Inoculative Freezing and the Problem of Winter Survival for Freshwater Macroinvertebrates
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Source: *Journal of the North American Benthological Society*, Vol. 16, No. 3 (Sep., 1997), pp. 635-650
Published by: University of Chicago Press on behalf of the Society for Freshwater Science
Stable URL: http://www.jstor.org/stable/1468150
Accessed: 08-03-2016 13:49 UTC

REFERENCES

J. N. Am. Benthol. Soc., 1997, 16(3):635-650

Inoculative freezing and the problem of winter survival for freshwater macroinvertebrates

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Abstract. Due to the thermal buffering of their environment, aquatic invertebrates are less likely than their terrestrial counterparts to face temperatures substantially below 0°C. Aquatic invertebrates may not be able to avoid internal freezing by supercooling (remaining unfrozen at temperatures below the freezing point of their body fluids), however, because when their body temperatures reach the freezing point of body fluids, these organisms will likely be in contact with external ice, which may induce formation of internal ice (i.e., inoculative freezing). In this study, a variety of winter-collected, aquatic invertebrates (a clam, *Sphaerium* sp.; an isopod, *Lirceus fontinalis*; a mayfly, *Stenonema femoratum*; a belostomatid, *Belostoma flumineum*; 2 dytiscids, *Ilybius oblitus* and *Agabus disintegratus*) and, for comparison, a terrestrial beetle (*Hippodamia convergens*) were studied with respect to their low temperature tolerance. No species appeared to lower its freezing point appreciably by accumulating colligatively active solutes in body fluids, and all aquatic species supercooled moderately (−5 to −7°C), but significantly less than the terrestrial beetle (−16°C), before freezing when chilled in a dry environment. However, when chilled in contact with external ice, all animals froze at their melting points or just below (as low as ~−2°C for the beetles), showing that they are susceptible to inoculative freezing. All aquatic species readily survived exposures to sub-zero temperatures when supercooled, but succumbed to the same conditions when inoculated by external ice. Survival of low temperatures by temperate zone aquatic invertebrates appears to depend upon thermal buffering provided by the aquatic environment, slow progress of ice formation in bodies of water at high sub-zero temperatures, avoidance of contact with external ice, and tolerance of high sub-zero temperatures when freezing does not occur in the animals themselves.

Key words: crystallization temperature, supercooling, ice encasement, ice nucleation, freezing point, melting point, cold tolerance, winter ecology.

Cold-hardy ectotherms use strategies of freeze avoidance or freeze tolerance to survive sub-zero temperatures (Storey and Storey 1988, Duman et al. 1991, Lee 1991, Costanzo and Lee 1995, Costanzo et al. 1995). Freeze-avoiding animals typically minimize exposure to low temperatures behaviorally and may depress their freezing points through production of colligatively active antifreeze chemicals, such as sugars and polyols, that accumulate in blood and other tissues (Sømme 1964, Storey and Storey 1988). In addition, freeze-intolerant animals may facilitate supercooling (remaining unfrozen at temperatures below the freezing point of their body fluids) by removing ice-nucleating agents from the gut and body fluids in preparation for exposure to low temperatures (Neven et al. 1986, Lee et al. 1993, 1995). Some animals produce proteins and glycoproteins that act in a non-colligative fashion to promote supercooling of the body fluids (i.e., they depress the freezing point of body fluids without affecting their melting point) (Duman and Horwath 1983, Wu et al. 1991).

Freeze-tolerant animals typically use ice-nucleating agents to promote freezing at relatively high sub-zero temperatures (Zachariassen and Hammel 1976, Duman et al. 1985, Layne et al. 1990, Lee et al. 1991, Mugnano et al. 1996). These animals avoid damage, in part, by promoting extracellular freezing, thereby enhancing the supercooling capacity of cell contents and avoiding intracellular freezing. Other biochemical adjustments, generally made concomitantly, enable cells to avoid damage from the resulting dehydration and intracellular concentration of solutes (Duman et al. 1985, Storey and Storey 1988, Costanzo et al. 1995).

Terrestrial insects employ diverse strategies to survive low winter temperatures (Lee 1989, 1991, Duman et al. 1991). Due to their small size, and hence small water volume, some supercool extensively and remain unfrozen to −25°C or lower. Others tolerate internal freezing that begins at high sub-zero temperatures, while some are capable of both supercooling and tolerating freezing (see tables in Sømme 1982 and Lee 1991).

The winter environment of aquatic invertebrates differs in important ways from that of their terrestrial counterparts and thus influences the types of low-temperature strategies the aquatic organisms employ (Moore and Lee 1991). First, because of its high specific heat, water in ponds or streams acts as an effective thermal buffer. When freezing does begin, the heat of crystallization that accompanies the phase change is released, slowing the cooling of underlying water. The layer of ice on the surface of the body of water insulates and further retards cooling of subsurface water. Thus, aquatic invertebrates face sub-zero temperatures less often than do terrestrial invertebrates. Nevertheless, organisms within benthic substrates may be exposed to sub-zero temperatures when temporary ponds dry, or during extremely cold weather, when ice may extend into the substrate. In lotic systems, currents help to retard ice formation, but in extremely cold weather subsurface anchor and frazil ice may form (Oswood et al. 1991). When aquatic organisms do face sub-zero temperatures, they are likely to do so while in contact with, or even encased in, ice. Thus, a 2nd difference afforded by an aquatic environment is that aquatic organisms may be subjected to mechanical stress that their terrestrial counterparts normally avoid.

A 3rd way in which a winter aquatic environment differs from a terrestrial one is that supercooling is less likely to be an effective strategy, if it is possible at all. A supercooled organism is in a metastable state. Once an ice crystal is initiated, it propagates throughout the body fluids. Terrestrial insects that supercool appreciably generally do so in a dry habitat where contact with an external crystal of ice, which could initiate internal ice formation (inoculative freezing), is unlikely. Aquatic habitats at subzero temperatures are replete with ice crystals. Thus, aquatic organisms will freeze unless they possess special adaptations that allow them to resist inoculation or internal ice crystal growth. Antarctic fishes, which inhabit water at temperatures below their melting points (i.e., the highest temperature at which ice crystals can persist in the body fluids), produce special antifreeze proteins that inhibit ice crystal growth in their body fluids (DeVries 1983, Costanzo et al. 1995). Some terrestrial insects produce particularly active antifreeze, or thermal hysteresis, proteins that may depress the freezing point (i.e., temperature at which ice crystals can grow) by 5°C or more below the melting point of body fluids (Duman and Horwath 1983, Duman et al. 1991).

