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The role of direct chilling injury and inoculative freezing in cold tolerance of *Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis*

CHARLES S. BURKS, RICHARD L. STEWART, Jr,*
GLEN R. NEEDHAM* and RICHARD E. LEE, Jr
Department of Zoology, Miami University, and *Acarology Laboratory, Department of Entomology, The Ohio State University, U.S.A.

Abstract. Supercooling points and chill tolerance were compared among nymphs and adults of the ixodid ticks *Dermacentor variabilis*, *Amblyomma americanum* and *Ixodes scapularis* (Acari: Ixodidae). Supercooling points in the range of <–22 to –18°C were observed for nymphs, and –22 to –8°C for adults. The lower lethal temperatures observed under dry conditions, –14 to –10°C, were warmer than the supercooling points, but still much colder than –4.8°C, the lowest temperature recorded from a likely tick habitat in southwestern Ohio. Based on our experiments, spontaneous freezing and direct chilling injury are not significant mortality factors in these species in the field. Mortality was observed between –5 and –3°C for *A.americanum* and *D.variabilis* nymphs chilled for 2 h while in direct contact with ice. This mortality is probably due to inoculative freezing. Given the requirement for a rather humid microhabitat for off-host survival, these findings suggest that inoculative freezing is an important cause of overwintering mortality in these medically important species.

Key words. Ixodidae, hard tick, cold hardiness, freezing injury.

Introduction

Three-host ixodid ticks take a single blood meal during each of three postembryonic stages and spend the greatest proportion of their lives off the host (Oliver, 1989). These extended off-host intervals have provided selection pressure favouring water and energy conservation (Needham & Teel, 1991). One unusual adaptation is the ability to absorb water from sub-saturated air via the mouthparts (Knulle & Rudolph, 1982). This ability to absorb water is energy dependent, and in some species is impaired by low temperatures (McEnroe, 1971; Sauer & Hair, 1971). In a variety of animal species, adaptations for water conservation also enhance overwinter survival (Costanzo *et al.*, 1993; Ring & Danks, 1994).

Most hard ticks in the U.S.A. of medical and veterinary importance overwinter off-host. This includes the lone star tick, *Amblyomma americanum* (L.), the American dog tick, *Dermacentor variabilis* (Say), and the Eastern blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae). *A.americanum* typically overwinters in a photoperiodically-induced diapause as a nymph (Barnard *et al.*, 1985; Pound & George, 1988), whereas *D.variabilis* may overwinter in any stage, depending on geographical and climatological factors (Sonenshine, 1979; McEnroe, 1982). *I.scapularis* uses a 2-year life cycle in the north, overwintering in the nymphal and adult stages (Yuval & Spielman, 1990; Platt *et al.*, 1992). These three species have overlapping distributions in the U.S.A., with *A.americanum* in the southeastern states north to the Ohio River valley (Barnard *et al.*, 1992), *D.variabilis* in the eastern U.S.A. west to the great plains and as far north as southern Canada (Sonenshine, 1979) and *I.scapularis* with a disjointed distribution, with one population in the Gulf States and another, formerly known as *I.dammini*, found along the northern Atlantic coast and around the Great Lakes except Ohio (Strickland *et al.*, 1978; Centers for Disease Control, 1992). These distribution patterns suggest that the range of these species may be limited, in part, by climatic factors (Sonenshine, 1979; Strey *et al.*, 1999).

Studies of insect cold hardiness have differentiated among freeze injury, direct chilling injury, and indirect chilling injury as causes of mortality (Lee, 1991). These types of mortality can be distinguished by comparing the temperature of crystallization (loosely synonymous with the supercooling point) with the lowest temperature at which survival is observed (lower lethal temperature, LLT). As operational definitions, direct chilling injury induces death in response to chilling episodes of less than 2 h, whereas indirect chilling injury occurs when much longer

Correspondence: Dr Charles S. Burks, USDA US Grain Marketing Laboratory, 1515 College Ave., Manhattan, KS 66502, U.S.A.

exposure, often days to weeks, is required to induce death (Morris & Watson, 1984). Inoculative freezing due to contact with external ice can increase the temperature at which ice forms in body fluids. Enhanced cold tolerance, or cold hardening, often occurs in response to decreased ambient temperature or shorter photoperiods, and has been noted in many insect species (Lee, 1991).

