation, vitrification, *Cucujus clavipes.*

Introduction

Insects that overwinter in regions where they are exposed to subzero temperatures must adapt by either becoming freeze tolerant (able to survive freezing of their body fluids) or freeze avoiding (Zachariassen, 1985; Bale, 1987; Storey and Storey, 1988; Block, 1990; Danks, 1991; Duman et al., 1991; Lee and Denlinger, 1991). Suites of adaptations involving high concentrations of polyols (such as glycerol) functioning as antifreezes or cryoprotectants, antifreeze proteins (AFPs; Duman, 2001), ice nucleators (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) can be important contributors to surviving subzero environments. These adaptations might be expected to be exaggerated in insects from arctic and subarctic regions, where temperatures can reach –60°C or lower (Danks, 1981; Miller, 1982; Ring, 1982; Sömme and Block, 1991; Barnes et al., 1996).

The beetle *Cucujus clavipes* (Cucujidae) has a broad latitudinal range, from North Carolina (~35°N) to northern interior Alaska above the Arctic circle (~67°30′ N). Consequently, this species presents the opportunity to study overwintering physiology over a large latitudinal expanse, including one of the coldest environments in North America. Previous studies of overwintering adaptations of an Indiana population of *C. clavipes* demonstrated the activity of AFPs

and mean lower lethal temperatures in winter ranging from –18 to –25°C (Duman, 1979, 1984). Interestingly, *C. clavipes* were freeze tolerant during the winter of 1979, but by 1983 they had altered their overwintering mechanism to freeze avoidance (Duman, 1984). In contrast to the Indiana population, Miller (1982) reported lower lethal temperatures of *C. clavipes* from interior Alaska of –55°C or colder. The supercooling points of the Alaskan insects were not reported, and whether they were freeze tolerant or freeze avoiding was not known. Miller did not screen the Alaskan insects for the presence of AFPs. The goal of our present study was to compare the overwintering adaptations of populations of *C. clavipes* from the northern limit of their range in arctic and subarctic Alaska with those near the southern end of their range in northern Indiana. Special attention was given to the role of antifreeze proteins because AFPs had not previously been studied in Alaskan insects, even though they are now known to be common in Alaskan terrestrial arthropods (Duman et al., 2004).

Materials and methods

Study sites

Three primary collecting sites in the USA were used in these studies: near (1) South Bend, Indiana (northern Indiana and southwestern Michigan), (2) Fairbanks, Alaska and (3) Wiseman, Alaska. *C. clavipes* larvae collected from each site are referred to as 'Indiana', 'Fairbanks' and 'Wiseman', respectively. Minimum winter temperatures in the South Bend area (~41°45′ N; 86°15′ W) are typically –20 to –30°C, although recent winters have been relatively mild. Since Fairbanks (~64°72′ N; 147°47′ W) is located in the interior of Alaska and has a continental climate, it provides a site with very low temperatures. Minimum winter temperatures often reach –50°C. Wiseman, Alaska (67°30′ N, 150°11′ W) is approximately 100 km north of the Arctic circle. Latitudinal tree line is approximately 50 km north of Wiseman, so this provides a site near the northern limit of *C. clavipes*. (In fact, despite considerable collecting time, we have not found *C. clavipes* beyond ~5 km north of Wiseman.) Altitudinal tree line is only ~150 m on the ridges around Wiseman. The climate here is slightly more extreme than Fairbanks. Both Alaska sites are in boreal forest while the Indiana site is in eastern deciduous forest.

Cucujus clavipes (Coleoptera: Cucujidae) larvae were collected from each of these populations at various times in the year between 2001 and 2004 and studied for seasonal changes in supercooling point (SCP), hemolymph thermal hysteresis activity (THA), polyols, water content and respiration rate. Air and microhabitat temperatures were monitored using Hobo Pro Series data loggers along with BoxCar Par 4 software (Onset Computer Corporation, Bourne, MA, USA).

Outdoor enclosures and indoor acclimations

Although field collections of larvae were made during all seasons, to ensure sufficient material for mid-winter experiments, *Cucujus* larvae were collected around Fairbanks in September and placed in plastic food storage containers (20×15×10 cm; *N*=20–50 per box) with moist bark from their native trees. To simulate field conditions, some boxes were placed in an outside enclosure in a wooded area on the University of Alaska, Fairbanks (UAF) campus either on small logs at ground level or ~0.5 m above ground to reduce the insulating effect of snow cover and expose them to colder winter air temperatures. Larvae collected in September from Fairbanks were also placed in box enclosures outdoor in Indiana, and September-collected Indiana larvae were placed outdoors in box enclosures in South Bend and Fairbanks. Larvae collected near Wiseman were also placed into containers in the field. Temperature data loggers (see below) were used to monitor enclosures and air temperatures at these sites. Boxes were retrieved in either mid-winter (January) or late winter/spring (March or April). *Cucujus* survival, SCPs, thermal hysteresis activity, water content and respiration rates were determined as described below. Additional boxes of insects collected in September were cold-acclimated in a Tenney Series 942 environmental chamber at the University of Notre Dame according to the following protocol. On days 1–3 the insects were held at 0°C, days 4–6 at –1°C, days 7–9 at –2°C, days 10–14 at –3°C, days 15–21 at –4°C and days 22–30 at –4.5°C.

Supercooling points

To determine SCPs, thermocouples were fixed to the dorsal surface of individual larvae using a small amount of beeswax, and larvae were suspended in 1.5 ml plastic tubes that were placed inside a larger glass container that was submerged in a cooling bath. Once equilibrated to 0°C, the container temperature was reduced at a rate of 0.2 deg. min^{-1}. The lowest larval temperature recorded before the release of the latent heat of fusion of body water, as evidenced by an exotherm, was recorded as the SCP (Lee and Denlinger, 1991). To determine their susceptibility to inoculative freezing during different seasons, SCPs were also determined on larvae in contact with ice. In these cases, larvae were equilibrated at –2 to –5°C to ensure that surrounding water was frozen before further cooling.

Thermal hysteresis activity

THA is an indication of the presence and activity of antifreeze proteins (AFPs) and was determined according to the method of DeVries (1986). Hemolymph samples (~2–6 μl) were drawn from punctured individual larvae when possible or pooled from larvae as necessary and sealed in glass capillary tubes. The sample was partially frozen by freezing the outside of the capillary tube with a spray freeze (Fisher Brand Super Friendly Spray Freeze; Fisher Scientific, Pittsburgh, PA, USA) and the temperature slowly raised to melt the ice until the ice crystal was just visible or disappeared under the microscope (= melting point). Beginning again with a seed crystal ~0.25 mm in diameter, the temperature was lowered very slowly until it was observed to grow (= freezing point). In the absence of AFPs, if the temperature is lowered 0.01–0.02°C below the

melting point the crystal will immediately grow (i.e. melting point = freezing point). However, if AFPs are present, the crystal will not grow until the temperature has been lowered to the hysteretic freezing point, whereupon the crystal grows rapidly (i.e. melting point and freezing point are not equal). The difference between melting point and freezing point is taken as the THA.