Observations on aquatic macroinvertebrates have included studies in which ice and frozen substrates from winter habitats have been brought into the laboratory, thawed, and the freed animals scored for survival. A variety of taxa survive such ice encasement (Table 1). The limitation of these studies is that while they demonstrate survival in real field conditions, it cannot be determined if the animals themselves were actually frozen, or merely ice encased. (Although extensive ice can form at about 0°C in freshwater, whose freezing point is near 0°C, no ice can form in invertebrates until the environmental temperature reaches their freezing point, which is often −0.4°C or below.) Conditions can be monitored more closely in the laboratory, but such studies also present significant difficulties. For example, freezing organisms in water-filled containers will probably produce unnatural mechanical stress on the organisms and freezing organisms out of water may subject them to crucial dehydration stress (Oswood et al. 1991). An additional logistical problem is that cooling animals in the presence of substantial external water makes it difficult to detect when (and if) actual freezing of the organism begins because freezing of external water will release heat that masks the exotherm produced by the specimen. (When liquid water changes to ice, the heat of crystallization is released. For a supercooled organism that begins to freeze, the sharp rise in recorded temperature is termed an exotherm (Fig. 1).) Laboratory tests of freezing survival in aquatic organisms typically have produced high mortalities (Table 2).

Another approach has been to investigate the physical properties of the animals themselves divorced from their natural habitat. Moore and Lee (1991) measured crystallization temperatures, sometimes termed supercooling points, of

a variety of aquatic insects frozen out of water and compared these with crystallization temperatures of terrestrial insects. They found that aquatic insects froze at much higher temperatures, and that there was less variability in their freezing points. These results are consistent with 2 scenarios. Aquatic organisms will most likely encounter external ice crystals in their environment and be vulnerable to inoculative freezing, in which case supercooling is not likely to work as a strategy. Alternatively, since water acts as an effective thermal buffer, aquatic animals are not likely to face as great a range of temperatures as are terrestrial organisms.

Improvements in the understanding of winter survival of aquatic invertebrates can be made if tests can be designed that correct for some of the limitations described above. Specifically, individually instrumented test subjects would allow precise assessment of animal temperature and state (frozen or supercooled). Second, while measures of crude physiologic capacities (e.g., Can these organisms supercool?) provide some insights, it is also important to test ecologically relevant capacities (e.g., Are these organisms likely to remain unfrozen in the presence of ice in nature?). Third, survival tests should separate the effects of sub-zero temperature from those of internal ice formation. Finally, because the majority of tests to date have been performed on arctic or subarctic species, it will be instructive to examine temperate species, which experience less extreme, but, nonetheless potentially problematic, sub-zero conditions.

We further investigated the physical properties and survival capacities of temperate aquatic macroinvertebrates at low temperatures. First, we determined crystallization temperatures for a variety of taxa in dry test conditions. We also tested these organisms for their capacity to resist inoculative freezing by using a technique that encased the organism in ice crystals without producing mechanical damage, and also allowed detection of organismal freezing. Finally, we tested the survival ability of these macroinvertebrates while supercooled; while unfrozen, but in contact with ice; and while frozen and ice-encased.

Methods

Animals

Test subjects were collected from streams (isopods, *Lirceus fontinalis*; mayflies, *Stenonema femoratum*; clams, *Sphaerium* sp.) or from a temporary pond (adult belostomatids, *Belostoma flumineum*; adult dytiscids, *Ilybius oblitus* and *Agabus disintegratus*) in Oxford, Ohio, during the winter and spring of 1994–1995. These species were chosen to provide a broad taxonomic spectrum for tests. Isopods, mayflies, clams, and belostomatids were encountered frequently in winter aquatic habitats. Two species of early spring-collected dytiscids were included because these individuals over-wintered as adults and they contributed to the taxonomic breadth of the study. All aquatic invertebrates were kept at 4°C in pond or stream water until use (1–5 d). Field-collected terrestrial beetles (lady beetles) (coccinellids, *Hippodamia convergens*) were purchased commercially (The Ladybug Company, Berry Creek, California) and provided a comparison group. Lady beetles were held in darkness at 4°C (simulating winter conditions) for several months prior to use. Live masses of animals were determined to the nearest 0.1 mg after gently blotting their surface. Dry masses were similarly determined after animals had dried at 60°C for 24 h. Osmolality of body fluids was determined on a Wescor 5500 vapor pressure osmometer. These samples were obtained by collecting fluid from a puncture wound either directly into a microcapillary tube, or from a microcentrifuge tube after first centrifuging the animal for 5 min at 1000–2300 RPM. For clams, the viscera were removed from the shell and centrifuged to separate body fluids. When possible, determinations were made on samples withdrawn from individual animals, but samples from several individuals were pooled when necessary (clams, mayflies).

Crystallization temperatures

Crystallization temperatures (T_cs) were determined for animals either in a dry environment or in contact with ice. In both cases, an individual was placed in contact with a 36-gauge copper–constantin thermocouple connected to an Omega 12- or 20-channel data logger. For T_c determinations in a dry environment, the animal was lightly blotted dry before wrapping it and the thermocouple in a strip of dry paper towel. For T_c determinations in contact with ice, the animal and thermocouple were wrapped in a water-saturated strip of paper towel. The wrapped animal (dry or wet) was fitted snugly into a

TABLE 1. Survival of aquatic invertebrates thawed from field collected ice samples.

Taxon	Sample	Locale	Survival (%)	Reference
Nematoda	Frozen lake water and sediment	Northwest Territories, Canada	45	Andrews and Rigler 1985
Tardigrada	Frozen lake water and sediment	Northwest Territories, Canada	61	Andrews and Rigler 1985
Annelida				
Oligochaeta				
"Oligochaetes"	Frozen lake water and sediment	Northwest Territories, Canada	63	Andrews and Rigler 1985
Tubificidae	Frozen river water and sediment	Sweden	100	Olsson 1981
Arthropoda				
Copepoda				
"Copepods"	Frozen lake water and sediment	Northwest Territories, Canada	13	Andrews and Rigler 1985
Harpaticoida	Frozen lake water and sediment	Northwest Territories, Canada	35	Andrews and Rigler 1985
Ostracoda	Frozen lake water and sediment	Northwest Territories, Canada	78	Andrews and Rigler 1985
Isopoda				
Asellus aquaticus	Frozen river water and sediment	Sweden	2	Olsson 1981
Insecta				
Plecoptera	Anchor ice, river bottom	Montana, United States	100	Brown et al. 1953
	Sheet ice, solid to river bottom	Montana, United States	0	Brown et al. 1953
Ephemeroptera	Anchor ice, river bottom	Montana, United States	100	Brown et al. 1953
	Sheet ice, solid to river bottom	Montana, United States	0	Brown et al. 1953
Odonata				
Coenagrion angulatum	Pond ice	Alberta, Canada	98–27[a]	Daborn 1971
Coenagrion resolutum	Pond ice	Alberta, Canada	98–27[a]	Daborn 1971
Enallagma boreale	Pond ice	Alberta, Canada	98–27[a]	Daborn 1971
Trichoptera	Anchor ice, river bottom	Montana, United States	100	Brown et al. 1953
Diptera				
"midges"	Anchor ice, river bottom	Montana, United States	100	Brown et al. 1953
"midges"	Sheet ice, solid to river bottom	Montana, United States	0	Brown et al. 1953
Chironomidae	Frozen lake water and sediment	Northwest Territories, Canada	84	Andrews and Rigler 1985
	Frozen pond mud	Alaska, United States	~100	Scholander et al. 1953
Chironominae	Frozen river water and sediment	Sweden	97	Olsson 1981
Tanypodinae	Frozen river water and sediment	Sweden	70	Olsson 1981
Orthocladiinae	Frozen river water and sediment	Sweden	86	Olsson 1981

TABLE 1. Continued.

