Ecology of Mountain Pine Beetle (Coleoptera: Scolytidae) Cold Hardening in the Intermountain West

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ABSTRACT The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, spends the majority of its life cycle within the phloem of pine trees, experiencing exposure to temperatures below −30°C in many parts of their expansive range. To better understand cold tolerance capabilities of this insect, seasonal patterns of cold-hardiness, as measured by supercooling points in the laboratory, were compared with seasonal patterns of host tree phloem temperatures at several geographic sites for 2 beetle generations. Larvae were found to be intolerant of tissue freezing, and supercooling points measured appear to be a reasonable estimate of the lower limit for survival. Of the compounds analyzed, glycerol was found to be the major cryoprotectant. No differences in supercooling points were found among instars or between larvae collected from the north and south aspect of tree boles. Both phloem temperatures and supercooling points of larvae collected from within the phloem were found to be different among the geographic sites sampled. Mountain pine beetle larvae appear to respond to seasonal and yearly fluctuations in microhabitat temperatures by adjusting levels of cold hardening.

KEY WORDS *Dendroctonus ponderosae*, bark beetle, supercooling point, freeze intolerant

THE MOUNTAIN PINE beetle, *Dendroctonus ponderosae* Hopkins, which overwinters under the bark of pine trees in a nonmobile stage, is unable to escape low temperature exposure. When escape from low temperatures is unavoidable, many insect species respond by adjusting physiological and biochemical processes that enhance their tolerance to freezing temperatures. Freeze tolerant species are able to withstand the formation of ice in the extracellular body fluid, whereas freeze intolerant species must avoid freezing of body tissues (Salt 1961). Freeze avoidance is accomplished through cold-hardening. The supercooling point refers to the temperature at which spontaneous nucleation of body water occurs and ice crystals begin to form in the insect tissue (Lee 1989). For those species that cannot survive tissue freezing, the supercooling point represents a lethal temperature threshold, although death may also result as a consequence of exposure to temperatures above the supercooling point (Lee 1991). The cold-hardening capacity of an insect may vary with the developmental stage, nutritional status, and duration of exposure to specific low temperatures.

Many physiological mechanisms involved in the cold-hardening process have been identified including ice-nucleating proteins, lipoproteins and antifreeze proteins, evacuation of the gut to remove potential ice nucleating agents, ice nucleating bacteria, and accumulation of low molecular weight cryoprotectants such as polyhydric alcohols (polyols) and sugars (Hamilton et al. 1985, Lee and Denlinger 1991). Polyol synthesis is known to be triggered by low-temperature exposure, with increasing rates at lower temperatures (Baust 1982, Storey and Storey 1983). Most likely, thermoperiodic cues, represented by some threshold length of time at or below a particular temperature, promote accumulation of an adequate concentration of cryoprotectants before the time they are needed (Storey and Storey 1991). To more fully understand the role of cold-hardening in mountain pine beetle population dynamics, therefore, it is necessary to relate supercooling capacities as determined in the laboratory to the thermal history of the microhabitat where larvae reside (Bale 1991).

The microhabitat of the mountain pine beetle is the phloem of living pines, wherein the majority of the life cycle is spent typically overwintering as larvae. In the northernmost part of their range, winter temperatures below −30°C are not uncommon. Mountain pine beetle eggs and pupae are considered the least cold tolerant life-stages (Reid 1963, Reid and Gates 1970, Amman 1973), whereas the large larvae are thought to be the most cold tolerant (Yuill 1941, Wygant 1942, Somme 1964). Given that effects of temperature on developmental rate are stage-specific (Bentz et al. 1991), cold-hardening capabilities may also depend on life-stage, with one stage more adapted for overwintering than another. Although temperature has previously been assigned as the most important mortality factor of the mountain pine beetle (Safranyik 1978, Cole 1981), little is understood about the timing of low temperatures and their effect on the population dynamics of this nondiapausing species. Specific objec-

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Table 1. Site collection information

