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Freeze tolerance in an arctic Alaska stonefly

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SUMMARY

Most aquatic insects do not survive subzero temperatures and, for those that do, the physiology has not been well characterized. *Nemoura arctica* is a species of stonefly widely distributed throughout arctic and subarctic Alaska. We collected nymphs from the headwaters of the Chandalar River, where we recorded streambed temperatures as low as –12.7°C in midwinter. When in contact with ice, autumn-collected *N. arctica* cool to –1.5±0.4°C before freezing, but individuals survived temperatures as low as –15°C, making this the first described species of freeze-tolerant stonefly. *N. arctica* clearly survive freezing in nature, as winter-collected nymphs encased in ice demonstrated high survivorship when thawed. In the laboratory, 87% of *N. arctica* nymphs frozen to –15°C for 2.5 weeks survived and, within one month of thawing, 95% of the last-instar nymphs emerged. *N. arctica* produce both glycerol and ice-binding factors (e.g. antifreeze protein) in response to low temperature. Hemolymph glycerol concentrations increased from 3 mmol l^{-1} to 930±114 mmol l^{-1} when temperatures were decreased from 4°C to –8°C, and *N. arctica* continued to produce glycerol even while frozen. Although the hemolymph of individual cold-acclimated nymphs occasionally exhibited more than a degree of thermal hysteresis, typically the hemolymph exhibited only hexagonal crystal growth, indicating a low concentration of ice-binding factor. Hemolymph of nymphs acclimated to subzero temperatures had recrystallization inhibition. These results demonstrate that, in the face of freezing conditions, *N. arctica* exhibit overwintering adaptations similar to those of terrestrial insects.

Key words: insect, freeze tolerance, antifreeze protein, stonefly, aquatic nymph, glycerol.

INTRODUCTION

Aquatic insects are generally protected from the extreme temperatures that terrestrial insects experience because the high specific heat of water thermally buffers the insect's environment, promoting more gradual seasonal changes (Danks, 1971). Even at high latitudes, where winter air temperatures can dip well below –20°C for extended periods, many streams retain perennial flow in the hyporheic zone or at the location of groundwater springs, which can serve as thermal refugia for aquatic invertebrates (Irons et al., 1993; Oswood et al., 1991). Most aquatic insects are able to avoid subzero temperatures altogether by being located in water that will not freeze or by migrating in advance of the freezing front (Olsson, 1981; Irons et al., 1993). However, other small arctic streams freeze into the substratum periodically or even on an annual basis (Huryn et al., 2005). Insects overwintering under these conditions must possess adaptations to survive subzero temperatures, although it is known that most aquatic insects do not even survive exposure to subzero temperatures above their supercooling points (Irons et al., 1993). Some species of aquatic insects, including species of caddisflies (Limnephilidae), dance flies (Empididae), mosquitoes (Culicidae) and midges (Chironomidae) (Danks, 2007), have been reported to survive subzero temperatures. However, the physiology employed by these aquatic insects is poorly understood. With more than 100 species reported, representing 40 genera and four subfamilies, the Chironomidae is the best-represented family in surveys of aquatic insects capable of surviving in frozen substrates (Danks, 1971). More than 25 of these species are able to survive freezing of their extracellular fluids (Danks, 2007). Various species of Chironomids are tolerant of conditions associated with winter survival, including desiccation (Leader, 1962), hypoxia, anoxia (Hilsenhoff, 1991) and freezing (Scholander et al., 1953).

In Alaska, Chironomidae and Nemouridae are the only two families of aquatic insects that increase their proportional representation with increasing latitude (Oswood, 1989), suggesting that individuals in these families possess physiological adaptations to survive the rigors of arctic winters. Even though nemourid stoneflies are associated with streams that are known to freeze (Huryn et al., 2005) and have been shown to survive in frozen sediments (Irons et al., 1993), almost nothing is known about their overwintering physiology or the extent of their cold tolerance.

One reason for the lack of detailed knowledge on the overwintering physiology of aquatic insects is that much of our understanding of overwintering physiology is dominated by research on terrestrial insects, which exhibit two distinct physiological strategies to survive subzero temperatures: freeze avoidance and freeze tolerance (Bale, 1987; Storey and Storey, 1988; Block, 1990; Lee and Denlinger, 1991). We presume that cold hardy aquatic insects will have similar physiological adaptations to terrestrial species. Freeze-avoiding insects do not survive freezing of their body fluids and typically supercool beyond the lowest environmental temperature they are likely to naturally experience (Duman, 2001). Supercooling, cooling below the freezing point without freezing, is enhanced through a variety of adaptations, including the removal of ice nucleators (Neven et al., 1986), dehydration (Lundheim and Zachariassen, 1993; Holmstrup and Sömme, 1998), production of low molecular mass antifreezes and ice-binding factors (IBFs) such as antifreeze proteins (AFPs) (Duman, 2001). IBF is a generic term that includes all relatively high molecular mass (>2 kDa) molecules, such as AFPs and antifreeze glycoproteins, that interact with the surface of ice and

perturb its growth. In a solution with a small ice crystal, IBFs (AFPs) depress the freezing point without significantly affecting the melting point. The resulting difference is termed thermal hysteresis (TH) and is diagnostic for the presence of IBFs (AFPs) (DeVries, 1986). Only proteins and glycoproteins are known to produce TH.

In contrast to freeze-avoiding insects, freeze-tolerant ones survive freezing of extracellular fluid, but they are generally intolerant of intracellular ice. Many freeze-tolerant insects prevent the formation of intracellular ice by producing extracellular ice nucleators (Zachariassen and Hammel, 1976) that promote ice formation at high subzero temperatures, reducing both rate of ice growth and the probability of intracellular freezing (Fahy, 1995). Ice growth in the extracellular fluid excludes solute, increasing the osmotic pressure. This causes an osmotic flux of water out of the cells and results in intracellular desiccation that may eventually pose a challenge to survival (Zachariassen, 1992). To mitigate the effects of intracellular dehydration, freeze-tolerant insects produce low molecular mass cryoprotectants, such as glycerol. These cryoprotectants reduce the proportion of frozen water and have a stabilizing effect on proteins and membranes (Storey and Storey, 1992).

We investigated the overwintering physiology of the widely distributed (Stewart and Oswood, 2006) Alaskan stonefly, *Nemoura arctica* Esben-Peterson (Plecoptera: Nemouridae). We report on several parameters related to the cold hardiness of this insect, including habitat temperature, supercooling capacity, TH and recrystallization inhibition. In addition, survivorship was tested both *in situ* and in laboratory acclimations, allowing us to make an initial characterization of the extent of cold tolerance in high-latitude *N. arctica.*

MATERIALS AND METHODS

Study site and nymph collections

The West Fork of the North Fork of the Chandalar River is a small headwater stream located just south of the Atigun Pass in the Brooks Range in Alaska, USA (~68°8′N; 149°29′W). In early autumn, the main channel is approximately 2.5 m wide by 20 cm deep and is characterized by extensive riffles and fast flowing runs. Nemourid stoneflies, chironomids and tipulids were the most abundant insect taxa encountered in the stream in early autumn (K.R.W., unpublished).