Taxon	Sample	Locale	Survival (%)	Reference
Mollusca				
Gastropoda				
Gyraulus acronicus	Frozen river water and sediment	Sweden	77	Olsson 1984
Gyraulus acronicus	Frozen river water and sediment	Sweden	82	Olsson 1981
Bivalvia				
Pisidium spp.	Frozen river water and sediment	Sweden	82	Olsson 1981

[a] Survival declined from 98% on 1 December to 27% on 30 January 1967

plastic microcentrifuge tube. This assembly was then lowered into a test tube immersed in an alcohol-filled low-temperature bath (Forma Scientific). Test subjects were allowed to reach thermal equilibrium at −0.5 to −0.7°C before further processing.

Ice crystal formation was initiated in wet paper towels either by dropping a small crystal of ice onto the top of the paper towel, or by sending a small puff of aerosol coolant (Histofreeze) down the mouth of the test tube. Inoculative freezing of water in the paper towel was verified by observing a rise in and plateauing of the temperature trace at −0.1 to −0.2°C (Fig. 1). The system was allowed to reach equilibrium again at −0.5 to −0.7°C before the bath temperature was decreased, cooling animals at −0.15 to −0.19°C/min. Internal freezing of animals was detected by the appearance of an exotherm in the recorded temperature (Fig. 1). T_c was defined as the lowest temperature reached prior to the exotherm (Lee 1991, Fig. 1). Frequently, for animals in contact with ice (frozen, wet paper towels), body temperature (T_b) did not decrease sharply when the bath temperature was lowered. Rather, T_b plateaued, indicating continued formation of ice with no prior supercooling (Fig. 1). For these animals, the early plateau temperature was recorded as T_c.

Survival tests

The low-temperature tolerance of aquatic invertebrates was tested under a variety of conditions. Some animals were cooled to temperatures approximately equal to the melting point of their body fluids and tested for survival after an exposure period in a dry, wet, or frozen paper towel wrap. Other animals were cooled well below the melting point of their body fluids and tested for survival in a supercooled or frozen state. In all cases, animals were wrapped in contact with a thermocouple in either a dry or water-saturated paper towel strip and placed in a low temperature bath, as described above. In the contact-with-ice and frozen treatments, freezing of water in the moist paper towel was initiated with a crystal of ice or a puff of Histofreeze. In other treatments, the absence of an exotherm verified that freezing did not occur (Fig. 2). Duration of the exposures ranged from 2 to 24 h. After the exposure, animals were warmed to 4°C in the low-temperature bath, carefully re-

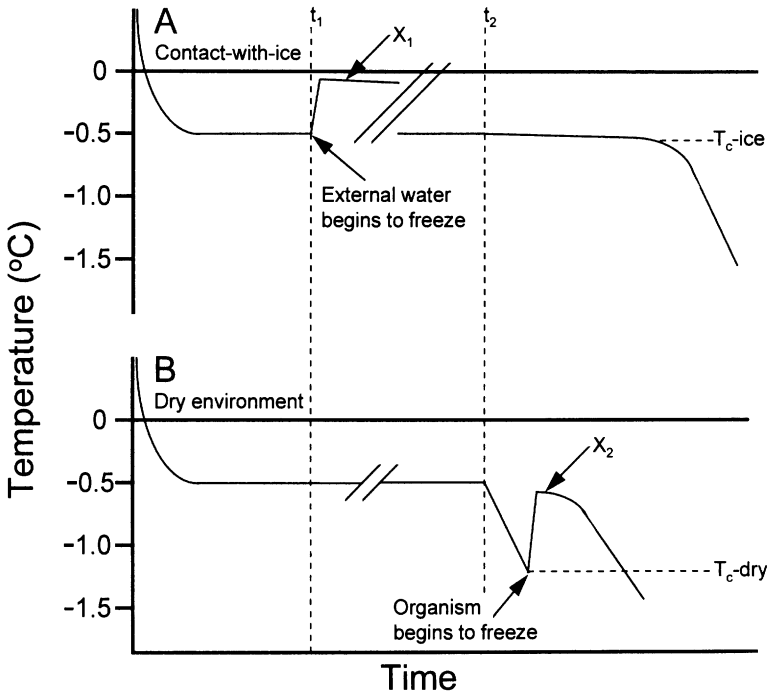

FIG. 1. Schematic diagrams showing the procedure for determining crystallization temperatures in aquatic invertebrates in contact with external ice crystals (A) and in a dry environment (B). In both cases, preparations were allowed to reach thermal equilibrium at −0.5°C. Those surrounded by wet paper toweling were inoculated at time t_1 (A). The temperature of the preparation rose (termed an exotherm) as freezing water in the toweling released the heat of crystallization (X_1). When thermal equilibrium was reestablished at −0.5°C, the bath temperature was lowered (t_2). Animals in a dry environment cooled below the melting point of their body fluids (supercooled) and then produced an exotherm (X_2) as freezing spontaneously initiated. Animals in contact with ice crystals exhibited a prolonged temperature plateau at the approximate melting point of their body fluids, indicating that freezing occurred as soon as the temperature reached that point.

moved from their wrapping, and placed on moist paper towels at 4°C. After 24 h they were checked for survival by observing whether they moved normally when placed in water. Clams could be reliably assayed because dead clams did not close their shells upon reintroduction to water.

Statistics

Statistical analyses were performed using Minitab (release 10Xtra. Minitab, Inc. State College, Pennsylvania). Body parameters (body water concentration = [BW] = μL/mg dry mass; crystallization temperature, dry = T_c-dry; crystallization temperature, ice = T_c-ice) of mayflies collected in May were compared with those of mayflies collected in February using separate Mann-Whitney tests. Body parameters ([BW], body fluid osmolality = HEM, T_c-dry, T_c-ice) were compared among species using separate 1-way ANOVAs followed by Tukey's multiple comparisons. The experimentwise error rate for the multiple comparisons was 0.05. Results in tables and elsewhere are presented as mean ±1 SE.

Results

The 6 species of aquatic animals and 1 species of terrestrial beetle that were analyzed ranged in average size from 16 to 225 mg. Body parameters and crystallization temperatures of these animals are summarized in Table 3. Mayflies (*Stenonema femoratum*) collected in February and May 1995 did not differ in body water concentration or crystallization temperature when dry ($p > 0.1$ in both cases). When cooled in contact

with ice, T_c-ice was slightly lower in February-collected animals (−0.8 vs. −0.5°C, $p = 0.02$). Although statistically significant, the small difference in T_c-ice between samples seems unlikely to have biological meaning. Thus, the 2 mayfly samples were pooled for the remaining analyses.