Site	Host	Elevation, m	Latitude	Site aspect	Basal area, ft^2/acre
			1992–1993		
Logan, UT	LPP	2,043	41° 56′	NE	210
Ranch, ID	LPP	2,061	44° 07′	SW	140
Moose, WY	LPP	2,121	42° 45′	N	100
Galena, ID	LPP	2,348	43° 54′	W	170
			1994–1995		
Ranch, ID	LPP	2,061	44° 07′	SW	170
Beaver, ID	LPP	2,247	43° 59′	W	160
Cedar, UT	PP	2,622	37° 37′	E	270

LPP, logdepole pine (*Pinus contorta* Douglas); PP, ponderosa pine (*P. ponderosa* Laws).

tives of this study were the following: to determine supercooling points of field collected mountain pine beetle larvae and associated microhabitat temperature regimes, to determine the physiological mechanisms that allow cold tolerance in mountain pine beetle larval stages, and to characterize the pattern of low temperature tolerance among geographic regions.

Materials and Methods

Supercooling Point Determination. Mountain pine beetles of different instars were collected from several infested trees at 4 sites during the 1992–1993 generation, and 3 sites during the 1994–1995 generation (Table 1). Individuals were collected from 1 site, Ranch, both years. All larvae were collected above the snow line from either the north or south aspect of the bole. Samples were collected 6 times during each generation from the same trees. On each sample date, larvae were removed live from under the bark, placed in petri dishes with moistened filter paper, and the dishes packed in insulated coolers for transportation to the laboratory. We also recorded the proportion of larvae that were dead at the time of collection. In 1992–1993, larvae were shipped via overnite express mail to Virginia Polytechnic Institute and State University (VPI&SU), Blacksburg, VA, for supercooling point determination. In 1994–1995, all supercooling point analysis was performed at the Forestry Sciences Laboratory in Logan, UT. In 1994–1995, a subsample from 3 sample dates were shipped to VPI&SU for polyol analysis.

The same technique for determining supercooling points was used both years. Using forceps, individual larvae were positioned between 2 copper constantan thermocouples (COC0–003, Omega Technologies, Stamford, CT) resting on the surface of a cold plate (Stir-Kool Cold plate [SK-31], Thermoelectrics Unlimited, Wilmington, DE) connected to a Fisher Isotemp Circulator (13-874-72A, Pittsburgh, PA) that had been cooled to ≈0°C. Eight larvae were analyzed at 1 time. Temperatures among thermocouples were stabilized by placing an insulating box over the cold plate. The temperature of the cold block was reduced by ≈1.5°C per minute. Measurements of top and bottom larval temperature were automatically recorded every second using a data micrologger (21× Datalogger, Campbell Scientific, Logan, UT). A sudden temperature increase (≥0.5°C), which is associated with formation of an ice lattice in insect tissue, indicated the supercooling point of each individual instar. After the supercooling point was determined, each larvae was placed at room temperature and the head capsule width measured to determine instar (Logan et al. 1998). After several hours at room temperature, larvae were gently probed to evoke a response. If larvae are freeze-tolerant, they can survive the freezing process and therefore would respond to probing after warming up. Otherwise, if larvae are dead after the freezing process, we assumed they were freeze-intolerant.

To assess if larvae were alive at temperatures above the measured supercooling point, 16 larvae were cooled (as above) to ≈4°C above the mean supercooling point determined for 16 additional larvae taken from the same tree. The larvae were slowly returned to room temperature and allowed to sit for 24 h. After this period, larval survival was determined.

Supercooling data were analyzed separately for each generation. Larvae from each site were pooled by month of collection. A three-way analysis of variance (ANOVA) with subsampling, blocked by site, was performed using month, aspect on the tree, and instar as the factors (SAS, PROC GLM, SAS Institute 1989). Although larvae were collected from 2 to 3 trees at each site on each sample date, this separation was not maintained in the supercooling analysis and a measure of tree replication at each site was therefore not available. An experimental error term was calculated as the interaction between site and the treatment effects (aspect, instar, and month). This experimental error term included the error caused by subsampling and was used in calculating the F statistic. Interaction terms that included cells with empty data were excluded from the analysis. The Student–Newman–Keuls method was used to compare treatment means.