Laboratory studies have also examined aspects of cold hardiness in ixodid tick species. Crystallization temperatures have been determined for unspecified stages of *D.albipictus*, *D.variabilis* and *I.scapularis*, and none survived testing (Schmid, 1986). The Antarctic tick *I.uriae* has crystallization temperatures ranging from –21 to –7°C in post-embryonic stages, with no indication of freeze tolerance or increases in cold tolerance following acclimation to low temperature (Lee & Baust, 1987). Half of *A.americanum* adults exposed to –12.5°C for 2 h survived, whereas the more southerly species, *A.cajennense*, did not survive exposure longer than 90 min at this temperature (Strey *et al.*, 1996). In a separate study, crystallization temperatures in *A.americanum* ranged from –22°C in adults to –13°C in nymphs, and over 50% of adults survived exposure to –15°C for 2 h (Needham *et al.*, 1995).

In the present study we compared crystallization temperatures and lower lethal temperatures of *A.americanum* and *D.variabilis* nymphs and adults, and *I.scapularis* nymphs with microhabitat temperatures from likely tick hibernacula in southwestern Ohio. We also evaluated the potential for increased cold hardiness in each species and stage in response to chilling and short photoperiods, and examined survival of ticks chilled in direct contact with ice. Results of these experiments were compared to the threat posed to these three tick species in their overwintering microhabitat.

Materials and Methods

Ticks. All ticks were maintained in closed chambers with a supersaturated solution of potassium nitrate, providing a relative humidity (r.h.) of approximately 93% (Winston & Bates, 1960). *A.americanum* and *D.variabilis* nymphs were obtained from adults fed on sheep in laboratory colonies maintained at Oklahoma State University, Stillwater, Oklahoma (Patrick & Hair, 1976). *I.scapularis* nymphs were progeny of ticks obtained from central Illinois. The larvae were fed on a gerbil, allowed to moult and resulting nymphs were maintained in a 93% r.h. chamber. 'Unacclimated' ticks were held for up to several weeks following moulting under L:D 15:9 h at room temperature (approximately 23°C). 'Cold-acclimated' ticks were held for several weeks under long photoperiods at room temperature, and then switched to a cold room (4°C) and held under L:D 10:14 h for 4 weeks (*A.americanum*, *D.variabilis* nymphs and adults) or 7 weeks (*I.scapularis*).

Determination of temperature of crystallization. Crystallization temperature was determined by attaching ticks with transparent tape to 30- or 36-gauge copper–constantan thermocouples. This assembly was placed in a 1.8 ml microcentrifuge tube plugged with foam rubber and inserted into a 19 × 150 mm glass tube, which was suspended in a refrigerated circulating bath (Neslab RTE-8). The thermocouples were attached to a twelve-channel scanning chart recorder (Omega OM500). Specimens were then chilled at a rate of ≤0.2°C/min to a low of –23 to –21°C. An exotherm, or transient increase of temperature due to heat released as water crystallized within ticks, was identified on the chart recording. The lowest temperature recorded prior to the exotherm was considered the temperature of crystallization.

Determination of lower lethal temperature. Acute chill tolerance was assessed by determining a lower lethal temperature for a 2 h exposure (LLT_{2h}). For this study, lower lethal temperature is considered the lowest integer value on the Celsius scale at which any survivorship was observed. Ticks were secured directly in a microfuge tube plugged with foam rubber and placed in a 19 × 150 mm glass tube, which was suspended in the refrigerated bath. Tube temperature was monitored using a thermocouple in a microfuge/glass tube assembly without ticks. Because it took approximately 10 min for the temperature inside the microfuge tube to equilibrate, an incubation time of 2 h 10 min was used for these experiments. After the chill period, tubes containing ticks were removed from the bath and held for several minutes at room temperature. Specimens were placed in a 95% r.h. chamber and held 24 h before evaluating survival. Ticks that moved normally were categorized survivors, and those that were dead or obviously impaired were classified dead. A one-way analysis of variance (ANOVA) was performed on the arcsine transform of the proportional survivorship for *A.americanum* and *D.variabilis* using the program Instat™ (Graphpad) on a Macintosh™ computer.

To determine the LLT_{2h} of ticks in contact with ice, 0.5 ml of water was frozen in 1.8 ml microfuge tubes, which were placed in glass tubes immersed in the alcohol bath, and chilled to subfreezing temperatures. Microfuge tubes were then briefly removed, ticks placed on the ice, and the tubes were transferred back into the refrigerated bath for 2 h. A thermocouple placed in a blank assembly monitored temperature. Following the chill period, specimens were dropped out of the microfuge tube with ice onto laboratory tissues, transferred into a clean dry microfuge tube, and allowed to recover in a humid chamber. Procedures for evaluation were as previously described.