In general, aquatic invertebrates had body water concentrations of about 3 μL/mg dry mass (Table 3), but there was significant variation among groups ($F_{6,93} = 14.6$, $p < 0.001$). Two species of beetles (*Agabus disintegratus* and the terrestrial *H. convergens*) had the lowest body water concentration, while a 3rd species of beetle (*Ilybius oblitus*) had the highest (Table 3).

Body fluids also differed in their osmolality among the 4 species tested ($F_{3,18} = 76.9$, $p < 0.001$, Table 3). The melting point of a solution is depressed by solutes at the rate of −1.86°C/1000 mOsm. Based on the colligative properties of body fluids alone, the animals tested would have melting points ranging from −0.1°C for the clam to −0.7°C for the belostomatid. Dry animals typically froze at much lower temperatures than these, however, revealing that they had supercooled before freezing (Table 3). Species differed significantly in their mean T_c-dry ($F_{6,38} = 94.0$, $p < 0.001$). The terrestrial beetle froze at a significantly lower temperature than did the aquatic animals.

Crystallization temperatures also differed among groups when animals were cooled in contact with ice ($F_{6,36} = 19.5$, $p < 0.001$, Table 3). The 2 aquatic beetles, terrestrial beetle, and bug appeared to resist freezing somewhat (T_c-ice = −1.2 to −2.2°C) even though they were in contact with ice crystals, while the other groups apparently began freezing when the wet paper towel around them was inoculated.

All groups survived exposure to a high sub-zero temperature that was near, but above, their own melting points (i.e., −0.5°C, Table 4). Survival was high at this temperature regardless of whether exposure was of short (2 h) or long (24 h) duration, although mortality was extensive for some groups when in dry conditions for 24 h (i.e., mayflies, isopods). All groups tolerated contact with ice at these high sub-zero temperatures. Survival at lower temperatures varied considerably among species. Belostomatids and clams survived 24 h exposures to −4.0°C and −3.5°C, respectively, when supercooled, but succumbed to the same exposure when frozen. Similarly, isopods and mayflies survived shorter exposures (~2 h) to −3.5°C or −3.0°C when supercooled, but not when frozen. The dytiscid *I. oblitus* survived a 24-h exposure to −1.0°C dry, and to a lesser extent ice encased, but did not survive (11 out of 12 died) a 24-h exposure to −3.0°C, in either a frozen or supercooled state.

Discussion

The aquatic invertebrates we tested appeared not to be making major biochemical adjustments in their body fluids that would lower their freezing points. Osmolality of body fluids ranged from 61 mOsm for clams to 355 mOsm for belostomatids. Dietz and coworkers reported blood-solute concentrations of 45 to 55 mOsm for 3 species of freshwater bivalves (Graves and Dietz 1980, McCorkle and Dietz 1980, Scheide and Dietz 1982). Sutcliffe (1962) reported hemolymph osmolalities ranging from 212 to 422 mOsm for a variety of aquatic insects. Frisbie and Dunson (1988) reported hemolymph osmolalities of 350–450 mOsm for seasonally collected dytiscid adults. Thus, the values reported in the present study are similar to those reported for other aquatic invertebrates. Based on the colligative properties of their body fluids, the animals tested here should have had melting points ranging from −0.1°C to −0.7°C. However, all animals tested froze at temperatures several degrees lower than this (−4.7 to −7.9°C), when cooled in a dry testing environment. Our results are consistent with those of Moore and Lee (1991), who found crystallization temperatures of −3.3 to −7.4°C for aquatic insects in seven orders, and Oswood et al. (1991) who reported T_cs of −3 to −7°C for winter-collected plecopterans, trichopterans, and ephemeropterans. These authors also reported, however, that empidids had much lower T_cs (−22.6°C). Chironomids are reported to have moderate supercooling abilities (−5.1 to −11.1°C, Danks 1971b, Oswood et al. 1991).

From an ecological perspective, it is, perhaps, unsurprising that aquatic invertebrates generally do not supercool appreciably before freezing since they are much less likely to encounter temperatures substantially below 0°C in nature than are their terrestrial counterparts. In our study, the single terrestrial animal that we tested supercooled to a far greater extent (*H. convergens*, −16°C) than did any of the aquatic animals, in-

TABLE 2. Survival of some aquatic and marine invertebrates exposed to sub-zero temperatures.

Taxon	Temperature (°C)	Duration	Conditions	Survival (%)	Reference
Arthropoda					
Insecta					
Plecoptera					
Nemouridae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
Perlodidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
"stoneflies"	−18.0	12 h+	water	0[a]	Brown et al. 1953
Ephemeroptera					
Baetidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
Heptageniidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
"mayflies"	−18.0	12 h+	water	0[a]	Brown et al. 1953
Odonata					
Enallagma boreale	−1.0	20 h	water	48	Duffy and Liston 1985
	−4.0	20 h	water	5	Duffy and Liston 1985
	−10.0	20 h	water	0	Duffy and Liston 1985
Coenagrion angulatum	−5 to −6	overnight	water	50[b]	Sawchyn and Gillott 1975
Coenagrion resolutum	−5 to −6	overnight	water	50[b]	Sawchyn and Gillott 1975
Trichoptera					
Limnephilidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
Rhyacophilidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
"caddisflies"	−18.0	12 h+	water	0[a]	Brown et al. 1953
Diptera					
Tipulidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
Simuliidae	−9.0, −2.0, −0.5	3 d	water and gravel	0	Irons et al. 1993
"midges"	−18.0	12 h+	water	0[a]	Brown et al. 1953
Chironomidae	−16, −20, −32	?	wet filter paper	~100[c]	Scholander et al. 1953
Einfeldia synchrona	−4	1 d	wet filter paper	23[d]	Danks 1971b
Polypedilum simulans	−4	1 d	wet filter paper	92[e]	Danks 1971b
Mollusca					
Gastropoda					
Pulmonata					
Gyraulus acronicus	−4.0	10 d	water and substrate	68[f]	Olsson 1984

TABLE 2. Continued.