Temperature Measurements. Beetle flight, host colonization, and brood emergence were monitored at each site. Upon successful host colonization, temperature probes were placed in the phloem of 4 infested trees at each site, 1.4 m above the ground on the north and south side of each tree. Temperature probes consisted of 30-gauge chromega/constantan thermocouple wire (Type E-nickel-chromium and copper-nickel wire, Omega Technologies, Stamford, CT) connected

Table 2. Results of ANOVA used to determine differences in daily maximum, minimum, and average phloem temperatures among sites and between tree bole aspects

Source	Between N and S tree bole aspect		Among sites	
	F (df = 1, 3,037)	P	F (df = 3, 3,037)	P
	1992–1993			
Max	31.84	0.001	5.09	0.002
Min.	0.46	0.498	32.56	0.001
Avg	2.14	0.144	13.85	0.001
	1994–1995			
Max	51.42	0.001	27.05	0.001
Min.	0.83	0.363	119.91	0.001
Avg	4.35	0.037	52.88	0.001

via a 20-gauge chromega/constantan wire to a micrologger (see above). Phloem temperatures were measured every 15 min and averaged and recorded for each hour, beginning just after successful colonization and ending during beetle emergence and flight the following year (≈365 d). Ambient air temperatures were measured 1.4 m above the ground, using a shielded temperature probe placed on the north side of a tree. Hourly temperatures of all 4 trees at a site were first averaged by north and south bole aspect. Daily maximum, minimum, and average temperatures were then computed for a site, and by tree aspect within a site.

Polyol synthesis is often dependent on temperature acclimation that is a complex combination of the duration and intensity of temperatures experienced in the insect's microhabitat. To relate recorded phloem temperatures and polyol synthesis, cooling and heating units were calculated using trapezoid integration above a lower threshold. Because polyol synthesis is typically triggered by temperatures around 0–5°C (Baust and Miller 1970, Storey and Storey 1991), these values were used as lower thresholds in calculating temperature units. Heat (>0 or 5°C) and cold (<0 or 5°C) units were calculated for each day then summed over the life of the generation. Accumulation for all sites and both generations was begun on Julian date 224, an average start date for oviposition at all sites. ANOVA was performed to test for significant differences in accumulated cooling and heating units across sites and months, separated by generation year.

Polyol Analysis. Several techniques were employed to measure hemolymph polyols of mountain pine beetle larvae. Initial high-performance thin-layer chromatgraphy analysis identified relative positions for sorbitol, dulcitol, and glycerol. Gas chromatography (GC) combined with mass spectrometery (MS) were then employed to more accurately measure which compounds were present. Mountain pine beetle larvae were collected from infested lodgepole pine trees at the following 3 times during the life cycle: (1) 23 November 1994, (2) 11 January 1995, and (3) 8 March 1995. In the field, larvae were place in petri dishes with slightly moistened filter paper, packed in coolers with ice, and shipped to VPI&SU via overnite express mail. Upon arrival, larvae were immediately frozen.

Each sample was weighed and placed in microcentrifuge tubes with 20 μl of 70% ethanol: 30% distilled water. A sample consisted of 3–4 small larvae in the November collection, and a large single larva in the January and March collections. The samples were homogenized with a probe tip, and the probe rinsed with 150 μl of the 70% ethanol: 30% distilled water solution. The samples were then centrifuged for 3 min at 5,600 × *g*. A 150-μl aliquot of the extracting solvent was then transferred to a 0.5-ml reaction vial and evaporated to dryness at room temperature using a nitrogen evaporator. Under a stream of nitrogen, 0.1 ml of the derivatizing agent (MTBSTFA + TBDMCS, 99:1 [N-(tert-Butyldimethylsilyl)-N-methyl-trifluoracetamide, with 1% TBDMSCI]; Aldrich, Milwaukee, WI) and 24 ml of acetonitrile were added to the reaction vial, vortexed, and placed on a heat block that had been heated to a temperature of 100°C. After 2 h the samples were removed and allowed to cool. Each sample was again evaporated to dryness at room temperature using a nitrogen evaporator. Hexane was added to attain a volume of 0.15 ml. The samples were then diluted as necessary for GC-MS analysis. Standards for glycerol, sorbitol, and dulcitol were derivatized using the same technique. Results are reported in μg/g of live wet tissue weight.