Monitoring soil surface temperature. Soil surface temperature was monitored in a farm wood in Butler County, Ohio, from December 1993 to March 1994, using a single thermistor attached to a HoboTemp™ temperature logger (OnSet Instruments). The thermistor was calibrated by measuring melting ice water, and was then placed on the soil surface under leaf litter. Temperature measurements were taken every 48 min. Data were periodically down-loaded using a Macintosh™ Powerbook computer.

Results

Under dry conditions, all *A.americanum* nymphs froze at less than –18°C, and ten of eleven *D.variabilis* nymphs froze at less than –20°C (Fig. 1). None of the eleven *I.scapularis* nymphs froze when they were chilled to –21.7°C and held at that temperature for 3 h (data not shown). For all three species, no nymphs froze at temperatures higher than –14°C under dry conditions. Among adults, crystallization temperatures as high as –9.7°C were observed for *A.americanum* females, but all crystallization temperatures for *D.variabilis* females and males were less than –14°C (Fig. 1).

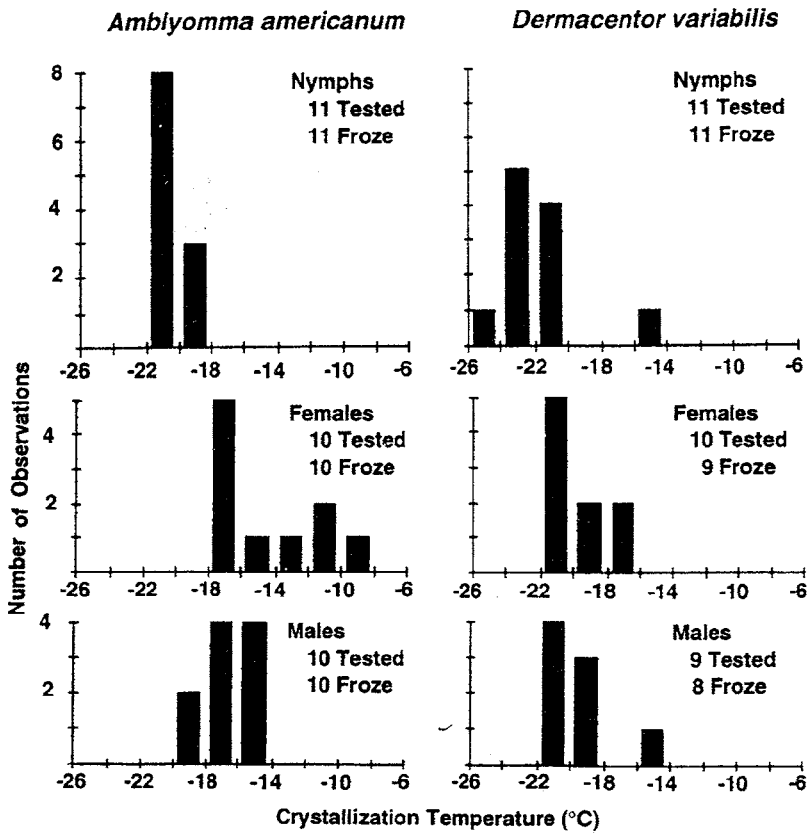

Fig. 1. Distribution of crystallization temperatures in *Amblyomma americanum* and *Dermacentor variabilis*.

The LLT_{2h} for unacclimated nymphs was −12°C for *A.americanum*, −14°C for *D.variabilis*, and −10°C for *I.scapularis* (Figs 2 and 3). A LLT_{2h} of −11°C was observed for *I.scapularis* nymphs acclimated with several weeks exposure to 4°C and short photoperiods (Fig. 3). Acclimation resulted in a shift toward increased survivorship for *A.americanum* nymphs and slightly decreased survivorship for *D.variabilis* nymphs. These trends were not significant ($P > 0.5$) when examined by one-way ANOVA (data not shown). The LLT_{2h} for unacclimated *A.americanum* adults was −10°C for females and −11°C for males (Table 1). Among both sexes of acclimated *A.americanum* adults some survivorship was noted at −12°C, the lowest temperature tested. A LLT_{2h} of −12°C was observed for unacclimated *D.variabilis* females and one of −13°C was observed for males (Table 1). Acclimation effects were not tested for *D.variabilis* adults.