Taxon	Temperature (°C)	Duration	Conditions	Survival (%)	Reference
Melampus bidentalis	−15	4.5 h	moist towel	0[g]	Hilbish 1981
	−10	48 h	moist towel	~90[g]	Hilbish 1981
Ctenobranchiata					
Valvata piscinalis	−4.0	10 d	water and substrate	0[f]	Olsson 1984
Bivalvia					
Mytilus edulis	−10	24 h	air; mantle cavity sea water	100[h]	Williams 1970
Venus mercenaria	−6	24 h	air; mantle cavity sea water	100[h]	Williams 1970
Modiolus demissus	−12	12 h	air; mantle cavity sea water	50[i]	Murphy and Pierce 1975

[a] No survival when water in containers froze solidly to the bottom
[b] Temperatures represent estimated LT_{50}s. March-collected individuals. May-collected individuals did not survive temperatures <0°C
[c] In some tests, larvae were frozen and thawed repeatedly. The "large red chironomid larva" could never be frozen in ice without killing it
[d] Larvae frozen in cocoons. Those frozen without cocoons had 8% survival
[e] Larvae frozen in cocoons. Those frozen without cocoons had 39% survival
[f] Winter collected samples
[g] Individuals collected in Massachusetts and Delaware
[h] Did not survive longer and/or colder exposures
[i] Taken from an LD_{50} determination of the effect of salinity on survival at low temperature

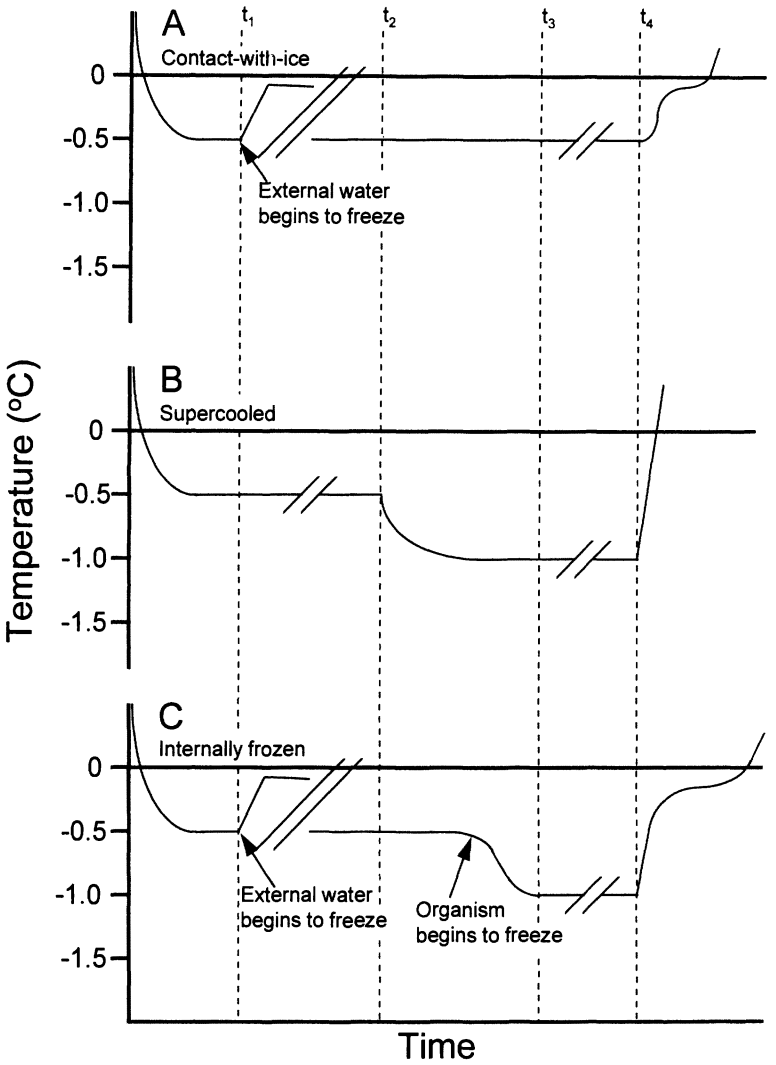

FIG. 2. Schematic diagrams showing the time course of contact-with-ice (A), supercooled (B), and internally frozen (C) treatments used in survival tests for aquatic invertebrates. All preparations were thermoequilibrated at −0.5°C. The wet paper toweling surrounding animals in (A) and (C) was inoculated at time t_1. When the systems were again at thermal equilibrium at −0.5°C (t_2), the bath temperature was set to the desired exposure temperature (−0.5°C for (A), −1.0°C for (B) and (C), in this figure). When preparations reached the target temperature (time t_3), the exposure period began. Note the appearance of a temperature plateau after t_2 in (C), indicating that additional ice (in the animal) was being formed. No such plateau is visible in (B), indicating that the animal was supercooled. The exposure period was terminated at time t_4. Note the temperature plateaus in (A) and (C) as the preparations absorbed the heat of crystallization during ice melting. The animal in (B) shows no such plateau, indicating that it remained unfrozen during the exposure period.

cluding 2 species of aquatic beetles. On the other hand, small volumes of water generally supercool readily (Lee 1989, 1991, Lee et al. 1993). Thus, from a physical perspective, it is surprising that the aquatic animals did not supercool more extensively than they did in dry test conditions. Some terrestrial arthropods increase their supercooling capacity by eliminating nucleators from their systems in preparation for winter (Duman et al. 1991). For an aquatic invertebrate, however, the powerful inoculative action of external ice would render such adjust-

ments ineffectual. Thus, elevated supercooling points in these animals may be the result of retaining compounds that incidently have ice nucleating activity.

What, then, do these organisms do when faced with low temperatures in a natural setting? Our method of testing animals in the presence of ice ensured that the animals would not be mechanically injured by external ice formation. We also monitored freezing of the animal itself independent of surrounding ice. Several points are worth noting about the method. First, even the small amount of water used to moisten the paper towel squares (~270 μL) took a substantial period of time to freeze at −0.5°C (10 h or more). Animals that subsequently remained in contact with frozen toweling for 24 h at −0.5°C did not themselves freeze. Thus, when animals are observed in contact with ice in the field, it cannot be assumed that they are frozen. Second, although the laboratory system reached equilibrium at −0.5°C before the temperature of the cooling bath was decreased further, additional freezing of water in the paper toweling occurred when the bath temperature was lowered. This additional freezing was evidenced by a continued plateau or a gentle downward slope of the temperature trace of water-saturated toweling, which contrasted sharply with the abrupt, linear downward slope of dry-towel preparations. Thus, the system reached equilibrium at −0.5°C with only some of its water frozen. A decrease in temperature froze additional water, causing the release of additional heat of crystallization. This observation underscores the idea that ice formation takes time and that the equilibrium quantity of ice formed will increase as the temperature is lowered. Thus, survival will depend on the duration and temperature of exposure.

Our data show that a variety of aquatic invertebrates are not resistant to inoculative freezing. Salt (1963) noted that ice readily propagates through insect cuticle and Danks (1971b) showed that wet chironomid larvae froze 2 to 8°C higher than did dry ones. In our study, clams, isopods, and mayflies apparently began freezing as soon as they reached their freezing points when in contact with external ice. This result is in stark contrast to their supercooling ability when isolated from external ice (−4.7 to −5.8°C), and should serve as a reminder that, although T_cs provide information about the physical properties of organisms, they are not, in themselves, reliable indicators of when organisms are likely to freeze in nature (Bale 1987, Layne et al. 1990, Lee 1991). In contrast, the belostomatid and 3 species of beetles appeared to resist inoculative freezing to a limited extent. They may possess just enough resistance to inoculation by external ice to delay the onset of freezing, which would decrease the amount of ice formed during short exposures. Thus, even slight resistance to inoculative freezing may be sufficient to allow these organisms to survive high sub-zero exposures of short duration.