Results

Daily maximum phloem temperatures on north and south aspects of tree boles were significantly different at all sites both years (Table 2). Daily average temperatures were significantly different between the north and south aspects of tree boles in 1994–1995, although not in 1992–1993. Daily minimum phloem temperatures between tree bole aspects were not significantly different at any site either year. There were significant differences in daily maximum, minimum, and average phloem temperatures (north and south aspect averaged) among all sites, both years (Table 2) (Figs. 1 and 2). Cumulated heating and cooling units were also significantly different among sites and sample months both years (Table 3). Winter phloem temperatures at the Ranch site were colder in 1992–1993 than in 1994–1995. A total of 1,060 cold units was accumulated at the Ranch site in 1992–1993 (Fig. 3B) compared with 847 in 1994–1995 (Fig. 4B), and the average winter supercooling point in 1992–1993 (-34.0°C) was significantly lower than in 1994–1995 (-29.9°C) ($t = -3.466$, df = 52, $P < 0.000$).

As a result of developmental progression with time and sampling error, not all instars were represented in all samples (Tables 4 and 5). For both generations, supercooling points among sample months and sites were significantly different (Table 6). The significant interaction between site and month in 1992–93 implies that the observed differences varied among the sites depending on sample month (Fig. 3C and 4C). In 1992–93, mean comparisons showed that average supercooling points among all sites were different (df = 428, $P < 0.05$). In 1994–95, only the average supercooling point at the Cedar site was significantly dif-

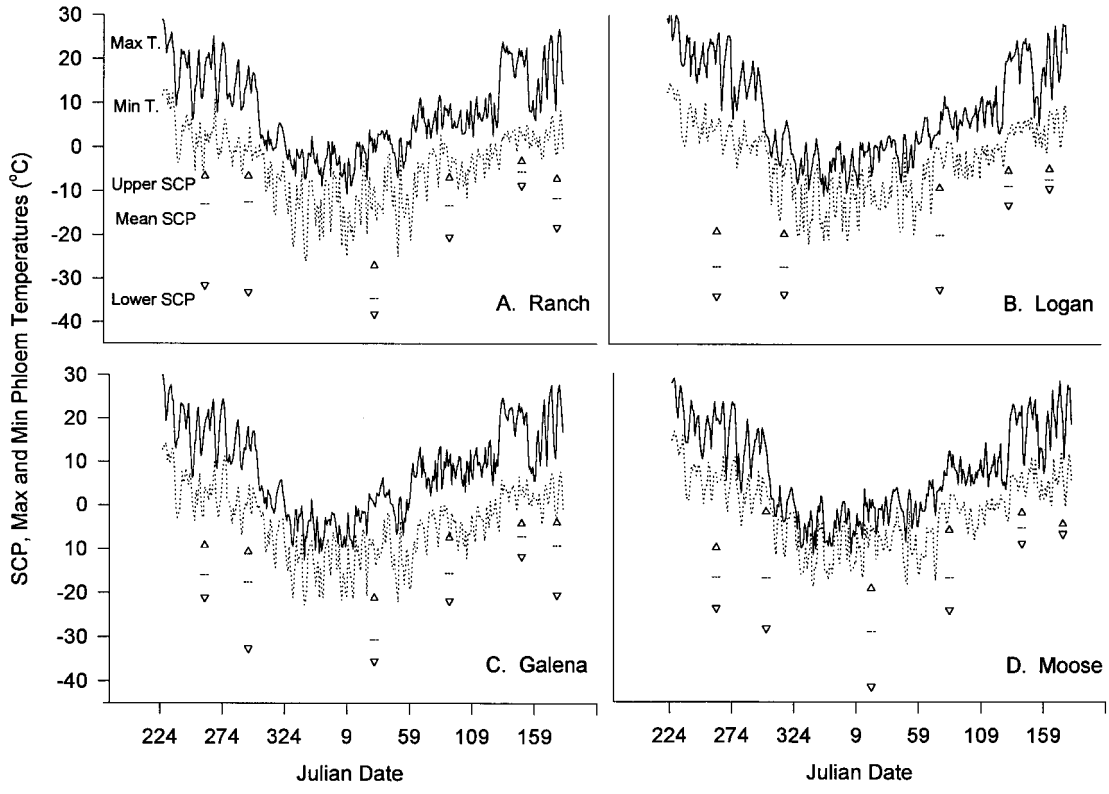

Fig. 1. Maximum and minimum phloem temperatures (T, °C) at 4 sites (A–D) in 1992–1993 with the mean (—) and range (△, ▽) of associated larval supercooling points (SCP) (°C).