To examine the potential impact of inoculative freezing on survival, chill tolerance was examined in unacclimated

Table 1. Survivorship of *Amblyomma americanum* and *Dermacentor variabilis* adults 24 h following exposure to various subzero temperatures for 2 h. Chill tolerance for *Amblyomma americanum* is compared between unacclimated ticks and those acclimated by exposure for 4 weeks to 4°C and LD 10:14 h.

	Amblyomma americanum				*Dermacentor variabilis*	
	Unacclimated		Acclimated		Unacclimated	
Chill temperature (°C)	Females	Males	Females	Males	Females	Males
−14	nd	nd	nd	nd	0	0
−13	nd	nd	nd	nd	0	2
−12	0	0	3	1	2	3
−11	0	1	4	2	5	5
−10	4	1	4	2	nd	5
−9	4	5	nd	nd	nd	nd

nd = not determined.

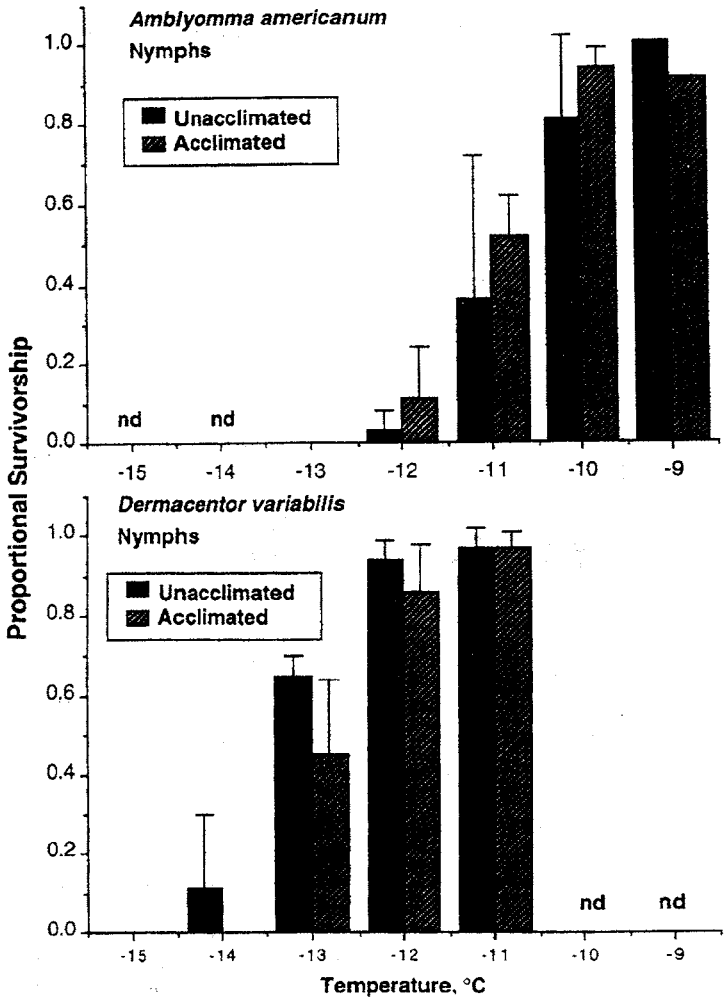

Fig. 2. Survivorship of *Amblyomma americanum* and *Dermacentor variabilis* nymphs chilled for 2 h at sub-zero temperatures. Nymphs were either unacclimated, or were acclimated by exposure for 4 weeks to 4°C and LD 10:14 h. Each bar represents the average of three replicates, with one replicate comprising the proportional survivorship of ten to twelve ticks. nd = not determined.

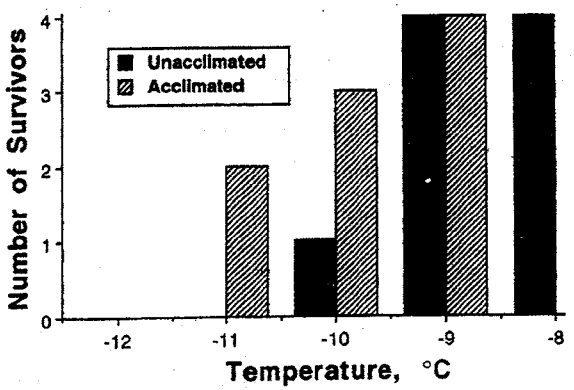

Fig. 3. Survivorship of *Ixodes scapularis* nymphs chilled for 2 h at sub-zero temperatures. Nymphs were either unacclimated, or were acclimated by exposure for 7 weeks to 4°C and LD 10:14 h.