N. arctica nymphs were collected from August through October when the stream was unfrozen. The nymphs were abundant. Typically, four to six researchers could collect several hundred nymphs within an hour using kick screens or by removal from upturned individual rocks. Collected insects were transported to the University of Alaska Fairbanks in stream water maintained near 0°C. The water was aerated when possible. In mid-winter, nymphs were collected from the stream by breaking off pieces of ice with a large axe. The ice pieces were transported in a cooler packed with snow. Survivorship, as defined by coordinated movement (e.g. walking, feeding, etc.), was evaluated 24 h after complete thawing of the ice at 4°C.

Stream temperature collection

Air temperatures and stream temperatures were recorded in high-resolution mode using Hobo Pro Series data loggers (part # H08-031-08; Onset Computer Corporation, Bourne, MA, USA) along with BoxCar Par 4 software (Onset Computer Corporation). Each logger has an internal temperature sensor located in the logger housing rated from –30°C to 50°C, in addition to an 1.82 m-long external sensor rated from –40°C to 100°C. The accuracy of the logger varies with temperature; from 0°C to 40°C the accuracy is ±0.25°C, at –20°C the accuracy is ±0.44°C and at –40°C the accuracy is ±0.75°C. The logger housing was fixed to the stream bank and the external sensor was secured approximately one-third of the way across the stream channel at the water/substrate (cobble and gravel) interface by attaching it to a large stone.

Water temperatures were measured in mid-August and late September with a Physitemp Model BAT-12 (Physitemp Instruments, Clifton, NJ, USA) digital laboratory thermometer, accurate to ±0.1°C between 0°C and 50°C.

Fresh mass and body water determinations

Nymphs were blotted dry with a Kimwipe™ prior to measuring the fresh mass to the nearest one-hundredth of a milligram. Nymphs were subsequently dried at 60°C for 48–72 h before the dry mass was measured (Rojas et al., 1986). Percent body water was determined for summer-collected, autumn-collected, cold-acclimated (at 0°C, –3.7°C and –8°C) and warm-acclimated (4°C) nymphs (N=16–23).

Freezing point determinations

The temperature of inoculative crystallization ($T_{c(inoc)}$), defined as the freezing point of stonefly nymphs in contact with ice, was determined by joining a thermocouple wire to the dorsal surface of a late September collected nymph using petroleum jelly. The insect was then placed in contact with ice in the bottom of an Erlenmeyer flask submerged (~90%) in a cooling bath. The ice was maintained slightly below zero as indicated by a second embedded thermocouple. The insect's temperature was permitted to stabilize for 3–5 min. The bath temperature was then reduced at 0.2°C min^{-1} until freezing was indicated by the release of the latent heat of fusion as recorded with Isothermex v. 3.2 computer software (Columbus Instruments, Columbus, OH, USA). The lowest temperature before the exotherm was recorded as the freezing point (Lee and Denlinger, 1991). Upon freezing, the cooling was halted and the insect was warmed to 0°C. The insect was removed from the flask and placed in water at 0°C and assessed for survivorship.

Nymph supercooling points were also determined in the absence of ice. In this case, nymphs were blotted dry with Kimwipes™ and placed in individual 1.5 ml microcentrifuge tubes. The thermocouple wire was wedged in contact with the insect body using a piece of foam. The 1.5 ml tubes (7–16 individuals per run) were placed inside a large glass container immersed in an alcohol bath. After the temperature was equilibrated at 0°C, the bath was cooled at 0.2°C min^{-1}. Each nymph was removed from the bath at its supercooling point and placed in 0°C water for 24 h to assess survivorship.

Survivorship treatments

Nymphs maintained at 0°C were placed into a sealed plastic sandwich bag ($13 \leq N \leq 106$) containing approximately 150 ml of stream water, which was placed into a second bag to prevent leakage. Insects were held at –1.5°C, –4°C, –5°C, –6.2°C, –8°C, –10°C and –15°C for a period of time ranging from two hours to one week. For warmer treatments (–1.5°C, –4°C), the nymphs were placed in an alcohol bath set to the treatment temperature. For treatment temperatures of –5°C or colder, the bath was set to –4°C until the water froze, then the temperature was reduced by 0.2°C min^{-1} to the final treatment temperature. Timing began when the insects reached the treatment temperature, as indicated by a thermocouple embedded in the ice. The nymphs were thawed at 4°C and survivorship, indicated by coordinated movement, was assessed 24–48 h after complete thawing.

Cold acclimation

Nymphs (N=191) collected in late September were kept in a 2.84-liter plastic container filled with 2 liters of water, aerated with an aquarium air pump. A layer of leaves collected from the stream was placed in the container. The nymphs were maintained at 0°C in darkness for three weeks and then acclimated in a Tenney Series 942 environmental chamber (Thermal Product Solutions, New Columbia, PA, USA) according to the following schedule: –0.2°C, 1 week; –2°C, 1 week; –4°C, 1 week; –6°C, 2 weeks; –8°C, 1.5 weeks; –10°C, 1 week; –12°C, 1 week; –14°C, 1 week; –15°C, 2.5 weeks. All temperature changes in the acclimation occurred gradually over a 24 h period. After 2.5 weeks at –15°C, the temperature in the chamber was adjusted to +1°C and held at this temperature for four days. The thawing process was completed at 4°C. Survivorship, as described above, was evaluated 48 h after thawing. Twenty last-instar nymphs that survived freezing to –15°C were placed on a 16 h:8 h light:dark photoperiod to evaluate the ability of these nymphs to emerge as adults.

Polyol determinations

^{13}C NMR was used to test for the presence of polyhydric alcohols or other potentially important compounds accumulated by *N. arctica* during cold exposure. A group of nymphs (N≈30) acclimated to +4°C (replicated twice) or –8°C were homogenized separately, each with 1 ml of phosphate buffer (pH 7.53) per gram fresh mass. The homogenates were maintained on ice. The homogenates were sonicated with a W-385 Sonicator (Heat systems-Ultrasonics, Farmingdale, NY, USA) for two 30 s intervals at power level 3 and subsequently heated in a boiling water bath for five minutes to inactivate enzymes. After heating, the homogenate was centrifuged at 9000 ***g*** for 20 min. The supernatant was transferred to a 5-mm NMR tube (Wilmad, Buena, NJ, USA) containing 65 µl of $^{2}H_2O$. The final volume was made up to 650 µl with phosphate buffer. The $^{13}C\{^{1}H\}$ spectra were obtained on a Varian Unity Plus 600 MHz (150.86 MHz for ^{13}C) NMR spectrometer equipped with a Varian probe (Palo Alto, CA, USA). The data acquisition parameters were as follows: 10,000 transients; 3 s recycle time; 295.15 K; –15 to 230 p.p.m. spectral window. The free induction decays were zero filled, and a line-broadening function (0.5 Hz) was applied prior to Fourier transformation. While these conditions do not yield quantitative ^{13}C NMR spectra, the peak height is an indication of relative abundance. The spectra were externally referenced using a solution of 1-[^{13}C]mannose dissolved in $^{2}H_2O$; the most intense signal in the spectrum, the C1 signal of D-α-mannopyranose, was set to 95.52 p.p.m.