ferent from the Ranch and Beaver sites ($df = 517$, $P < 0.05$). No significant differences in supercooling points were observed among instars nor among individuals collected from the north and south bole aspect in either generation (Table 6). Although the proportion of 1st and 2nd instars in winter samples of lodgepole pine hosts were lower than proportions of 3rd and 4th instars at the same time, all instars were observed in samples throughout the winter and into May and June in both hosts.

There was no response from any individual allowed to warm to room temperature after supercooling point determination. Observed survival was 100% for the 16 larvae that were cooled to temperatures just above the supercooling point.

A similar seasonal trend was observed in mean supercooling points in both generations, for all instars, at all sites except Logan. Values were approximately −10 to −20°C in early fall, dropped to −25 to −35°C in January, and increased again in the spring and early summer (Figs. 1 and 2). At all sites except Beaver, the associated daily minimum phloem temperatures, a representation of the microhabitat of larvae, were above the average supercooling point for larvae on each sample date (Figs. 1 and 2). Around Julian day 1 at the Beaver site, minimum phloem temperatures were slightly below the average supercooling point measured for individuals 8 d later. No mortality was observed at this time. Assigning mortality factors in the field was difficult, however, because of the rapid desiccation and decline of larval carcasses. Consequently, in both generations we observed very little mortality during field sampling. During the spring especially, many individual supercooling points were warmer than minimum phloem temperatures experienced at that time (Figs. 1 and 2). Supercooling points in the fall were always lower than −10°C despite the absence of any temperatures below a threshold of 0 or 5°C (Figs. 3B and 4B). In the middle of February (around Julian day 45), small increases in temperature units above 0°C were associated with a raising of the supercooling point. Although cooling units were still being accumulated, the rate of accumulation during this time was decreasing, whereas heat units were constant or only slightly increasing.

The major polyol found in larval mountain pine beetles was glycerol, with negligible concentrations of sorbitol or dulcitol. There were no significant differences found in glycerol levels of larvae collected from the north and south aspects of tree boles. Glycerol levels (mean ± SE) in the 3 samples collected in November (6.8 ± 3.0 μg/g), January (8.6 ± 4.0 μg/g),