Water content

Total body water content was determined according to Rojas et al. (1986). Individual larval fresh mass was determined to the nearest 0.1 mg. Larvae were then dried at 60°C to constant dry mass (~48 h). Body water content was calculated as the percentage of initial fresh mass lost during drying. The absolute body water content of larvae was also calculated (g water g^{-1} dry mass; Hadley, 1994).

Polyol determinations

^{13}C NMR was used to determine the presence of polyols and other potentially important solutes in the hemolymph of cold acclimated *C. clavipes* larvae. The $^{13}C\{^{1}H\}$ NMR spectrum was obtained on a Varian Unity *Plus* 600-MHz NMR spectrometer equipped with dual $^{1}H/^{13}C$ 3-mm microprobes (Nalorac, Martinez, CA, USA), operating at 150.86 MHz for ^{13}C. The hemolymph sample (250 μl) was diluted with 30 μl of $^{2}H_2O$ and transferred to the NMR tube prior to data collection. Data acquisition conditions were as follows: 31 000 transients; 2.5 s recycle time; 303 K; 1-230 p.p.m. spectral window. The resulting free induction decay (FID) was zero-filled (yielding a final digital resolution of 0.14 Hz per point), and a 1-Hz line-broadening function was applied prior to Fourier transformation. Chemical shifts were referenced to the most intense C1/C3 signal of glycerol (64.2 p.p.m.) observed in the spectrum (Kukal et al., 1988).

Glycerol concentrations in the hemolymph were determined using a colorimetric assay (Boehringer Mannheim/R-Biopharm, Marshall, MI, USA) (Kreutz, 1962).

Respirometry

Insect resting rates of CO_2 production were measured using a flow-through respirometry system (Sable Systems International, Las Vegas, NV, USA) with a LiCor model LI-6252 CO_2 analyzer (Lincoln, NB, USA). The incoming air stream (baseline) was scrubbed of water vapor and CO_2 using Molecular Sieve™ and Ascarite™ and magnesium perchlorate, respectively. Air flow rate was 25 ml min^{-1}. Carbon dioxide production by individual larvae was recorded for 20 min with 10 min of baseline recording between larvae. Summer larvae were removed from food for 24 h before recordings to clear the digestive tract. This was not necessary for winter larvae since they had ceased feeding. Each larva was recorded at ambient temperatures of 0, 10 and 20°C and allowed to equilibrate in each condition for one hour before recording. To prevent desiccation between recordings, insects were flushed with air humidified to 83% relative humidity by bubbling the air through a 20% KOH solution at a rate of 25 ml min^{-1} (Solomon, 1951). Insects were weighed to the nearest 0.1 mg both before and after respirometry and typically showed less than 10% change in fresh mass. Those that lost more than 10% mass or that defecated while in recording chambers were excluded from analysis. Recordings were analyzed using DataCan (Sable Systems International) or LabGraph (developed by Oivind Toien, University of Alaska, Fairbanks). For each larva, mean CO_2 production (μl h^{-1} g^{-1} dry mass), corrected for standard temperature and pressure, assuming a respiratory quotient of 0.85, was calculated from the most stable 2–5 min of the 20 min recording to exclude fluctuations due to animal movement. (Note that even the use of respiratory quotient values very different from 0.85 would not affect the results of this study.)

Results

Microhabitat characteristics

In winter, as well as summer, *C. clavipes* larvae inhabit the layer under slightly loose bark of recently dead trees, both standing and fallen. In Alaska they are found almost exclusively in poplar (*Populus* spp). In northern Indiana and southern Michigan, where poplar is less abundant, *C. clavipes* are also present in other species (ash, maple and oak). While larvae may be dispersed through a large part of the tree in summer, in standing trees in winter in Alaska they tend to move to within ~30 cm of the base of the tree, perhaps because here they are generally insulated by snow for most of the winter. This is not the case for larvae in Indiana and Michigan, where winter larvae are commonly found above 30 cm from the base of standing dead trees. Larvae in fallen logs do not move away from exposed portions of the log to better insulated regions closer to the ground. As a consequence, temperatures experienced by *C. clavipes* in any given area can vary considerably over the course of a winter, depending on their microhabitat.

Air and microhabitat temperatures were monitored over the course of three years in the three primary collecting sites: near South Bend (Indiana) and Fairbanks and Wiseman (Alaska). Fig. 1 illustrates the diversity of temperatures among these climates and permits the physiological adaptations to be placed into context. Fig. 1A demonstrates that larvae in a fallen log (near Fairbanks) that was suspended off the ground, and therefore had little insulation, experienced low temperatures similar to air, a minimum of –37°C during winter 2001–2002. Fig. 1B shows winter air and microhabitat temperatures at Wiseman, Alaska, near the northern limit of the range of *C. clavipes*. Air temperatures here are somewhat lower than at Fairbanks, and the winter is longer. However, snow depths are generally greater and in these circumstances provide additional, prolonged insulation for those larvae under the snow. Consequently, larvae in this well-insulated site experienced a minimum temperature of only –13°C in October prior to heavy snow cover. Microhabitat temperatures in other sites varied according to the amount of insulation provided by snow (data not shown). Fig. 1C illustrates the shorter and less

severe winters experienced by *C. clavipes* near South Bend, Indiana. During the winter of 2002–2003, the minimum temperature experienced by larvae in this 'above ground' log was –13°C, the same minimum temperature in the insulated site at Wiseman (Fig. 1B). (It should be mentioned that recent winters at all three sites were warmer than average.) These three examples illustrate the variation in microhabitat temperatures at the collecting sites. Although air temperatures are lower in Alaska, the temperatures experienced by *C. clavipes* depend on their microhabitat temperatures, and these can vary drastically within a given region. Obviously, the duration of the winter is much longer in Alaska.