Glycerol concentrations in the hemolymph were measured using a spectrophotometric assay (Boehringer Mannheim/R-Biopharm, Marshall, MI, USA) (Kreutz, 1962). The procedure was scaled down to a microplate assay so that glycerol concentrations could be determined from the hemolymph of individual nymphs. This was possible for only cold-acclimated nymphs, as the hemolymph was diluted from 100- to 500-fold. The hemolymph from nymphs acclimated to 4°C was pooled and assayed without dilution. For each 96-well plate, the hemolymph samples or dilutions thereof were run in triplicate alongside a standard curve made from dilutions of the standard provided with the kit.

Nymphs acclimated below zero for polyol determinations were sandwiched between ice in the bottom of a 50 ml tube and frozen moistened Kimwipes™. This permitted the quick removal and processing of the insects, since polyol levels are likely to be sensitive to temperature, and protracted thawing at 0°C could affect the results. Nymphs were acclimated stepwise to progressively lower temperatures until the final treatment temperature was attained. Initially, nymphs were acclimated at 0°C for 3 weeks, then to –3.7°C for 2 weeks and finally to –8°C for 2 weeks. All temperature transitions occurred at 1°C h^{-1}.

Thermal hysteresis, crystal morphology and recrystallization inhibition

The TH of *N. arctica* hemolymph was determined for nymphs acclimated at 4°C, 0°C, –3.7°C and –8°C (acclimated in stepwise manner described above). Nymphs were blotted dry and a pulled glass micropipette was used to pierce the cuticle and remove a small sample of hemolymph (≤0.25 µl). A micrometer syringe then delivered between 25 and 100 nl of hemolymph into heavy mineral oil located in the sample well of a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) (Chakrabartty and Hew, 1991). The sample was frozen by cooling to –40°C and then warmed until a single ice crystal was attained. The melting and freezing points of the ice crystal, as well as its growth morphology, were determined at 300× magnification. Crystal growth morphology is a sensitive assay for the presence of IBFs (Griffith and Yaish, 2004). At very low concentrations, IBFs do not produce measurable TH but still perturb crystal growth, typically resulting in hexagonal growth morphology. In the absence of IBFs, crystals grow as round disks.

Recrystallization inhibition (RI) is also a very sensitive assay for the presence of IBFs. For instance, the hemolymph of *Dendroides canadensis*, a freeze-avoiding beetle larvae, can be diluted over 10,000-fold before RI is lost (Knight and Duman, 1986). The RI technique was modified from Knight and Duman (Knight and Duman, 1986). Nymphs assayed for RI were acclimated at –5.7°C in a stepwise manner, as described for the glycerol determinations, and were bled immediately upon thawing. Two microliters of pooled hemolymph (N=10) or control buffer were sandwiched between two glass slides placed on the stage of a Linkam BCS 196 cryostage microscope (Linkam Scientific Instruments, Waterfield, Tadworth, UK). To prevent desiccation, the sample was sealed between the two slides with a small amount of mineral oil placed around the periphery. The control buffer consisted of 25 mmol l^{-1} Tris buffer, 1 mg ml^{-1} bovine serum albumin (BSA) and 0.5 mol l^{-1} glycerol. The melting point of the hemolymph and the control buffer were approximately equal. The sample was frozen by cooling at 40°C min^{-1} to –30°C. The initial picture was taken upon warming the sample to –6°C. The sample was then allowed to anneal for 6 h before the second picture was taken. A large increase in the average crystal size during annealing indicates the absence of RI.

RESULTS

Stream temperature data

The stream began to freeze in late September/early October, but did not freeze completely until approximately 3 weeks later (Fig. 1). Once the available water froze, the temperature began to drop below zero. The lowest recorded streambed temperature for the 2005–2006 and 2006–2007 winter seasons was –9.12°C and –12.7°C, respectively. Unfortunately, the 2005–2006 data are truncated due to a logger failure in early December. The hydrology of the stream appears to be dominated by melt water from the Brooks Range, as no aufeis was observed in winter. In 2007, the stream began thawing around early to mid-April, although the streambed temperature remained near 0°C during the first week of May.

The water temperature measured 4.4°C at five different locations within the stream on 12 August 2008. On 23 September 2008, stream temperatures varied between 1.0°C and 1.4°C at the time of collection.

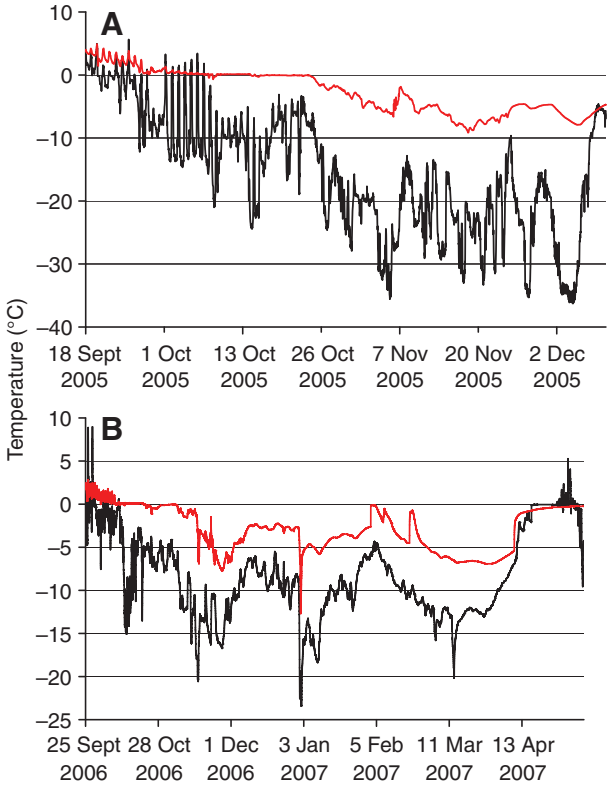

Fig. 1. Stream temperatures measured at the sediment–water interface (red) and the air or ground level temperatures (black) from the headwaters of the Chandalar River located near the Atigun Pass in the Brooks Range, AK, USA. (A) Temperature data from the winter of 2005–2006 was truncated due to a logger failure. Black tracing represents air temperature. (B) Winter 2006–2007. Black tracing indicates temperature at ground level; midwinter ground-level temperatures are higher than air temperatures due to the insulating effect of snow cover.