It is interesting to note that the organisms that possessed the greatest resistance to inoculative freezing are species that breathe air. Both belostomatids and dytiscids rise to the water surface to acquire oxygen and thus do not need to have a permeable membrane in contact with water. In addition, adult belostomatids and adult dytiscids periodically migrate from ponds and must be able to withstand terrestrial conditions. Both have rather tough integuments, and dytiscids are covered by thick cuticle and wax. In contrast, the other species tested are obligate aquatic organisms and employ aquatic gas exchange through gills or tracheal gills which likely are sites of penetration by external ice crystals. Clams have extremely hard, and seemingly impenetrable, external coverings. However, the fact that these organisms had little capacity to resist inoculative freezing indicates that they were unable to, or did not, seal their shells effectively against penetrating ice crystals.

Other authors have detected seasonal variation in low-temperature-related characteristics of aquatic invertebrates. Frisbie and Dunson (1988) documented clear seasonal changes in hemolymph osmolality in a large species of dytiscid and showed that this change could be brought on by exposure to low temperature. Olsson (1984) found that the gastropod *Gyraulus acronicus* was freeze-tolerant in winter, but not in summer. Sawchyn and Gillott (1975) reported that damselfly larvae that survived encasement in ice at −6°C in March, lost this ability in May. Although we did not specifically test seasonal adaptation to cold in our study, we found no evidence of seasonal effects. Mayflies that were collected in February and May did not differ in body composition ([BW]) or T_c-dry. Although they did differ marginally and statistically in T_c-ice, it is

TABLE 3. Body parameters and crystallization temperatures of several aquatic invertebrates and a terrestrial beetle. Values are mean ± 1 SE (*n*). [BW] = body water concentration (μL/mg dry body mass); T_c-dry = crystallization temperature of dry, live animal; T_c-ice = crystallization temperature of live animal surrounded by ice. Within a column, values followed by a common superscript do not differ significantly.

Species	Season	Live mass (mg)	Body water (μL)	[BW] (μL/mg DM)
Mollusca				
Bivalvia				
Sphaerium sp.	Feb	65.2 ± 4.7 (12)	47.2 ± 3.2 (12)	2.6 ± 0.1^{bc} (12)
Arthropoda				
Isopoda				
Lirceus fontinalis	Feb	66.2 ± 6.2 (12)	48.5 ± 4.7 (12)	2.7 ± 0.1^{cd} (12)
Insecta (aquatic)				
Stenonema femoratum	Feb	56.9 ± 1.6 (18)	42.7 ± 1.1 (18)	3.1 ± 0.2* (18)
	May	64.5 ± 2.3 (18)	48.4 ± 1.6 (18)	3.1 ± 0.1^{cd} (18)
Belostoma flumineum	Nov	224.5 ± 12.9 (8)	168.4 ± 9.5 (8)	3.1 ± 0.3^{cd} (8)
Ilybius oblitus	Jun	83.1 ± 3.0 (10)	64.2 ± 2.7 (10)	3.7 ± 0.5^{d} (10)
Agabus disintegratus	Jun	29.9 ± 0.7 (10)	17.0 ± 0.9 (10)	1.7 ± 0.5^{ab} (10)
Insecta (terrestrial)				
Hippodamia convergens	com†	16.3 ± 0.9 (12)	8.7 ± 0.5 (12)	1.2 ± 0.0^{a} (12)

* Mayfly samples were pooled for analysis of variance on [BW], T_c-dry, and T_c-ice

† Lady beetles were field-collected animals purchased from a commercial vendor. They were held in darkness at 4°C until use

doubtful that this difference (−0.8 in February to −0.5°C in May) plays a significant role in avoiding freezing in nature. However, our latest collected organisms (mayflies and *I. oblitus*) performed relatively poorly in tests of survival of supercooling. A more careful assessment is needed before seasonal changes in low-temperature survival are ruled out.

The critical question is whether or not aquatic organisms survive sub-zero temperatures. Our survival data suggest that a variety of aquatic invertebrates cannot survive internal ice formation at relatively modest sub-zero temperatures (≥−4°C). On the other hand, all groups tested survived 24 h exposures to high sub-zero temperatures when in contact with ice. Our data suggest that if these aquatic organisms can withstand the physical forces generated by ice formation, they are capable of surviving ice encasement in nature. Field survival data also demonstrate that a variety of aquatic invertebrates can survive ice encasement (Table 1). An interesting pattern is apparent in the data of Brown et al. (1953), who reported 100% survival of plecopterans, ephemeropterans, and dipterans when thawed from anchor ice, but no survival when these same animals were thawed from sheet ice. Since anchor ice forms when disk-shaped plates of frazil ice agglomerate on underwater surfaces (Oswood et al. 1991), animals trapped therein may be subjected to more modest physical forces than those trapped by actual crystal growth as surface sheet ice expands downward. Two species of mollusks also readily survived ice encasement (Table 1). In these cases, the hard shell may afford protection from the physical forces of external ice formation. On the other hand, a variety of soft-bodied

TABLE 3. Extended.

Hemolymph (mOsm)	T_c-dry (°C)	T_c-ice (°C)
61 ± 3[a] (3)	−5.4 ± 0.2[de] (6)	−0.4 ± 0[d] (5)
268 ± 19[b] (5)	−4.7 ± 0.5[e] (5)	−0.6 ± 0[cd] (6)
—	−5.4 ± 0.1* (6)	−0.8 ± 0.1* (5)
330 ± 15[c] (6)	−5.8 ± 0.2[de] (6)	−0.5 ± 0.1[cd] (6)
355 ± 6[c] (8)	−7.9 ± 0.6[b] (5)	−1.2 ± 0.2[bc] (5)
—	−6.2 ± 0.1[cd] (5)	−2.0 ± 0.1[a] (5)
—	−7.1 ± 0.2[bc] (5)	−2.2 ± 0.4[a] (5)
—	−16.1 ± 0.2[a] (6)	−1.5 ± 0.1[ab] (6)

taxa also survive encasement in ice and sediments (Table 1).

Beyond the physical stresses caused by external ice formation, the temperature reached by aquatic animals is critical to their survival. Our results suggest that low temperature itself is not the important variable, because most species tested survived 24 h exposures to temperatures of −3.0°C or lower, as long as they remained supercooled. Some of the mortality seen in supercooled mayflies and isopods may reflect desiccation rather than cold stress. The single species of beetle tested under these conditions (*I. oblitus*) did not survive supercooling to −3.0°C, as 5 of 6 tested died. It would be interesting to determine if a winter sample of the same species survived better. Death occurred in all cases when animals were cooled in the presence of ice below the melting point of their body fluids, and they presumably froze. An interesting case in this regard is *I. oblitus*, which had only 50% survival at −1.0°C when in contact with ice. This temperature is very close to the melting point of the insect's body fluids (~−0.7°C). During the 24-h exposure, it is possible that inoculation did occur in some of the individuals and ice formed inside them. In contrast, 5 of 6 *I. oblitus* survived a 24-h, −1.0°C exposure in dry conditions. Thus, the critical phenomenon appears to be whether or not ice forms within the animal's tissues.