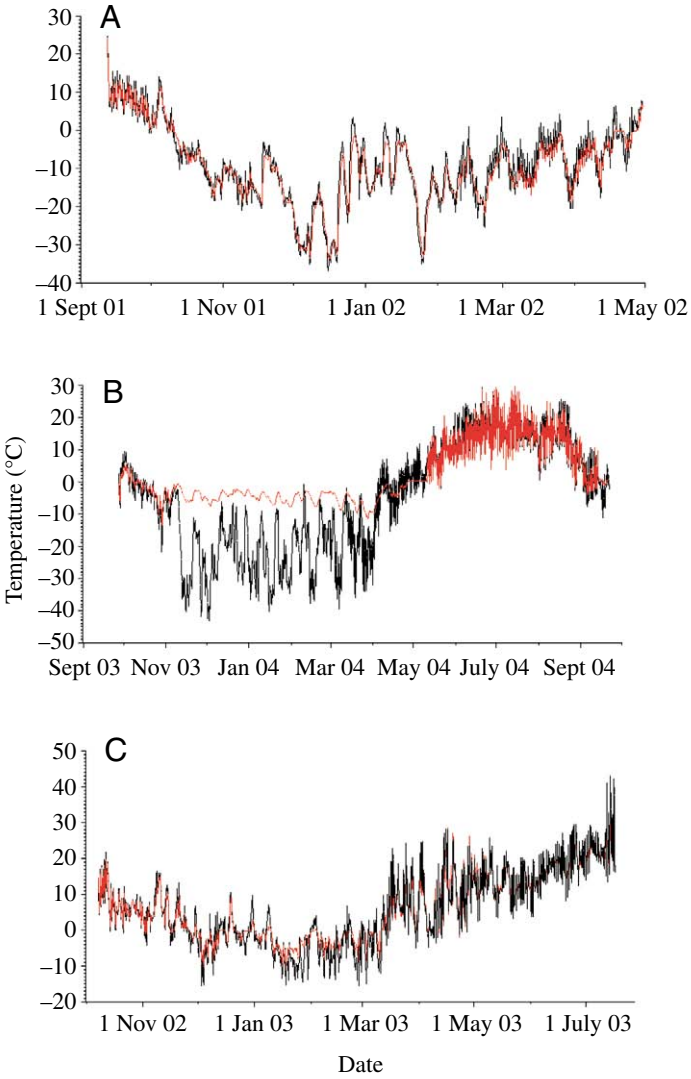

Fig. 1. Representative microhabitat (red) and air (black) temperatures experienced by *C. clavipes* in Alaska and Indiana over the three years of the study. Examples were chosen to illustrate the extremes of insulated and uninsulated microhabitats. Note how microhabitat temperatures in poorly insulated sites (in logs above snow levels) closely track air temperatures, while those in well-insulated sites (in logs below snow level) generally remain warmer than air temperatures after snow arrives in winter. (A) Temperatures in a poorly insulated log (off the ground) near Fairbanks, Alaska. (B) Temperatures at a well-insulated site near Wiseman, Alaska. (C) Temperatures in a poorly insulated log near South Bend, Indiana.

Populations of *C. clavipes* must be adapted to survive a range of minimum temperatures, depending on the air temperatures occurring during a given winter and the extent of insulation provided by a given microhabitat. This ability is illustrated by an experiment where larvae collected from habitats near Fairbanks in October 2002 were placed into two enclosures, one placed at ground level and the other above the eventual snowline. Temperatures experienced by the two groups are shown in Fig. 2. In early January, larvae in the ground level box insulated by snow experienced a minimum temperature of –15°C while the group above the snow had a minimum temperature of –35°C. In spite of these large differences, both groups had >90% survivorship when the boxes were retrieved in mid-January (95.7% in the ground box and 93.4% in the high, uninsulated box).

Supercooling points and mortality

Winter *C. clavipes* larvae from all sites died when frozen, even for a brief period, at their supercooling points. In contrast, larvae cooled and held at just above the SCP exhibited no mortality. Consequently, the SCP appears to be the lower lethal temperature for the larvae in winter. Representative SCPs of field-collected Alaska larvae during various seasons are shown in Fig. 3. Summer larvae, such as those collected near Fairbanks on 17 August 2002, had high SCPs (mean, –7.2°C); however, by early October the mean SCP had decreased to –25.3°C. SCP values continued to decrease through the autumn. Note especially the detailed data from autumn 2003. By early January 2003, the mean SCP in these larvae reached –42.2°C, with individual SCPs as low as –58°C. SCPs of larvae collected from either well or poorly insulated positions were similar, as illustrated by values from the two groups held in enclosures (mean, –34.9°C in the ground box and –36.9°C in the high box). In mid-March 2003, SCPs in Fairbanks larvae from the field were still quite low (mean, –35.1°C), and larvae from the ground and the uninsulated high box enclosures still

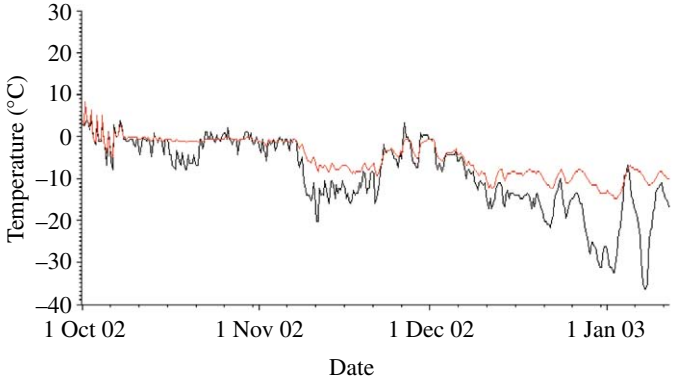

Fig. 2. Temperatures experienced by *C. clavipes* larvae in two box enclosures near Fairbanks, one near the ground and therefore well insulated by snow (red line) and the other above snow level (black line).

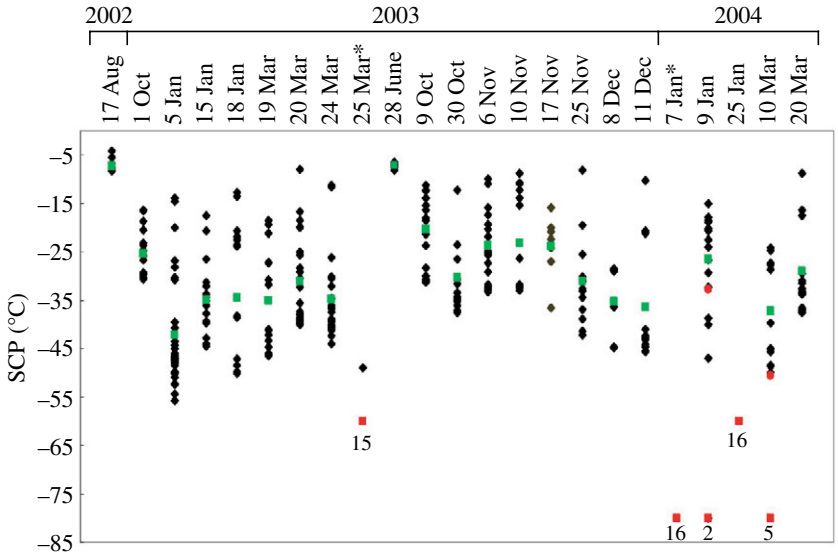

Fig. 3. Supercooling points (SCPs) of Alaska *C. clavipes* larvae from summer 2002 to spring 2004. Most are from the vicinity of Fairbanks, except those collection times identified by an asterisk, which are from near Wiseman. Black diamonds represent individual SCPs; however, there are numerous overlapping values. Green squares identify mean SCPs for a given date. Red squares identify larvae that were cooled to –60°C or –80°C without expression of a freezing exotherm. Numbers below the red squares indicate the number of individuals that did not freeze in that particular run. The red circles show the mean SCP when those individuals that did not freeze at –80°C were included in the mean calculation, using –80°C as the SCP of these individuals (9 January and 10 March 2004).

did not differ from one another (means, –31.2°C and –34.8°C, respectively).