Table 1. Mean glycerol concentrations and melting points of *N. arctica* hemolymph vary with acclimation temperature

Treatment temperature (°C)	Mean glycerol concentration (mmol l^{-1})*	Percent body water†	Mean melting point (°C)†
4	3 (pool of 16)	74±3^{D} (16)	–0.45±0.08^{E} (15)
0	267±137^{A} (12)	76±3^{D} (16)	–0.69±0.12^{E} (11)
–3.7	576± 66^{B} (11)	77±3^{D} (16)	–1.26±0.46^{F} (17)
–8	930±114^{C} (9)	75±3^{D} (16)	–1.92±0.59^{G} (11)

Values are means ± s.d. (*N*). Means that were statistically different from one another (P<0.05; Tukey's multiple comparison test) are indicated by different superscript uppercase letters. The mean glycerol concentration at 4°C represent measurements made on pooled hemolymph.
*Data collected autumn 2007.
†Data collected autumn 2008.

Fresh mass and body water

The fresh mass of summer-collected (12 August 2008) nymphs ranged from ~0.30 to 4.93 mg. The fresh mass of nymphs collected in early autumn (23 September 2008) ranged between ~0.32 and 7.89 mg, although greater than 90% of nymphs were less than 5.0 mg. The mean body water content varied slightly, but significantly, across some of the groups (ANOVA, P=0.003, N=103). Only the body water content of the summer-collected nymphs (74±3%; N=23) and autumn-collected nymphs (78±3%; N=16) were significantly different from one another (Tukey's multiple comparison test, P=0.001). The body water content did not vary significantly among any of the other treatments (Table 1).

Freezing points

The mean $T_{c(inoc)}$ (± s.d.) of nymphs was –1.5±0.4°C (N=19), approximately 1°C below their hemolymph melting point. When nymphs were not in contact with ice, the mean supercooling point, –7.8±1.5°C (N=16), was significantly lower (Student's t-test, P<0.0001). All autumn-collected nymphs survived transient freezing (≤2 min) at their supercooling point, regardless of whether they were in contact with ice.

Survivorship treatments

While *N. arctica* does not survive temperatures below its $T_{c(inoc)}$ in summer, it survives increasingly lower temperatures as the autumn progresses. In August, when the stream is at summer temperatures, the nymphs are susceptible to subzero temperatures: only 11% of nymphs survived freezing to –1.5°C (Fig. 2), their approximate $T_{c(inoc)}$. It is possible that the nymphs that survived remained supercooled and avoided freezing. Nymphs collected in late September, when the stream temperature was approximately 0°C, exhibited much greater cold hardiness. Greater than 80% of the nymphs survived while frozen in water at temperatures as low as –6.2°C for 1 week. However, these insects exhibited near 80% mortality at –10°C. Nymphs collected one month later exhibited over 90% survivorship at –10°C. October-collected insects did not exhibit high mortality (~80%) until exposure to –15°C.

Cold acclimation

Eighty-seven percent (166 of 191) of *N. arctica* nymphs survived gradual acclimation to –15°C. Of the surviving nymphs, 95% (19/20) of the last-instar nymphs emerged as adults within one month of thawing. Their emergence was well synchronized, occurring within a span of one week.

Winter collections

On 12 December 2004 and 18 January 2006, a total of two and eight stonefly nymphs, respectively, were found embedded in ice from the Chandalar River. Both insects collected from the ice in December 2004 survived. Seven of eight (88%) collected in January 2006 survived. The low number of nymphs collected results from the difficulty of removing ice from the frozen streambed and our inability to evaluate how many nymphs were encased in the ice at the time of collection. During the course of the freezing of the stream, the water level dropped, producing two to three layers of ice separated by air spaces. There was also a layer frozen in contact with the substratum. None of the nymphs was collected from the frozen substratum, as we could not effectively remove this layer.

Cryoprotective compounds

The comparison of the ^{13}C spectra from warm- (4°C) and cold-acclimated (–8°C) nymphs reveals that glycerol is the only carbon-containing cryoprotective compound accumulated at high concentrations. Glycerol cannot be identified in the homogenate from the warm-acclimated nymphs but it is the predominant compound found in the homogenate of cold-acclimated nymphs (Fig. 3A). Glycerol exhibits chemical shifts at 64.1 p.p.m. and 73.7 p.p.m. The former is twice the intensity of the latter because it represents two magnetically identical terminal carbons (C1,C3) whereas the chemical shift at 73.7 p.p.m. is due solely to C2.

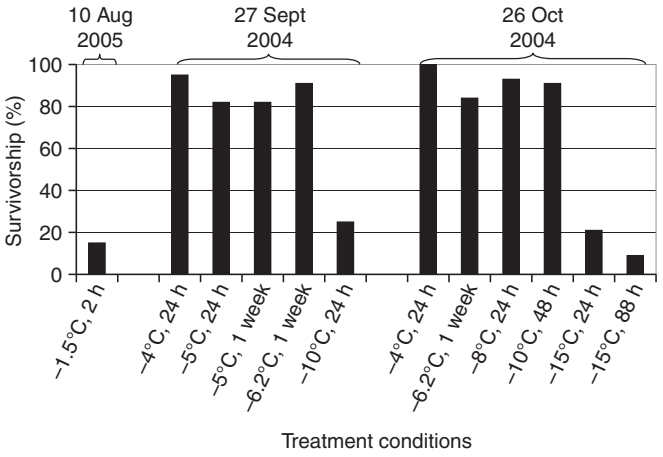

Fig. 2. Survivorship of *N. arctica* nymphs collected in late summer and early autumn. The collection dates are shown at the top of the figure. Nymphs were frozen in sealed plastic bags partially filled with water. The duration and temperature of the treatments (indicated on the *x*-axis) were varied according to survivorship of the nymphs collected on a specific date. Survivorship was assessed 24–48 h after complete thawing at 4°C.