Other tests of survival by aquatic organisms in low temperatures have not uncovered this pattern (Table 2). In most of these tests, there was little to no survival, even in cases where tests were run at several temperatures. There are 3 potential problems in comparing these data with ours. First, in cases where animals were frozen in water, mortality may have resulted from mechanical stress and not directly from exposure to low temperature. Second, in many cases, tests were run at temperatures substantially lower than those we chose. Thus, other authors have not specifically tested whether low temperature is sufficient to cause mortality, or whether ice formation in body tissues is the key factor. Finally, in most tests, animals were not individually instrumented, and one cannot be certain that animals exposed in air or on moist paper towels actually froze, as opposed to remaining supercooled. Nevertheless, several reports of survivorship of low temperatures stand out. Odonate larvae seem to withstand ice encasement at temperatures that should ensure their own freezing (Table 2). It is interesting that survival dropped dramatically when exposure temperatures were decreased. Chironomids also survived very low temperatures, at which supercooling would be unlikely. None of the aquatic invertebrates we tested can match this capacity.

Our tests were carried out on organisms collected in the temperate zone, where prolonged cold exposures occur haphazardly. Most other studies of low-temperature survival by aquatic organisms have been carried out in more northerly regions where extended periods of cold occur regularly (Table 1). Selection for low-temperature survival mechanisms should be more intense in these latter regions, and, thus, there may be significant geographic differences among populations. It would be interesting to compare low-temperature-related characteristics of a single species, or within a closely related

TABLE 4. Survival of several invertebrates exposed to sub-zero temperatures.

Species	Season	Temperature (°C)	Duration[a] (h)	Test condition[b]	Survival (alive/total)
Mollusca					
Bivalvia					
Sphaerium sp.	Feb	−0.5	24	Contact with ice	6/6
				Wet	6/6
				Dry	6/6
		−3.0	24	Frozen	0/6
				Supercooled, dry	6/6
Arthropoda					
Isopoda					
Lirceus fontinalis	Feb	−0.5	2	Contact with ice	5/6
				Wet	6/7
				Dry	7/7
		−0.5	24	Contact with ice	3/4
				Wet	2/4
				Dry	0/4
		−3.5	2	Frozen	0/8
				Supercooled, dry	3/4
Insecta					
Stenonema femoratum	May	−0.5	24	Contact with ice	4/6
				Wet	6/6
				Dry	2/6
		−2.0	2.5	Frozen	0/6
				Supercooled, wet	5/6
		−3.0	2	Frozen	0/6
				Supercooled, dry	3/6
Belostoma flumineum	Nov	−0.3	24	Contact with ice	6/6
				Wet	6/6
		−4.0	24	Frozen	0/6
				Supercooled, dry	6/6
Ilybius oblitus	Jun	−1.0	24	Contact with ice	3/6
				Supercooled, dry	5/6
		−3.0	24	Frozen	0/6
				Supercooled, dry	1/6

[a] Exposure time began when the system reached thermal equilibrium at the temperature listed

[b] Animals held in contact with ice were cooled to −0.5°C in a moist paper towel wrap. The towel was inoculated with a crystal of ice or a puff of Histofreeze. Animals in the wet and dry treatments were wrapped in moist and dry paper toweling, respectively. No ice formed in these treatments. Frozen animals were cooled below their melting points while in contact with moist toweling that had been inoculated at −0.5 to −1.0°C. Supercooled animals were wrapped in dry toweling and cooled below the melting points of their body fluids. No ice formed in this treatment since no exotherms were recorded.

taxonomic group, across a broad north–south distribution.

Several authors working in more northern regions have reported ice and sediment temperatures well below 0°C in streams and ponds (Danks 1971a, Irons et al. 1993, Olsson 1984, 1988). Under these conditions, the organisms we studied would not be able to survive. In more temperate regions, however, it is less likely that ice will form all the way to the bottom of waterways. Irons et al. (1993) suggested that many aquatic invertebrates actively move down, away from an advancing ice front. Our data suggest that a number of species could withstand circum-zero temperatures and survive in such conditions. Furthermore, even if freezing should begin around them, the invertebrates in our study could survive provided they remained unfroz-

en. This condition would be met if the temperature did not fall below ~−0.5°C and/or if the exposure were not too long. Since ice formation at relatively high sub-zero temperatures progresses slowly, it is probable that the temperatures at the bottoms of even very shallow temperate ponds do not get substantially below 0°C unless the cold spell lasts for several weeks. Some of the species we tested may be able to resist freezing for 24 h or more even when ice temperatures drop to −1.0°C or lower. In comparison with the cold tolerance strategies of some terrestrial invertebrates, these capacities are modest; but in the more thermally buffered aquatic environment, they may be enough of a hedge to get most aquatic invertebrates through all but extremely severe winters.

Acknowledgements

We thank Guenter Schuster and Bill Wolfe for taxonomic determinations and Jon Costanzo for his constructive review of an earlier version of the paper. This work was supported by a Research Opportunity Award Supplement from the National Science Foundation to REL (IBN-9305809) and by a sabbatical leave granted by Eastern Kentucky University to MPF.

Literature Cited

ANDREWS, D., AND F. H. RIGLER. 1985. The effects of an arctic winter on benthic invertebrates in the littoral zone of Char Lake, Northwest Territories. Canadian Journal of Zoology 63:2825–2834.

BALE, J. S. 1987. Insect cold hardiness: freezing and supercooling—an ecophysiological approach. Journal of Insect Physiology 33:899–908.

BROWN, C. J. D., W. D. CLOTHIER, AND W. ALVORD. 1953. Observations on ice conditions and bottom organisms in the West Gallatin River, Montana. Proceedings of the Montana Academy of Sciences 13:21–27.

COSTANZO, J. P., AND R. E. LEE. 1995. Supercooling and ice nucleation in vertebrate ectotherms. Pages 221–237 *in* R. E. Lee, G. J. Warren, and L. V. Gusta (editors). Biological ice nucleation and its applications. APS Press, St Paul, Minnesota.

COSTANZO, J. P., R. E. LEE, A. L. DEVRIES, T. WANG, AND J. R. LAYNE. 1995. Survival mechanisms of vertebrate ectotherms at subfreezing temperatures: applications in cryomedicine. FASEB Journal 9:351–358.

DABORN, G. R. 1971. Survival and mortality of coenagrionid nymphs (Odonata: Zygoptera) from the ice of an aestival pond. Canadian Journal of Zoology 49:569–571.

DANKS, H. V. 1971a. Overwintering of some north temperate and arctic Chironomidae. I. The winter environment. Canadian Entomologist 103:589–604.

DANKS, H. V. 1971b. Overwintering of some north temperate and arctic Chironomidae. II. Chironomid biology. Canadian Entomologist 103:1875–1910.