Of a group of March 2003 larvae collected from Wiseman and placed there in a box enclosure the previous September, only 1 of 16 showed an exotherm indicative of an SCP (at –48.9°C), while the other 15 failed to freeze when cooled to –64°C (the lowest temperature our equipment at that time was capable of reaching; Fig. 3). These larvae were then held at –64°C for ~10 h. After then being held at 4°C for several days, seven of 15 were alive, indicating that these had SCPs below –64°C. In early January 2004, none of the 32 Wiseman larvae tested exhibited exotherms when cooled to –80°C, the lowest temperature our equipment at that time was capable of recording (Fig. 3). By contrast, Fairbanks larvae in early January had a mean SCP of –26.5°C, considerably higher than in January of the previous year or in the Wiseman larvae of January 2004. However, by 25 January 2004, the Fairbanks larvae did not freeze when cooled to –60°C (32 of 32). Fairbanks larvae collected on 10 March 2004 had a mean SCP of –37.2°C, not including five individuals (of 16) that did not exhibit exotherms down to –80°C. The absence of freezing at such a low temperature as –80°C hints that the water in these larvae may have been vitrified rather than liquid.

Some individual larvae on most of the winter dates in Fig. 3 have SCPs that are suspiciously higher than might be expected (i.e. 9 January 2004), sometimes even higher than the ambient temperatures experienced by the larvae. At least some of these SCPs are probably real and therefore illustrate considerable variation in the population. However, they may also represent an artifact resulting from the difficulty encountered in the process of removing larvae from under the bark for collection. Considerable amounts of ice are present in this habitat, especially in the sites at the base of standing trees, and it is common for larvae to be partially, or completely, encased by ice. Consequently, the cuticle may be damaged when the larvae are collected, thereby affecting the SCP. Larvae with missing legs or antennae were not used, but more subtle damage, such as broken bristles or abrasions to the cuticle, may have been overlooked. As a consequence, the mean winter SCPs may well be lower than shown.

SCPs presented in Fig. 3 were gathered using ‘dry’ larvae, i.e. those not in contact with ice. However, condensation can occur as air in the container holding the insects is cooled during the SCP measurement. This water can freeze on the surface of the insects. To more closely approximate microhabitat conditions in which larvae are often in contact with ice, SCPs were recorded with Alaskan larvae (Fairbanks and Wiseman)

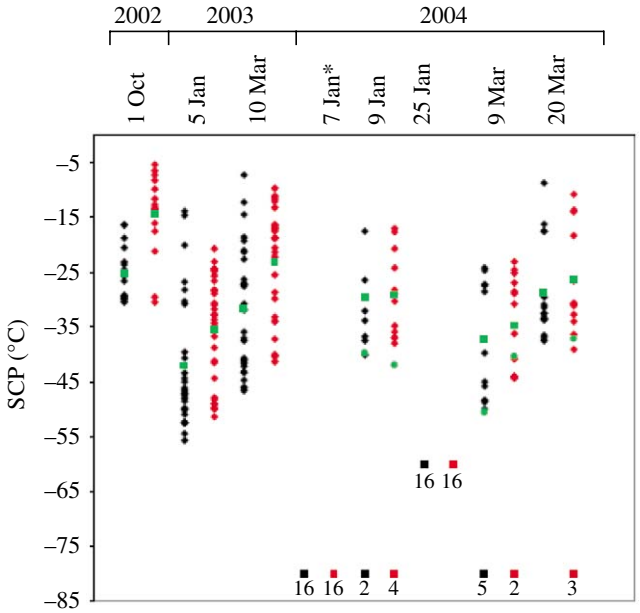

Fig. 4. Comparisons of supercooling points (SCPs) of Alaska *C. clavipes* larvae cooled in contact with ice (red) with those of larvae not in contact with ice (black). Green squares indicate means. Red or black squares indicate larvae that did not freeze at –80°C or –60°C. Green circles indicate means calculated with the inclusion of individuals that did not freeze at –80°C (using –80°C as their SCP).

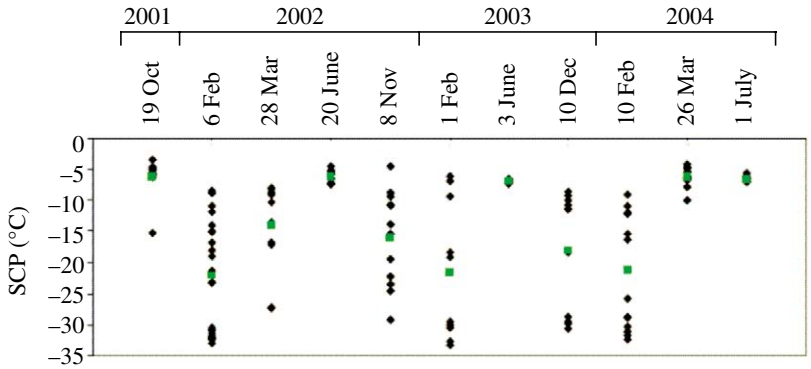

Fig. 5. Representative supercooling points (SCPs) of *C. clavipes* larvae from the vicinity of South Bend, Indiana collected between Autumn 2001 and Summer 2004. Black diamonds indicate individual SCPs; however, there are numerous overlapping values. Green squares identify mean SCPs for a given date.

both in contact with ice and dry (Fig. 4). SCPs of the two groups were not significantly different. Larvae either in contact with ice or dry were included among those cooled to –60 or –80°C without showing exotherms.

SCPs of *C. clavipes* larvae from Indiana measured in winter were much higher than those of Alaska larvae (Fig. 5). A few individual Indiana larvae had SCPs in February as low as –32°C, but mean values were considerably higher. SCPs of larvae in contact with ice did not differ from those of dry larvae (data not shown).