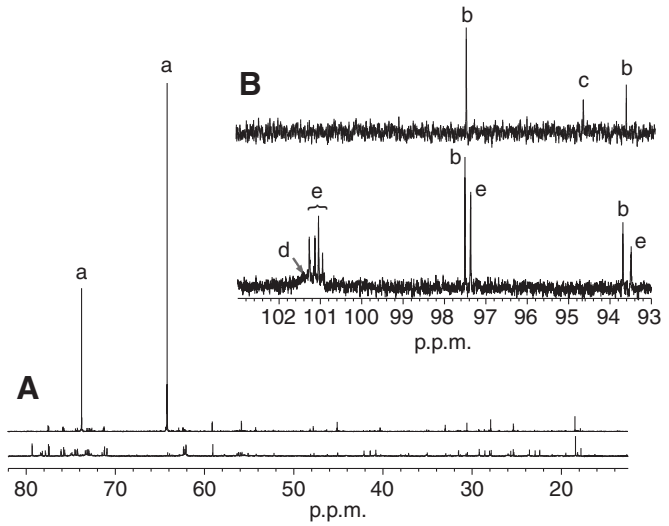

Fig. 3. Partial $^{13}C\{^1H\}$ NMR spectra (150 MHz) comparing the whole-body homogenates of warm-acclimated (4°C) and cold-acclimated (–8°C) insects. The lower spectrum in each alignment represents the homogenate from warm-acclimated nymphs (*N*=30) and the upper spectrum represents cold-acclimated nymphs (*N*=30). (A) Expansion of the region between 12.5 and 82 p.p.m. (B) Expansion of saccharide anomeric region. Reduced signal-to-noise ratio due to lower signal intensity. Assignment of the signals are as follows: a, glycerol; b, glucose (α and β anomers); c, α,α-trehalose; d, glycogen – broad hump in the baseline; e, tentative glucose oligomers produced from degradation of glycogen.

Many of the low-intensity peaks remain unidentified. However, the presence of proline was confirmed by spiking the homogenate of the cold-acclimated insects with a small volume of concentrated proline solution. The chemical shifts tentatively assigned to proline were enriched, confirming its presence. Proline appears to accumulate in the cold-acclimated nymphs; however, very low levels were also observed in the warm-acclimated homogenate. Trehalose was also identified in the homogenate from the cold-acclimated insects.

In 2007, hemolymph glycerol concentrations increased almost three orders of magnitude as acclimation temperature decreased (Table 1). Nymphs actively grew and emerged at 4°C, indicating that 3 mmol l^{-1} glycerol represented their basal hemolymph concentration. After acclimation at –8°C, the mean glycerol concentration in the hemolymph increased to 930±114 mmol l^{-1}. The mean glycerol concentrations for the 0°C, –3.7°C and –8°C treatments were all significantly different from one another ($P<0.05$, Tukey's multiple comparison test), indicating that nymphs continued to accumulate glycerol in spite of being frozen. The depressed melting points measured in 2008 (Table 1) indicate that glycerol concentrations increased as the acclimation temperature decreased.

Thermal hysteresis, crystal morphology and recrystallization inhibition

In 2005, hemolymph from nymphs collected in September when water temperatures were between 3°C and 4°C did not exhibit thermal hysteresis activity (THA) or hexagonal crystal growth, both indicators of IBFs (Griffith and Yaish, 2004). However, after just 4 days of acclimation to 0°C, the nymphs averaged 0.50±0.46°C of THA (Table 2), with some individuals exhibiting over 1°C. The large increase in TH in September 2005 was transient: after about 3 weeks of darkness at 0°C, most individuals showed little to no THA in the hemolymph (Table 2). The magnitude of the increase in THA in 2005 for the 0°C-acclimated nymphs was much larger than observed in the subsequent three years, when TH in the hemolymph, typically, did not increase as dramatically. The results from 2008 (Fig. 4) are typical of those of 2005–2006. Nymphs maintained at +4°C rarely exhibited measurable THA, although weak hexagonal crystal growth, indicating a low concentration of IBFs in the hemolymph, was observed ~40% of the time. Most nymphs did not exhibit increased TH after acclimation at 0°C, although a few individuals showed greatly increased TH in the hemolymph (Fig. 4). Relative to 4°C-acclimated nymphs, the proportion of nymphs exhibiting TH and hexagonal crystal morphology at subzero temperatures increased (Fig. 4); however, THA did not vary significantly across the various temperature treatments due to high variability (ANOVA, $P=0.08$, $N=63$).

In 2007, hemolymph from nymphs acclimated at –5.7°C inhibited recrystallization relative to the buffer control (Fig. 5). The crystals

Table 2. Thermal hysteresis (TH) activity and hexagonal crystal growth of hemolymph as a function of acclimation temperature and time

Acclimation status	Sample size (*N*)	Mean (± s.d. TH) (°C)*	Range of TH values (°C)*	% of nymphs with hexagonal crystal growth†
Field collected	5	0.00 ± 0.00^{A}	0.00–0.00	0
0°C (4 days)	7	0.50 ± 0.46^{B}	0.07–1.28	100
0°C (11–13 days)	9	0.12 ± 0.23^{A}	0.00–0.71	67
0°C (23–24 days)	9	0.00 ± 0.01^{A}	0.00–0.01	44

*The resolution of the osmometer is ~0.01°C, thus a difference between the melting and freezing points of 0.01 is reported as 0.00°C of TH.
†Nymphs were included if hexagonal crystal growth morphology or significant TH was observed.
All nymphs were collected on 18 Sept 2005. Water temperature ranged from 3.1°C to 4.2°C on 19 Sept 2005.
Distinct uppercase superscripts indicate statistically different TH values (Tukey's multiple comparison test; $P<0.05$).

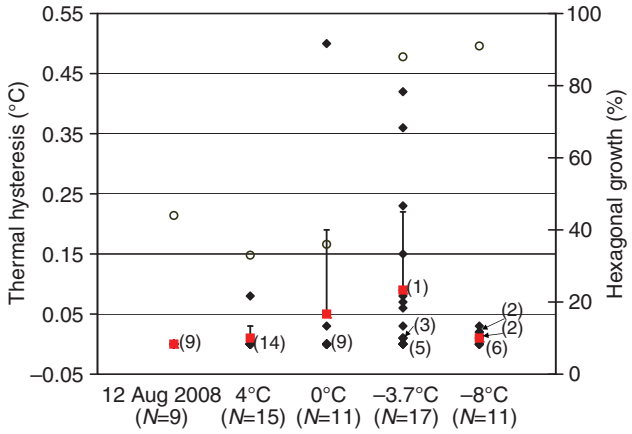

Fig. 4. Thermal hysteresis and percent hexagonal crystal growth *versus* acclimation or acclimatization. Insects were either field collected or acclimated to various temperatures, which are indicated along the *x*-axis. The insects were bled and their hemolymph was assayed for thermal hysteresis (TH) and hexagonal crystal growth morphology. Black diamonds represent individual TH measurements, and the numbers in parentheses indicate the number of overlapping data points. Red squares represent the mean TH, and error bars indicate 1 s.d. Open circles correspond to the secondary *y*-axis and indicate the percent of nymphs that exhibited hexagonal crystal growth morphology in their hemolymph.

in the *N. arctica* hemolymph were relatively static and remained small during the annealing period. By contrast, the average ice crystal size in the buffer control increased dramatically during the annealing period.