Table 2. Survivorship of *Amblyomma americanum* and *Dermacentor variabilis* nymphs 24 h following exposure for 2 h to subzero temperatures while in contact with ice. Five individuals were used for each determination.

Chill temperature (°C)	*Amblyomma americanum*		*Dermacentor variabilis*	
	n	Survivorship	*n*	Survivorship
–6	nd	nd	30	0.47
–5	14	0	30	0.33
–4	10	0.2	30	0.13
–3	10	1.0	10	0.80
–2	10	1.0	nd	nd

nd = not determined.

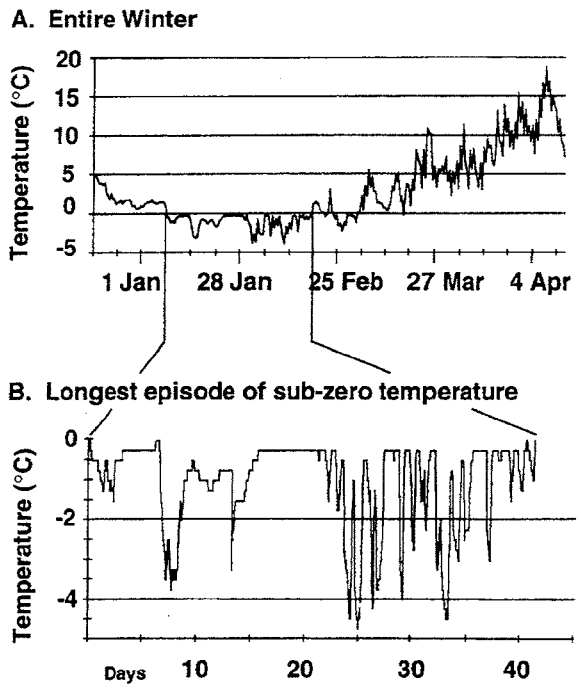

Fig. 4. Soil surface temperature beneath leaf litter in Southwest Ohio during the winter, 1993–94. (A) 17 December to 2 May. (B) Detail of longest period of continuous sub-zero temperature. This period lasted 42 days (8 January to 19 February). The longest periods during which temperatures were lower than –4°C were 11 h 12 min. This occurred on the evenings of 1–2 and 9–10 February.

A.americanum and *D.variabilis* nymphs in contact with ice. All *A.americanum* nymphs survived chilling in the presence of ice at –3 and –2°C (Table 2). Low survivorship was seen at –4°C and none survived chilling at –5°C. Mortality was slight for *D.variabilis* nymphs chilled at –3°C, the highest temperature for which inoculative freezing was tested. Mortality was higher at temperatures between –6 and –4°C, but there was no apparent correlation between chill temperature and survivorship, with some survivors observed in all tests.

To compare the crystallization temperatures and lower lethal temperatures with those occurring in nature, microhabitat temperatures in southwest Ohio were continuously monitored during the winter of 1993–94 (Fig. 4). The lowest temperature experienced was –4.8°C (Fig. 4A). The longest continuous period of temperature below 0°C was 42 days between (8 January to 19 February, Fig. 4B). During most of this period the temperature exceeded –2°C, although on four dates the temperature in leaf litter dropped below –4°C for 12 h or less.

Discussion

Thresholds for crystallization temperatures and direct chilling threshold temperatures for *A.americanum*, *D.variabilis* and *I.scapularis* nymphs and adults were systematically compared with temperatures these species might experience while overwintering. The $LLT_{2\,h}$ values observed were far higher than the temperature of crystallization values for all stages and species examined, with the exception of *A.americanum* females. Moreover the LLT_{2h}, a measure of the threshold for direct chilling injury, ranged from –10 to –14°C for all species and stages examined. This was substantially colder than the coldest temperatures observed by continuously monitoring the soil surface under leaf litter in a wood lot in southwest Ohio for a single winter. In the case of unacclimated *A.americanum* females one of ten individuals tested froze at a temperature greater than the observed LLT_{2h} of –10°C, but most *A.americanum* females froze at lower temperatures. Thus the temperature of crystallization has little biological relevance for these species, indicating that chilling injury rather than freezing was the cause of death in the ticks we tested.