In September of both 2002 and 2003, larvae collected in Indiana were taken to Fairbanks and placed in outdoor box enclosures on the ground, and Fairbanks larvae were placed in enclosures in Indiana. The following spring, mortality of Indiana larvae in Alaska was 100% in both years, while mortality of Fairbanks larvae in Indiana was under 10% in both years. Indiana larvae in box enclosures in Indiana had mortalities of <10%, while mortality of Fairbanks larvae in box enclosures in Fairbanks was 14.3% in 2003. In February 2003, SCPs (mean, –24.0°C) of *C. clavipes* larvae collected near Fairbanks in September 2002 and placed in box enclosures in Indiana were comparable to those of Indiana larvae placed in enclosures in Indiana but were significantly higher than those of Alaska larvae from January 2003 (compare with Fig. 3).

Water content and dehydration

When winter *C. clavipes* were first collected in Alaska (in January 2002), most of the larvae appeared dead, perhaps resulting from the near absence of insulating snow and a period of temperatures near –40°C in the previous month. The larvae were so desiccated that hemolymph samples could not be obtained. When warmed on moist paper towels to 4°C, or higher, most did not become mobile, even after several days. However, in March, mortality of larvae in the field was just 17.4%. Obviously, most of the larvae were not dead in January. Two factors may be responsible for the immobility of the larvae in midwinter. These are metabolic diapause (see below) and dehydration.

Mean water content of Alaskan *C. clavipes* larvae in summer was 64.5%, or 1.84 g water g^{-1} dry mass on 28 June 2003 (Fig. 6). However, in January 2004, the mean water content decreased to 27.6–40.3% (0.40–0.68 g water g^{-1} dry mass). By contrast, Indiana larvae during winter in Indiana maintained the same approximate water content as in summer. Also, Alaska larvae held in a box enclosure in Indiana had mean water contents of 61.6 and 64.6% (1.72 and 1.87 g water g^{-1} dry mass) in February 2003 and January 2004, respectively. The severe dehydration in Alaskan larvae in winter might contribute to the low midwinter SCPs by concentrating solutes (especially glycerol and antifreeze proteins) and by making

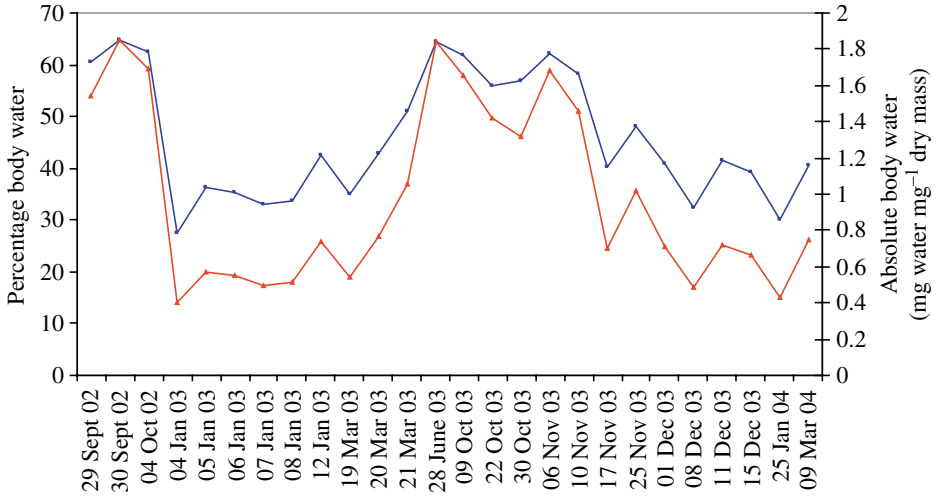

Fig. 6. Water content of *C. clavipes* larvae collected near Fairbanks between 29 September 2002 and 9 March 2004. Horizontal axis identifies collection dates. Vertical axes show percent body water (blue line) and absolute body water (red line).

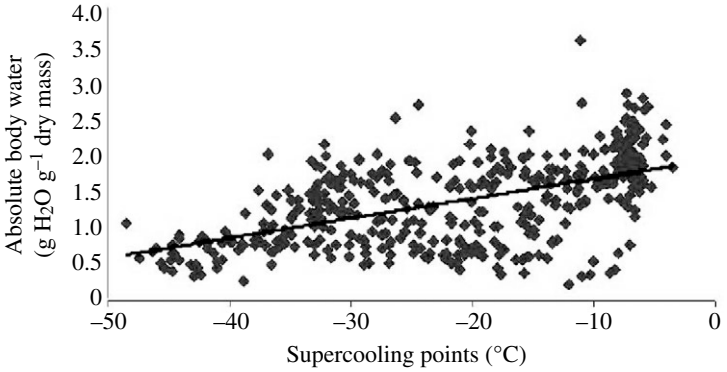

Fig. 7. Correlation between supercooling points and absolute body water of *C. clavipes* larvae. There was a moderate, but highly significant, association between absolute body water and SCP (r=0.6079, P<0.001, N=384), based on the Spearman rank correlation.

less water available for freezing. To determine whether a relationship exists between absolute body water content and supercooling point, SCPs and absolute body water contents of several hundred individuals were measured between September 2002 and November 2004. Fig. 7 presents the results of the Spearman Rank Correlation between absolute body water and SCP of larvae over this period. Absolute body water and SCP were not normally distributed. There was a moderate, but highly significant, association between absolute body water and SCP (r=0.6079, P<0.001, N=384). Absolute body water explains approximately 37% of the variation in SCP. Note that winter larvae for which an exotherm could not be measured when cooled to –60 or –80°C were not included in these calculations.

Antifreezes: glycerol and antifreeze proteins

Antifreezes are expected to be major factors in achieving the very low SCPs seen in winter *C. clavipes* larvae, especially in Alaska. In fact, the larvae produce both polyols and AFPs. Glycerol is the primary colligative antifreeze in both Alaska and Indiana larvae. A typical winter hemolymph glycerol concentration of Indiana larvae is ~0.5 mol l^{-1}, while that of Alaska larvae is considerably greater. Fairbanks larvae collected in late September and cold acclimated for 1 month to a final temperature of –4.5°C had a hemolymph glycerol concentration of 2.2 mol l^{-1}. However, the water content of these acclimated larvae was 63.1% (1.701 g H_2O g^{-1} dry mass), similar to that of summer larvae. Recall that we were unable to collect hemolymph from Alaska larvae in January because they were highly desiccated. The water content of Fairbanks larvae in January 2003 was 35.2% (0.532 g H_2O g^{-1} dry mass), a ~3.2-fold reduction in water content relative to summer. If the hemolymph of the cold-acclimated larvae with normal summer body water content and hemolymph glycerol concentrations of 2.2 mol l^{-1} was concentrated 3.2-fold, the glycerol concentration would be 7.0 mol l^{-1}. This value probably closely represents the true hemolymph glycerol concentration of midwinter Alaska larvae following dehydration. ^{13}C NMR of hemolymph from cold-acclimated Fairbanks larvae (Fig. 8) demonstrated that glycerol is the only solute present in unusually high concentrations, and consequently glycerol is the only polyol antifreeze produced by *C. clavipes* larvae. Trehalose is the next most abundant substrate. Proline is also present.