DISCUSSION

Stewart et al. reported distinct size cohorts in northern Alaska populations of *N. arctica* and concluded that completion of their life cycle requires at least two years (Stewart et al., 1990). The nymphs we collected in autumn exhibited a wide range of masses, confirming that our population of *N. arctica* is similar to those studied by Stewart et al. (Stewart et al., 1990). While semivoltinism may permit *N. arctica* to colonize streams with short growing seasons and low summer temperatures, it also precludes the possibility of reproduction without overwintering. The consistent abundance of *N. arctica* at our study site, where water in the stream completely freezes (Fig. 1), suggests that these insects are well adapted to survive subzero temperatures. Laboratory experiments conclusively demonstrate that *N. arctica* nymphs exhibit freeze tolerance beginning in autumn, as nymphs are able to survive freezing well below the temperature at which their bodies freeze.

The ecology of *N. arctica*, in addition to constraining its life cycle, also directly impacts its overwintering physiology. The mean whole-body freezing point of nymphs frozen in contact with ice is significantly higher than their supercooling point (SCP) under dry conditions, indicating that nymphs are inoculated with ice directly across the cuticle. Aquatic insects, in particular juvenile life stages, lack the thick cuticular wax coating of terrestrial insects (Beament, 1961), increasing their susceptibility to inoculative freezing. Overwintering in a stream that freezes into the substrata ensures contact with ice and results in inoculative freezing across the relatively permeable cuticle, effectively alleviating the need to produce ice nucleators. A limited ability to supercool is typical of freeze-tolerant organisms (Zachariassen and Hammel, 1976), and reliance on external ice to initiate freezing at high subzero temperatures is likely to be common among freeze-tolerant aquatic insects. This strategy has also been reported in some terrestrial species overwintering in moist microhabitats (Tursman et al., 1994). However, it should be noted that *N. arctica* may be capable of surviving freezing without inoculative freezing, as nymphs survived transient (≤2 min) freezing at the SCP when not in contact with ice.

Nymphs collected from the Chandalar River in January 2006 had experienced a long duration of subzero temperatures, yet demonstrated high survivorship despite being encased in pieces of ice that were forcefully fractured from the streambed. Unfortunately, due to a logger failure, we do not have a complete temperature profile for the 2005–2006 winter (Fig. 1A). However, two conclusions can be inferred from the temperature data. (1) The January-collected nymphs experienced temperatures at least as low as –9.2°C. Nymphs may have experienced even lower temperatures; the lowest

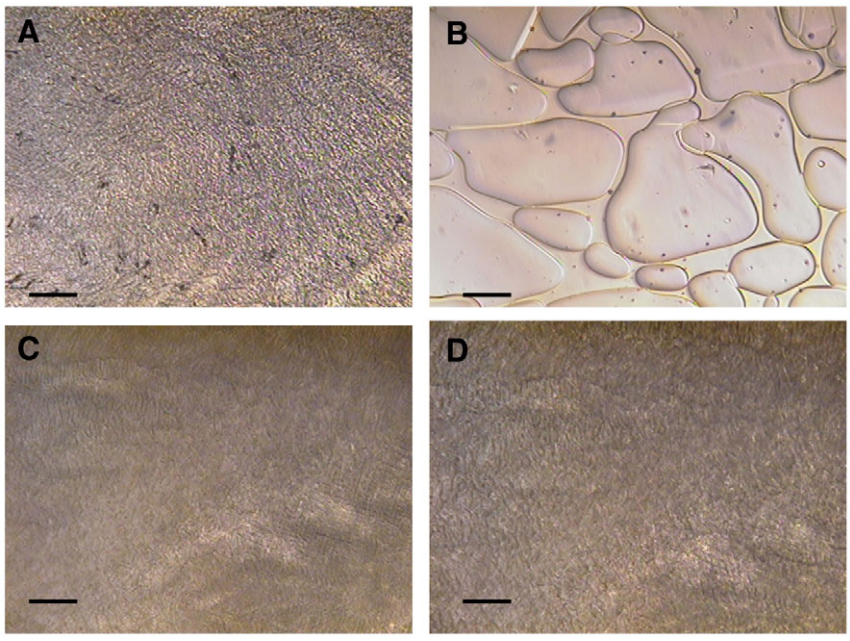

Fig. 5. Comparison of recrystallization of ice crystals in pooled hemolymph (*N*=10) from nymphs collected in September 2007 and acclimated to –5.7°C *versus* ice in a buffer control, consisting of 25 mmol l^{-1} Tris buffer, 1 mg ml^{-1} BSA and 0.5 mol l^{-1} glycerol. The melting point of the hemolymph and the control buffer were approximately equal. (A) Control buffer initial time point; (B) Control buffer after annealing; (C) *N. arctica* hemolymph at initial time point; (D) *N. arctica* hemolymph after annealing. Samples were frozen by cooling at 40°C min^{-1} to –30°C. The initial picture was taken after the sample froze and was warmed to –6°C. The sample was allowed to anneal at –6°C for 6 h before the second picture was taken. Scale bars, 100 μm.

streambed temperature of the 2006–2007 winter, –12.7°C, was not recorded until the first week in January. (2) The nymphs experienced subzero temperatures until they were collected, ~85 days below zero degrees. The streambed temperature began decreasing below zero on 24 October and remained consistently subzero at least until the data logger ceased recording in December. The nymphs collected in January were encased in ice and the streambed showed no signs of a winter thaw.

The ability of stonefly nymphs to penetrate deep into stream sediments is an important behavioral overwintering adaptation in northern stonefly populations (Stewart and Ricker, 1997), allowing nymphs to avoid freezing altogether in many habitats. Behavioral freeze avoidance may be advantageous, even for freeze-tolerant insects, because the mechanical stresses associated with being encased in ice (e.g. the pressure created by volume expansion of the surrounding water upon freezing) are thought to be a significant source of winter mortality for freeze-tolerant aquatic organisms in nature (Olsson, 1981). Upon thawing the –15°C-acclimated nymphs that were encased in ice, the guts of a few nymphs protruded through their abdominal walls, suggesting that they may have succumbed to the pressure exerted by ice. Even if nymphs ultimately cannot avoid freezing by moving deeper into stream sediments, they would still be likely to experience benefits including ameliorated temperatures and protection from flooding (Dole-Olivier et al., 1997) associated with the spring thaw. The migration of nymphs into the substrate may also explain the paucity of nymphs collected in midwinter, as we were not able to remove the frozen substrata.

The midwinter lower lethal temperature of *N. arctica* nymphs remains uncertain due to the difficulty of collecting nymphs once the stream freezes. However, it is certain that their LT_{50}, the temperature resulting in 50% mortality, is below –15°C. Laboratory freezing experiments of aquatic insects often do not achieve the same level of survivorship observed in the field (Olsson, 1981). Yet 87% of laboratory-acclimated nymphs survived freezing at –15°C in a large block of ice. The subsequent synchronized emergence of these last-instar nymphs indicates that freezing does not permanently disrupt their life cycle.