DEVRIES, A. L. 1983. Antifreeze peptides and glycopeptides in cold-water fishes. Annual Review of Physiology 45:245–260.

DUFFY, W. G., AND C. R. LISTON. 1985. Survival following exposure to subzero temperatures and respiration in cold acclimatized larvae of *Enallagma boreale* (Odonata: Zygoptera). Freshwater Invertebrate Biology 4:1–7.

DUMAN, J. G., AND K. HORWATH. 1983. The role of hemolymph proteins in the cold tolerance of insects. Annual Review of Physiology 45:261–270.

DUMAN, J. G., L. G. NEVEN, J. M. BEALS, K. R. OLSON, AND F. J. CASTELLINO. 1985. Freeze-tolerance adaptations, including haemolymph protein and lipoprotein nucleators, in the larvae of the cranefly *Tipula trivittata*. Journal of Insect Physiology 31: 1–8.

DUMAN, J. G., D. W. WU, L. XU, D. TURSMAN, AND T. M. OLSEN. 1991. Adaptations of insects to subzero temperatures. The Quarterly Review of Biology 66:387–410.

FRISBIE, M. P., AND W. A. DUNSON. 1988. Seasonal aspects of sodium, potassium, and water balance in the predaceous diving beetle *Dytiscus verticalis*. Canadian Journal of Zoology 66:1553–1561.

GRAVES, S. Y., AND T. H. DIETZ. 1980. Diurnal rhythms of sodium transport in the freshwater mussel. Canadian Journal of Zoology 58:1626–1630.

HILBISH, T. J. 1981. Latitudinal variation in freezing tolerance of *Melampus bidentatus* (Say) (Gastropoda: Pulmonata). Journal of Experimental Marine Biology and Ecology 52:283–297.

IRONS, J. G., L. K. MILLER, AND M. W. OSWOOD. 1993. Ecological adaptations of aquatic macroinvertebrates to overwintering in interior Alaska (U.S.A.) subarctic streams. Canadian Journal of Zoology 71:98–108.

LAYNE, J. R., R. E. LEE, AND J. L. HUANG. 1990. Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*). Canadian Journal of Zoology 68:506–510.

LEE, R. E. 1989. Insect cold hardiness: to freeze or not to freeze? BioScience 39:308–313.

LEE, R. E. 1991. Principles of insect low temperature tolerance. Pages 17–46 *in* R. E. Lee and D. L. Denlinger (editors). Insects at low temperature. Chapman and Hall, New York.

LEE, R. E., M. R. LEE, AND J. M. STRONG-GUNDERSON. 1993. Insect cold-hardiness and ice nucleating active microorganisms including their potential use for biological control. Journal of Insect Physiology 39:1–12.

LEE, R. E., M. R. LEE, AND J. M. STRONG-GUNDERSON. 1995. Biological control of insect pests using ice nucleating microorganisms. Pages 257–269 *in* R. E. Lee, G. J. Warren, and L. V. Gusta (editors). Biological ice nucleation and its applications. APS Press, St Paul, Minnesota.

LEE, R. E., J. M. STRONG-GUNDERSON, M. R. LEE, K. S. GROVE, AND T. J. RIGA. 1991. Isolation of ice nucleating active bacteria from insects. Journal of Experimental Zoology 257:124–127.

MCCORKLE, S., AND T. H. DIETZ. 1980. Sodium transport in the freshwater asiatic clam *Corbicula fluminea*. The Biological Bulletin 159:325–336.

MOORE, M. V., AND R. E. LEE. 1991. Surviving the big chill: overwintering strategies of aquatic and terrestrial insects. American Entomologist Summer 1991:111–118.

MUGNANO, J. A., R. E. LEE, AND R. T. TAYLOR. 1996. Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). Journal of Experimental Biology 199:465–471.

MURPHY, D. J., AND S. K. PIERCE. 1975. The physiologic basis for changes in the freezing tolerance of intertidal molluscs. Journal of Experimental Zoology 193:313–322.

NEVEN, L. G., J. G. DUMAN, J. M. BEALS, AND F. J. CASTELLINO. 1986. Overwintering adaptations of the stag beetle, *Ceruchis piceus*: removal of ice nucleators in the winter to promote supercooling. Journal of Comparative Physiology B 156:707–716.

OLSSON, T. I. 1981. Overwintering of benthic macroinvertebrates in ice and frozen sediment in a North Swedish river. Holarctic Ecology 4:161–166.

OLSSON, T. I. 1984. Winter sites and cold-hardiness of two gastropod species in a boreal river. Polar Biology 3:227–230.

OLSSON, T. I. 1988. The effect of wintering sites on the survival and reproduction of *Gyraulus acronicus* (Gastropoda) in a partly frozen river. Oecologia 74:492–495.

OSWOOD, M. W., L. K. MILLER, AND J. G. IRONS. 1991. Overwintering freshwater benthic macroinvertebrates. Pages 360–378 *in* R. E. Lee and D. L. Denlinger (editors). Insects at low temperature. Chapman and Hall, New York.

SALT, R. W. 1963. Delayed inoculative freezing of insects. Canadian Entomologist 95:1190–1202.

SAWCHYN, W. W., AND C. GILLOTT. 1975. The biology of two related species of coenagrionid dragonflies (Odonata: Zygoptera) in western Canada. Canadian Entomologist 107:119–128.

SCHEIDE, J. I., AND T. H. DIETZ. 1982. The effects of independent sodium and chloride depletion on ion balance freshwater mussels. Canadian Journal of Zoology 60:1676–1682.

SCHOLANDER, P. W., W. FLAGG, R. J. HOCK, AND L. IRVING. 1953. Studies on the physiology of frozen plants and animals in the Arctic. Journal of Cellular and Comparative Physiology 42:1–56.

SØMME, L. 1964. Effects of glycerol on cold-hardiness in insects. Canadian Journal of Zoology 42:87–101.

SØMME, L. 1982. Supercooling and winter survival in terrestrial arthropods. Comparative Biochemistry and Physiology 73A:519–543.

STOREY, K. B., AND J. M. STOREY. 1988. Freeze tolerance in animals. Physiological Reviews 68:27–84.

SUTCLIFFE, D. W. 1962. The composition of haemolymph in aquatic insects. Journal of Experimental Biology 39:325–343.

WILLIAMS, R. J. 1970. Freezing tolerance in *Mytilus edulis*. Comparative Biochemistry and Physiology 35:145–161.

WU, D. W., J. G. DUMAN, C-H. C. CHENG, AND F. J. CASTELLINO. 1991. Purification and characterization of antifreeze proteins from larvae of the beetle *Dendroides canadensis*. Journal of Comparative Physiology B 161:271–278.

ZACHARIASSEN, K. E., AND H. T. HAMMEL. 1976. Nucleating agents in haemolymph of insects tolerant to freezing. Nature 262:285–287.

Received: 9 December 1996
Accepted: 15 April 1997