As illustrated in Table 1, the level of thermal hysteresis indicative of antifreeze protein activity in winter is generally 3–4°C in both Alaska larvae prior to dehydration and in Indiana larvae. However, Alaska larvae produce antifreeze

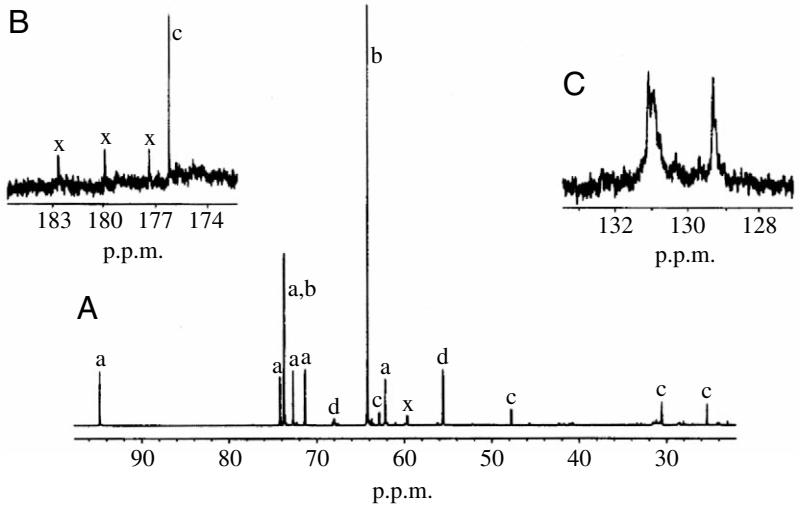

Fig. 8. The $^{13}C\{^1H\}$ NMR spectrum (150 MHz) of cold-acclimated *C. clavipes* hemolymph. (A) Expansion of the 22–98 p.p.m. region of the spectrum. Assignments of the major signals are as follows: a, α,α-trehalose; b, glycerol; c, proline; d, betaine. The signal at ~55.5 p.p.m. is a triplet and that at ~68 p.p.m. is a complex multiplet; assignment of these signals to betaine is tentative [other species bearing a $-N(CH_3)_3^+$ group may be responsible for these signals]. The signal marked with 'x' was unassigned. (B) Expanded carboxyl region of the spectrum showing assignment c of the carboxyl carbon of proline; x was unassigned. (C) Expanded aromatic region of the spectrum, showing two broad multiplets due to presently unknown species. Signals in B and C were very weak compared with those observed in A (note the reduced S/N in B and C relative to A).

Table 1. *Melting points, freezing points and thermal hysteresis activity (melting point – freezing point) of hemolymph from* C. clavipes *larvae collected periodically from Alaska and Indiana*

Date	Sample	*N*	Melting point (°C)	Freezing point (°C)	Thermal hysteresis (°C)
3 Sept 2001	Wiseman	6	–0.85±0.11	–3.98±0.83	3.13±0.86
7 Sept 2001	Fairbanks	8	–0.64±0.29	–3.36±0.25	2.72±0.24
Autumn 2001	Fairbanks cold accl.	8	–3.43±1.14	–6.68±1.22	3.25±0.49
30 Apr 2002	Fairbanks	10	–0.66±0.23	–3.92±0.50	3.26±0.54
21 Aug 2002	Fairbanks	10	–0.69±0.19	–0.97±0.25	0.28±0.12
30 Sept 2002	Fairbanks	6	–0.71±0.19	–4.65±0.91	3.95±0.74
Autumn 2002	Fairbanks cold accl.	9	–2.89±0.44	–6.74±0.55	3.85±0.79
Autumn 2002	Fairbanks cold accl.	Pool	–3.42	–6.70	3.28
Autumn 2002	Above, concentrated 3.2×	Pool	–10.83	–23.68	12.85
30 June 2002	Fairbanks	6	–0.55±0.06	–0.82±0.10	0.27±0.27
14 Feb 2003	Fairbanks, in Indiana	5	–2.84±0.73	–7.17±0.54	4.32±1.24
24 Oct 2001	Indiana	10	–1.03±0.37	–2.19±0.59	1.16±0.60
Autumn 2001	Indiana cold accl.	12	–1.57±0.57	–4.52±1.26	2.94±1.18
8 Feb 2002	Indiana	15	–2.12±0.08	–5.31±0.29	3.18±0.22
29 Mar 2002	Indiana	10	–0.60±0.10	–3.32±0.49	2.72±0.48
20 June 2002	Indiana	8	–0.54±0.10	–0.71±0.17	0.18±0.16
26 Sept 2002	Indiana	7	–0.60±0.15	–0.76±0.16	0.15±0.04
19 Feb 2003	Indiana	3	–2.18±0.25	–6.16±0.29	3.98±0.21
8 Oct 2003	Indiana	6	–0.57±0.11	–1.17±0.30	0.60±0.21
10 Dec 2003	Indiana	8	–0.90±0.16	–4.76±0.55	3.86±0.54
10 Feb 2004	Indiana	9	–2.80±0.50	–6.40±0.50	3.60±0.65
12 Aug 2004	Indiana	7	–0.51±0.14	–0.67±0.11	0.17±0.09
3 Nov 2004	Indiana	8	–0.73±0.17	–2.69±0.90	2.80±0.79

Values are means ± S.D.

proteins much earlier in the autumn and lose them much later in the spring than do Indiana larvae. Alaska larvae collected in early September already had THA nearly equal to that of mid-winter Indiana larvae, while Indiana larvae do not begin increasing THA until late September or October. THA in Alaska insects collected in winter cannot be directly assessed since hemolymph samples cannot be obtained from dehydrated midwinter larvae. Using the hemolymph of the cold acclimated (but not dehydrated) larvae mentioned above in relation to glycerol, we determined a reasonable thermal hysteresis activity of January larvae in the field. THA in a hemolymph pool from the cold-acclimated larvae was 3.28°C. After this hemolymph was concentrated 3.2-fold to reflect the dehydration of the January larvae in the field, the measured THA was 12.85°C (Table 1). This value is by far the highest THA ever measured in any organism. It is interesting that this concentrated THA value is more than 3.2-fold greater than the THA prior to concentration. The high AFP activity in combination with the high concentrations of glycerol and other solutes results in a freezing point depression of the concentrated hemolymph of approximately –24°C.