The cold-hardiness in *N. arctica* nymphs is associated with the accumulation of cryoprotective compounds. Certain low molecular mass solutes, such as proline and trehalose, that were identified in cold-acclimated *N. arctica* nymphs have previously been shown to interact directly with membrane phospholipids and stabilize the lipid bilayer during freezing (Rudolph and Crowe, 1985; Crowe et al., 1987). Polyols, such as glycerol, have been shown by a wide body of literature to protect from damage associated with freezing (Bale, 1987; Storey and Storey, 1988; Block, 1990; Lee and Denlinger, 1991; Storey and Storey, 1992). Accumulation of small molecular mass solutes increases the osmotic pressure of the nymph's physiological fluids, perhaps causing the small observed increase in body water content of the autumn-collected nymphs. The glycerol concentration reported here for *N. arctica*, 930±114 mmol l^{-1} (~9% of fresh mass) after acclimation to –8°C, is comparable to values reported for freeze-tolerant terrestrial insects. The glycerol concentration reported for some freeze-tolerant Alaskan species ranged from 3% to 21% of the fresh mass, with 14% being the average for the five reported species (Miller, 1982). However, glycerol continues to accumulate even while *N. arctica* is frozen (Table 1) and, thus, longer acclimation to lower temperatures may yield higher glycerol concentrations. In insects, the accumulation of glycerol occurs at the expense of glycogen stored in the fat body (Storey et al., 1990), explaining why glycogen was not observed in the homogenate of cold-acclimated (–8°C) nymphs (Fig. 3).

Even though IBFs (e.g. AFPs) have been found in diverse freeze-tolerant organisms including fungi, insects and plants, their functions in freeze tolerance are poorly understood (Duman, 2001). However, it is reasonable to assume that the physiological functions of IBFs in the context of freeze tolerance differ from those in freeze-avoiding species. For instance, we do not expect that IBFs promote supercooling as they do in freeze-avoiding species, since freeze-tolerant organisms typically do not exhibit increased supercooling capacity associated with cold acclimation (Zachariassen and Hammel, 1976) and the TH present in freeze-tolerant species is typically much lower than in freeze-avoiding species (Duman, 2001). Nonetheless, IBFs appear to promote cold-hardiness in the freeze-tolerant species, as they are seasonally regulated and have been shown to enhance the cellular cold tolerance of plants and arthropods (Sathyanesan, 1999; Tursman and Duman, 1995). Recrystallization inhibition (RI) is the only described function of IBFs in freeze-tolerant organisms (Urrutia et al., 1992; Tursman and Duman, 1995; Griffith and Yaish, 2004), with the exception of certain plant IBFs that exhibit dual functions, such as antipathogenic activity, in addition to their ability to interact with ice. The low level of THA typically encountered in the hemolymph of freeze-tolerant organisms is sufficient to prevent recrystallization, as was demonstrated here in *N. arctica* (Fig. 5). RI presumably prevents tissue damage associated with the growth of extracellular ice crystals. Tursman et al. reported that the hemolymph of the freeze-tolerant centipede, *Lithobius forficatus*, exhibited RI in winter but typically did not exhibit measurable TH (Tursman et al., 1994). However, individuals on rare occasions exhibited more than a degree of TH. These observations are consistent with patterns demonstrated for the hemolymph of cold-acclimated *N. arctica* nymphs. High variability in hemolymph TH associated with cold acclimation is likely due, in part, to the timing of the increase in TH since hemolymph levels appear to decrease over time (Table 2). One potential explanation for decreasing TH in the hemolymph is the ability of the IBF to associate with the cell membranes, which appeared to be the case for *L. forficatus* (Tursman et al., 1994).

Temperature is an important cue for cold adaptation in *N. arctica*. Autumn-collected nymphs were acclimated to ecologically relevant temperatures. A water temperature of 4°C is comparable to the daytime highs in mid-August, when nymphs are actively growing. In the laboratory, nymphs acclimated to 4°C in darkness actively grow and molt while showing no signs of cold adaptation. Nymphs emerge at this temperature when placed on a long (16 h:8 h light:dark) photoperiod. By contrast, a stable temperature of 0°C indicates that the stream has begun to freeze and will freeze completely within a few weeks (Fig. 1). Nymphs acclimated in darkness at 0°C exhibited increased glycerol (Table 1) and, less predictably, TH. Furthermore, *N. arctica* responds physiologically to subzero temperatures by producing glycerol. *N. arctica*, like some other freeze-tolerant insects, can continue to accumulate glycerol while frozen (Baust and Miller, 1970; Ring and Tesar, 1980), suggesting that frozen nymphs actively respond to their winter environment and are capable of reducing their lower lethal temperatures.

Nemoura arctica, the first described species of freeze-tolerant stonefly, is quite freeze tolerant for an aquatic insect. *Nemoura arctica* nymphs achieve freeze tolerance through a suite of physiological adaptations that includes, but is probably not limited to, the accumulation of small molecular mass cryoprotectants,

consisting of predominantly glycerol, and the production of IBFs. These physiological adaptations, which are at least partially cued by temperature, allow *N. arctica* nymphs to decrease their lethal temperature from their $T_{c(inoc)}$ in the summer to a temperature at least as low as –15°C in the winter. These results demonstrate that *N. arctica* nymphs are well adapted to survive the rigors of winter in a small arctic headwater stream that freezes into the streambed.

LIST OF ABBREVIATIONS

AFP	antifreeze protein
$^{13}C\{^{1}H\}$ NMR	proton decoupled carbon-13 nuclear magnetic resonance
IBF	ice binding factor
LT_{50}	temperature resulting in 50% mortality
RI	recrystallization inhibition
SCP	supercooling point
THA	thermal hysteresis activity

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REFERENCES

Bale, J. S. (1987). Insect cold hardiness: freezing and supercooling: an ecological perspective. *J. Insect Physiol.* **33**, 899-908.

Baust, J. G. and Miller, L. K. (1970). Variations in glycerol content and its influence on cold hardiness in the Alaskan Carabid beetle *Pterostichus brevicornis*. *J. Insect Physiol.* **16**, 979-990.

Beament, J. W. L. (1961). The waterproofing mechanism of arthropods: the permeability of the cuticle of some aquatic insects. *J. Exp. Biol.* **38**, 277-290.

Block, W. (1990). Cold tolerance of insects and other arthropods. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **326**, 613-633.

Chakrabartty, A. and Hew, C. L. (1991). The effect of enhanced α-helicity on the activity of a winter flounder antifreeze polypeptide. *Eur. J. Biochem.* **202**, 1057-1063.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Winstrom, C. A. (1987). Stabilization of dry phospholipid bilayers and proteins by sugar. *Biochem. J.* **242**, 1-10.

Danks, H. V. (1971). Overwintering of some north temperate and Arctic Chironomidae. II. The winter environment. *Can. Entomol.* **103**, 589-604.