Habitat data demonstrated that in a wood in southwest Ohio during the winter of 1993–94 the temperature was usually above –2°C and never went below –5°C (Fig. 4). Schmid (1986) reported that during years of normal snowfall the leaf litter microhabitat temperatures in central Minnesota do not usually go below –2°C. Thus ticks overwintering in northern temperate habitats in the US are not likely to experience temperatures low enough to cause death due to direct chilling injury.

Inoculative freezing, however, did occur in the temperature range observed for our field recordings. The tests of direct chilling injury to ticks indicated that, in all species and stages examined, mortality was negligible at temperatures above –9°C. This strongly implies that mortality observed at higher temperatures in ticks chilled in the presence of an ice nucleator is due to freezing injury. Exposure to –4°C for 2 h in the presence of ice proved fatal to a high proportion of both *A.americanum* and *D.variabilis* nymphs observed, and ticks overwintering in southwest Ohio might experience such temperatures several times during the winter. Therefore this study indicates that, at least in *A.americanum* and *D.variabilis*, inoculative freezing is probably the most important source of cold-induced mortality to overwintering ticks. The one-host tick, *D.albipictus*, generally remains on its host until after snow melt (Drew & Samuel, 1986). This is consistent with selective pressure to avoid inoculative freezing.

Previous studies indicate that relatively moist microhabitats are generally important to ixodid survival (Knulle & Rudolph, 1982; Needham & Teel, 1991). Most ixodids experience net water loss when exposed to less than 80% r.h. (Knulle & Rudolph, 1982), therefore a humid microhabit is necessary for long off-host survival. Our study suggests that the danger of desiccation while overwintering must be balanced against that of death due to inoculative freezing.

Ticks actively absorb water from subsaturated air, which augments moisture lost primarily via transpiration across the cuticle. The relative humidity at which the net weight is maintained is called the critical equilibrium humidity (Needham & Teel, 1991). A tick with a high rate of transpiration would presumably have a more permeable cuticle and therefore be more susceptible to inoculative freezing than a tick with a lower transpiration rate. *Amblyomma americanum* adults have a higher rate of transpiration than that observed for adults of *A.maculatum*

or *A.cajennense*, but such data are lacking for other species (Needham & Teel, 1991). However, both water vapour uptake and water movement across the cuticle are affected by temperature (Wharton, 1985), and both sorptive gain and transpirational loss of water decrease at cooler temperatures for *A.americanum* and *D.variabilis* adults (McEnroe, 1971; Sauer & Hair, 1971). Low temperature decreases transpirational water loss, aiding ticks stressed by sub-critical equilibrium humidity. A higher humidity would prevent transpirational water loss, but ice formation from condensed water vapour is likely to lead to death from inoculative freezing. Therefore transpirational water loss and inoculative freezing probably determine the upper and lower bounds of acceptable microhabitat temperature and humidity for overwintering ticks. Few data are available quantifying these important parameters in the physiological ecology of ixodids.

It should be noted that some ticks became completely encased in ice during the inoculative freezing study. One might question whether mortality in this study was due to this, rather than inoculative freezing. We have ruled this out because the treatment of *A.americanum* which allowed total survivorship included individuals that became encased in ice. Moreover, previous studies showed that *D.variabilis* can withstand submersion in water for several days (Smith *et al.*, 1946). Also, one might question whether chilling the ticks directly on ice adequately mimics field conditions. Patches of glare ice were commonly observed beneath the leaf litter while taking temperature recordings, suggesting that exposure to ice in our laboratory experiment may represent conditions experienced by overwintering ticks.

The present study suggests that acclimation may occur in *A.americanum* and *I.scapularis* nymphs. Such increased chill tolerance could result from an accumulation of low molecular weight cryoprotectants (Lee, 1991). Alternatively, induction of a quiescent state could afford additional chill tolerance (Hochachka, 1982; Storey, 1988). Previous studies failed to detect glycerol in populations of the circumpolar tick, *I.uriae* (Lee & Baust, 1987) overwintering in Antarctica, or in *A.americanum* in response to short exposures to 0–5°C (Needham *et al.*, 1995). Results from this study indicate that cold-related overwintering death is due to indirect chilling injury or to inoculative freezing. It is not clear whether susceptibility to direct chilling injury accurately predicts susceptibility to indirect chilling injury, although recent data from the fruit fly *Drosophila melanogaster* suggest that such an extrapolation may be valid for this species (Chen & Walker, 1994). There are few data available to predict whether or not acclimation improves the ability of arthropods to resistance inoculative freezing.

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