Respirometry

Based on CO_2 production rates (Fig. 9), it appears that Alaska, but not Indiana, *Cucujus* enter a winter diapause indicated by seasonal depression of metabolic rates. In an analysis of variance using a general linear, full-factorial model, significant between-subjects effects were detected for both population ($F_{1,45}$=4.86, P<0.05) and season ($F_{3,45}$=34.01, P<0.0001), indicating that both location and time of year when insects were collected influenced CO_2 production rates. There was also a significant interaction between population and season ($F_{2,45}$=23.23, P<0.0001), indicating that the seasonal effects differ between the Indiana and Alaska populations. Within subjects, a univariate repeated measures ANOVA (Greenhouse-Geisser corrected P-values) showed that temperature had a significant effect on CO_2 production rate as expected ($F_{1.3,58.0}$=208.87, P<0.0001). Significant interaction terms were detected between temperature and season ($F_{3.9,58.0}$=16.23, P<0.0001) and for the three-way interaction between temperature, season and population ($F_{2.6,58.0}$=22.71, P<0.0001), indicating that season altered the effect of temperature on CO_2 production rate and that this alteration also varied between populations. However, the temperature–population interaction was not significant ($F_{1.3,58.0}$=1.62, P=0.211), suggesting that temperature had a similar effect on CO_2 production rates in both Indiana and Alaska populations of *Cucujus*.

Due to the significance of these interactions, each population was further analyzed separately using univariate repeated measures ANOVA and Tukey *post-hoc* tests to determine where these differences lie. For the Indiana population of

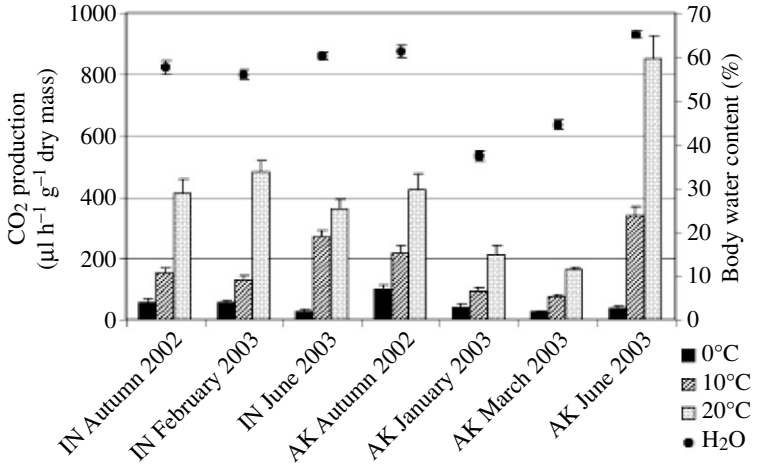

Fig. 9. Seasonal changes in metabolic rate (CO_2 production in µl h^{-1} g^{-1} dry mass) of *C. clavipes* larvae (*N*=6–11) from near South Bend, Indiana and Fairbanks, Alaska. Indiana larvae (IN) were collected on 8 November 2002 (autumn), 1 February 2003 and 3 June 2003. Alaska (AK) larvae were collected between 20 September and 3 October 2002 and either cold acclimated to –4.5°C for one month (AK autumn 2002; see text for details) or held in a box enclosure at ground level under Fairbanks field conditions until 16 January 2003 (AK January 2003 ground box). Alaska larvae were also collected on 19–22 March 2003 and 28–30 June 2003 and run immediately. Metabolic rates were determined at 20, 10 and 0°C. Body water content (% body water) is also shown for these larvae. Values are means ± S.E.M. See text for statements on statistical significance.

Cucujus, temperature significantly affected CO_2 production rate within subjects ($F_{1.4,23.0}$=161.68, P<0.0001), and this effect varied with season when insects were collected (temperature × season interaction; $F_{2.7,23.0}$=7.34, P<0.01). In between-subjects comparisons, there was no observed seasonal metabolic depression for Indiana *Cucujus*; i.e. autumn, winter and summer CO_2 production rates did not differ significantly from each other ($F_{2,17}$=0.30, P=0.75). However, for the Alaska population, both temperature and season significantly affected CO_2 production rate (temperature, $F_{1.3,35.3}$=115.62, P<0.0001; season, $F_{3,28}$=40.88, P<0.0001), and this variation differed with season when insects were collected (temperature × season interaction; $F_{3.8,35.3}$=26.81, P<0.0001). Summer CO_2 production rates were highest, winter rates were lowest, and autumn rates were intermediate, and all significantly differed from each other according to Tukey *post-hoc* test (P<0.001). CO_2 production rates of January- and March-collected Alaska *Cucujus* did not significantly differ from each other (P=0.89), suggesting that *Cucujus* were still in a late-winter diapausing state in March.

Discussion

Larval SCPs seem to reflect the lower lethal temperatures of winter (i.e. September to mid-May) *C. clavipes*, at least in the short term. One caveat is that the difficulty in reviving field-collected mid-winter Alaska larvae, apparently due to diapause and/or extensive dehydration, makes it difficult, at best, to assess mortality at this time. As expected, the SCPs of Alaska larvae were much lower than those of Indiana larvae. However, it is interesting that the temperatures experienced by Indiana larvae in more exposed sites (i.e., fallen logs above the snow) may not be much different from those of Alaska larvae overwintering in more insulated microhabitats. Of course, Alaska larvae must survive periodic extremely cold temperatures (–50°C or less) that may well coincide with a period of little or no snow that occurs early or late in the winter season. The lowest air temperature ever officially recorded in Alaska was –62°C in 1971 at Prospect Creek Camp, ~45 km south of the Wiseman collecting site. In spite of this, Fairbanks larvae placed in box enclosures in Indiana showed mid-winter SCPs similar to those of Indiana larvae. In contrast, Indiana larvae in box enclosures in Fairbanks failed to survive two separate winters, suggesting that these larvae cannot adapt to the harsher Alaska temperatures. However, since Alaska larvae produce AFPs much earlier than do Indiana larvae, an inability to adjust the timing of the onset of overwintering adaptations to the Alaska environment, rather than the actual extremes of the minimum winter temperature, could be at least part of the problem. Therefore, early autumn freezes might have been the actual cause of mortality of the Indiana larvae in Fairbanks.