Danks, H. V. (2007). How aquatic insects live in cold climates. *Can. Entomol.* **139**, 443-471.

DeVries, A. L. (1986). Antifreeze glycopeptides and peptides: interactions with ice and water. *Methods Enzymol.* **127**, 293-303.

Dole-Olivier, M. J., Marmonier, P. and Beffy, J. L. (1997). Response of invertebrates to lotic disturbance: is the hyporheic zone a patchy refugium? *Freshw. Biol.* **37**, 257-276.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu. Rev. Physiol.* **63**, 327-357.

Fahy, G. M. (1995). The role of nucleation in cryopresevation. In *Biological Ice Nucleation and its Applications* (ed. R. E. Lee, G. J. Warren and L. V. Gusta), pp. 315-316. St Paul, MN: American Phytopathological Society.

Griffith, M. and Yaish, M. W. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends Plant Sci.* **9**, 399-405.

Hilsenhoff, W. L. (1991). Diversity and classification of insects and Collembola. In *Ecology and Classification of North American Freshwater Invertebrates* (ed. J. H. Thorp and A. P. Covich), pp. 593-664. San Diego: Academic Press.

Holmstrup, M. and Sömme, L. (1998). Dehydration and cold hardiness in the Arctic collembolan *Onychiurus arcticus*. *J. Comp. Physiol. B* **168**, 197-203.

Huryn, A. D., Slavic, K. A., Lowe, R. L., Parker, S. M., Anderson, D. S. and Peterson, B. J. (2005). Landscape heterogeneity and the biodiversity of arctic stream communities: a habitat template analysis. *Can. J. Fish. Aquat. Sci.* **62**, 1905-1919.

Irons, J. G., 3rd, Miller, L. K. and Oswood, M. K. (1993). Ecological adaptations of macroinvertebrates to overwintering in interior Alaska (USA) subarctic streams. *Can. J. Zool.* **71**, 98-108.

Knight, C. A. and Duman, J. G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. *Cryobiology* **23**, 256-262.

Kreutz, F. H. (1962). Enzymatische Glycerinbestimmung. *Klin. Wochenschr.* **40**, 362-363.

Leader, J. P. (1962). Tolerance to freezing of hydrated and partially hydrated larvae of *Polypedilum* (Chironomidae). *J. Insect Physiol.* **8**, 155-163.

Lee, R. E. and Denlinger, D. L. (1991). *Insects at Low Temperature*. London: Chapman and Hall.

Lundheim, R. and Zachariassen, K. E. (1993). Water balance of overwintering beetles in relation to strategies for cold tolerance. *J. Comp. Physiol. B* **163**, 1-4.

Miller, L. K. (1982). Cold-hardiness strategies of some adult and immature insects overwintering in interior Alaska. *Comp. Biochem. Physiol.* **73A**, 595-604.

Neven, L. G., Duman, J. G., Beals, J. M. and Castellino, F. J. (1986). Overwintering adaptations of the stag beetle, *Ceruchus piceus*: removal of ice nucleators in winter to promote supercooling. *J. Comp. Physiol.* **156**, 707-716.

Olsson, T. I. (1981). Overwintering of benthic macroinvertebrates in ice and frozen sediment in a north Swedish river. *Holarct. Ecol.* **4**, 161-166.

Oswood, M. W. (1989). Community structure of benthic invertebrates in interior Alaska (USA) streams and rivers. *Hydrobiologia* **172**, 97-110.

Oswood, M. W., Miller, L. K. and Irons, J. G., III (1991). Overwintering of freshwater benthic macroinvertebrates. In *Insects at Low Temperature* (ed. R. E. Lee, Jr and D. L. Denlinger), pp. 360-375. New York: Chapman and Hall.

Ring, R. A. and Tesar, D. (1980). Cold-hardiness of the arctic beetle *Pytho armericanus* Kirby Coleoptera, Pythidae (Salpingidae). *J. Insect Physiol.* **26**, 763-774.

Rojas, R. R., Lee, R. E. and Baust, J. G. (1986). Relationship of environmental water content to glycerol accumulation in the freezing tolerant larvae of *Eurosta solidaginis* (Fitch). *Cryo Lett.* **7**, 234-245.

Rudolph, A. S. and Crowe, J. H. (1985). Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22**, 367-377.

Sathyanesan, S. (1999). Purification and identification of thermal hysteresis and other cryoprotective proteins in the bittersweet nightshade (*Solanum dulcamara*). PhD thesis, University of Notre Dame, Notre Dame, IN, USA, p. 157.

Scholander, P. F., Flagg, W., Hock, R. J. and Irving, L. (1953). Studies on the physiology of frozen plants and animals in the arctic. *J. Cell Physiol.* **42**, 1-56.

Stewart, K. W. and Ricker, W. E. (1997). Stoneflies (Plecoptera) of the Yukon. In *Insects of the Yukon* (ed. H.V. Danks and J. A. Downes), pp. 201-222. Ottawa, Ontario: Biological Survey of Canada.

Stewart, K. W. and Oswood, M. W. (2006). *The Stoneflies (Plecoptera) of Alaska and Western Canada*. Columbus, OH: The Caddis Press.

Stewart, K. W., Hassage, R. L., Holder, S. J. and Oswood, M. W. (1990). Life cycles of six stonefly species (Plecoptera) in subarctic and arctic Alaskan streams. *Ann. Entomol. Soc. Am.* **83**, 207-214.

Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. *Physiol. Rev.* **68**, 27-84.

Storey, K. B. and Storey, J. M. (1992). Biochemical adaptations for winter survival in insects. In *Advances in Low Temperature Biology* (ed. P. L. Steponkus), pp. 101-140. London: JAI Press.

Storey, K. B., Wilkie, D. R., Cairns, A., Thomas, H., Cowan, D. A., Baust, J., Pullin, A. S., Russell, N. J. and Jaenicke, R. (1990). Biochemical adaptation for cold hardiness in insects. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **326**, 635-654.

Tursman, D. and Duman, J. G. (1995). Cryprotective effects of thermal hysteresis protein on survivorship of frozen gut cells from the freeze-tolerant centipede *Lithobius forficatus*. *J. Exp. Zool.* **272**, 249-257.

Tursman, D., Duman, J. G. and Knight, C. A. (1994). Freeze tolerance adaptations in the Centipede, *Lithobius forficatus*. *J. Exp. Zool.* **268**, 347-353.

Urrutia, M. E., Duman, J. G. and Knight, C. A. (1992). Plant thermal hysteresis proteins. *Biochim. Biophys. Acta.* **1121**, 199-206.

Zachariassen, K. E. (1992). Ice nucleating agents in cold-hardy insects. In *Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Level* (ed. G. N. Somero, C. B. Osmond and L. Bolis), pp. 261-281. Berlin: Springer-Verlag.

Zachariassen, K. E. and Hammel, H. T. (1976). Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285-287.