The low SCPs of Alaska *C. clavipes* are noteworthy. The mean SCP in January 2003 was –42°C, with individual SCPs reaching as low as –58°C. However, even more interesting is the lack of freezing exotherms in Wiseman larvae cooled to –64°C in late March 2003 and the subsequent survival of half of these larvae. Likewise, 32 Wiseman larvae in early January 2004 and 32 Fairbanks larvae collected in late January 2004 failed to exhibit freezing exotherms when cooled to –80 and –60°C, respectively, even with half of the individuals in contact with ice. It is interesting that during the entire three years of the study no exotherms were recorded below –58°C. A possible explanation is that these larvae were vitrified, although there is no experimental evidence to prove this. Miller (1982) reported that two adult *C. clavipes* survived temperatures of –55°C. He speculated that they were freeze tolerant, although SCPs were not measured.

Other insects with extremely low SCPs have been identified. Larvae of the beetle *Pytho deplanatus* from high altitude in the Canadian Rocky Mountains had a mean SCP of –54°C, yet the larvae survived freezing (Ring, 1982). Populations of three species of willow gall insects from interior Alaska exhibited mean SCPs of –56 to –58°C (Miller, 1982). These were two species of Diptera (Cecidomyiidae: *Rhabdophaga strobiloides* and *Mayetiola rigidae*) and a hymenopteran (*Euura* sp.). These freeze-avoiding, gall-forming (and therefore uninsulated) species require extremely low SCPs as they are found primarily in low-lying areas where cold dense air pools in winter, and they are therefore exposed to very cold air temperatures.

However, failure to exhibit freezing exotherms was not reported in these species.

A number of studies of insects and other invertebrates have demonstrated a correlation between dehydration and ability to prevent freezing (Zachariassen, 1985; Lundheim and Zachariassen, 1993; Gehrken, 1989; Rickards et al., 1987; Worland, 1996; Block, 2003; Worland and Block, 2003; Danks, 2000; Holmstroup, 1995; Worland et al., 1998). Over the winter, it is not uncommon for dormant insects to experience water stress, since they typically do not eat or drink. This is especially true of freeze-avoiding species since at low temperatures in the presence of ice in the hibernaculum the partial pressure of water in the air is lower than that of the insect hemolymph. Thus, the insect loses water by evaporation (Lundheim and Zachariassen, 1993). This may be especially problematic for Alaska *Cucujus* because of the length and severity of the winters; in fact, Alaska *C. clavipes* exhibit severe dehydration in winter. Larvae desiccate from mean values of ~63–65% body water (1.70–1.85 g $H_2O\ g^{-1}$ dry mass) in summer to 28–40% body water (0.40–0.68 g $H_2O\ g^{-1}$ dry mass) in mid-winter. Although this several-fold reduction in water volume may cause water stress in the larvae, it should also promote supercooling by concentrating antifreezes and reducing the volume of freezable water. The hemolymph glycerol concentration in cold-acclimated autumn Fairbanks larvae was 2.2 mol l^{-1} in these non-dehydrated animals. Following a 3.2-fold reduction in water volume, the glycerol concentration would be at least 7 mol l^{-1}, if there were no downward adjustments in glycerol concentration during the desiccation process. (Actually, mean fold reductions as large as 4.7 were measured. This would result in a glycerol concentration of ~10 mol l^{-1}.) Likewise, the 3.2-fold dehydration of the cold-acclimated larval hemolymph produced THAs of nearly 13°C, much higher than has ever been reported. Therefore, although *C. clavipes* larvae appear to complete AFP synthesis during a short period in late summer, desiccation later in the season effectively concentrates the AFPs several fold. Consequently, Alaska *C. clavipes* may not need to synthesize more AFP than do Indiana larvae. Both cold-acclimated and field-collected Alaskan larvae in late autumn prior to desiccation have approximately the same levels of hemolymph thermal hysteresis as do Indiana larvae in mid-winter. We were unable to extract hemolymph samples from Alaska larvae after desiccation in mid-winter, but presumably the antifreeze concentrations reflect the 3–4-fold increases consistent with the levels of dehydration.

To achieve the extreme levels of supercooling characteristic of Alaska *C. clavipes* larvae requires (1) the inhibition of inoculative freezing initiated by external ice across the cuticle and (2) the removal and/or inactivation of potential ice nucleators. AFPs are known to assist supercooling by both of these mechanisms in larvae of the beetle *Dendroides canadensis* (Olsen et al., 1998; Olsen and Duman 1997a,b; Duman, 2002). It is important to note that the level of protection afforded to the insect by AFPs greatly exceeds the magnitude of thermal hysteresis activity measured in the insect hemolymph, both with respect to inhibition of inoculative freezing and masking of ice nucleators. The absence of freezing in some Alaska *C. clavipes* suggests that their AFPs are able to inhibit ice nucleators to very low temperatures and that they may even inhibit homogeneous nucleation, thereby promoting vitrification. The absence of endotherms between –58°C (the lowest SCP measured) and –80°C (and perhaps lower) indicates that there may be a threshold effect operating such that, beyond a certain level of dehydration (which concentrates the AFPs, glycerol and perhaps other factors leading to high viscosity), vitrification, rather than freezing, may occur.

Another overwintering adaptation present in Alaska, but not Indiana, larvae is diapause. While Indiana larvae may continue to feed well into November and resume feeding in March, the winter season is much more extended in Alaska. This may necessitate the reduced metabolic state in the Alaska larvae. In addition, the downregulated metabolism characteristic of larval diapause can also contribute directly to supercooling capacity. For example, the stag beetle *Ceruchus piceus* (Lucanidae) removes hemolymph lipoproteins with ice nucleating activity in winter, permitting them to supercool significantly without the production of antifreezes (Neven et al., 1986). It is unlikely that the normal lipid shuttle function of the hemolymph lipoproteins could be spared in winter without a concomitant reduction in metabolic rate in diapausing larvae.

In keeping with the more extreme temperatures experienced by Alaska populations of *C. clavipes*, Alaska larvae exhibited a considerably greater capacity to supercool than did Indiana larvae. As noted earlier, at certain times Alaska larvae failed to freeze even when cooled to –80°C, perhaps suggesting the involvement of vitrification. This level of supercooling may appear to be greater than necessary based on ambient temperatures measured at the Alaska collecting sites over the past three years, when minimum winter air temperatures did not exceed –42°C. However, these winters were abnormally mild. Air temperatures in the interior of Alaska commonly reach –50°C, sometimes for extended periods. In addition, such temperatures can occur at times (i.e. spring) when insulating snow cover is minimal. It appears that the combination of AFPs, glycerol, dehydration and diapause combine to produce extreme levels of supercooling, and perhaps vitrification, in Alaska *C. clavipes*.

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