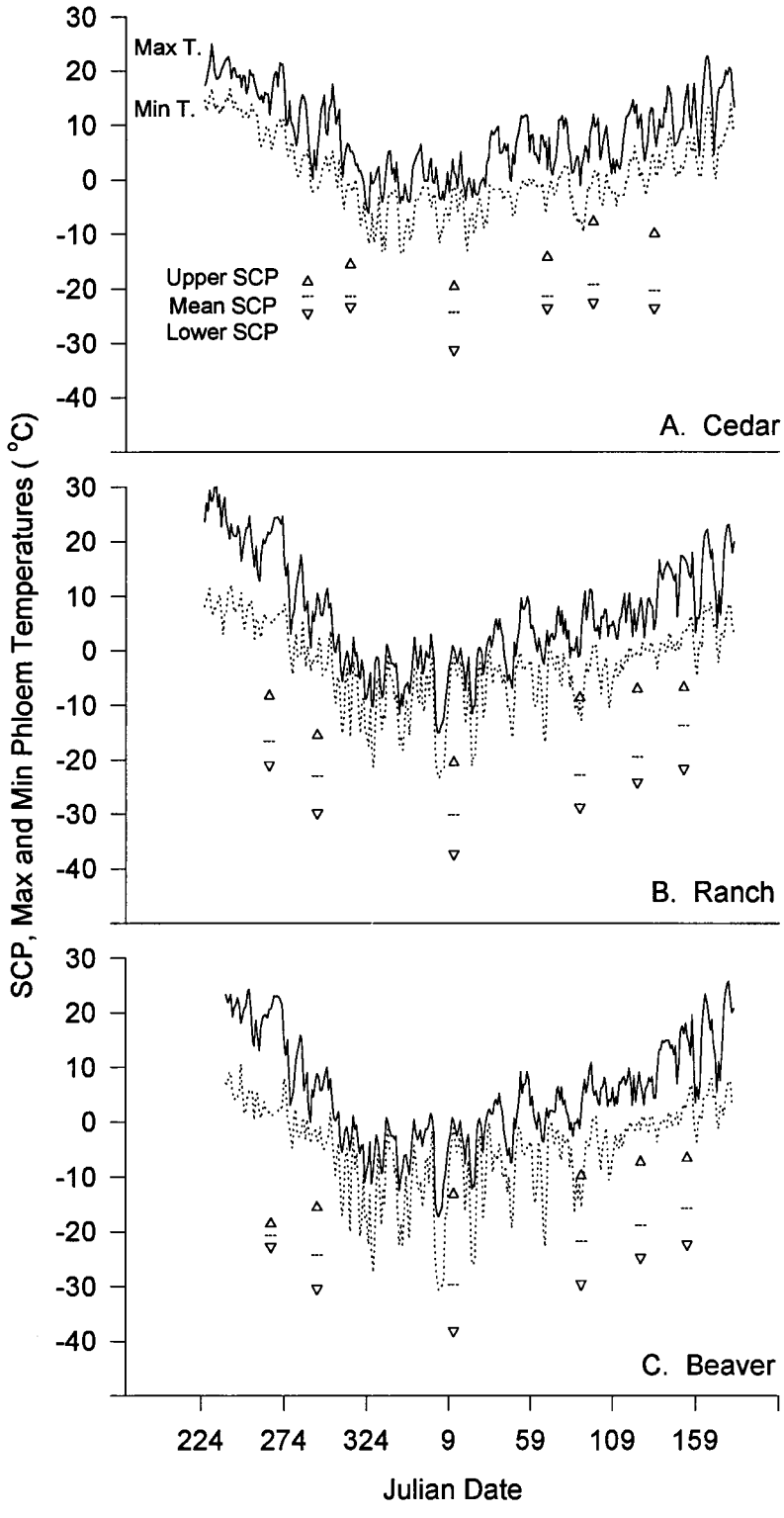

Fig. 2. Maximum and minimum phloem temperatures (T, °C) at 3 sites (A–C) in 1994–1995 with the mean (—) and range (△, ▽) of associated larval supercooling points (SCP) (°C).

Table 3. Results of ANOVA used to determine differences in cooling and heating units among sites and sample months

Source	Among sites		Among months	
	F (df = 3, 457)	P	F (df = 6, 457)	P
	1992–1993			
Cooling units	254.40	0.001	12,609.15	0.001
Heating units	394.61	0.001	6,988.80	0.001
	1994–1995			
Cooling units	3,124.54	0.001	1,517.73	0.001
Heating units	2,319.71	0.001	1,229.38	0.001

and March (10.6 ± 6.4 μg/g) were not significantly different.

Discussion

Larvae that were cooled to temperatures just above the measured supercooling point survived, suggesting that mortality above the measured supercooling point is minimal, and that the supercooling point is a reasonable measure of cold tolerance for this insect. To truly understand mortality above the supercooling point however, long-term effects will need to be assessed. Additional tests wherein larvae are kept at temperatures just above the supercooling point for extended periods will need to be performed to conclusively determine if larvae can indeed survive temperatures above the supercooling point. The lack of response from any individual allowed to warm to room temperature after supercooling point determination suggests that the mountain pine beetle is freeze intolerant and cannot survive tissue freezing. Several other scolytid bark beetles have been found to be freeze intolerant (Ring 1977, Gehrken 1984, Miller and Werner 1987).

All mountain pine beetle instars were found to overwinter, although higher proportions of 3rd and 4th instars were observed during winter months. Although Amman (1973) and Langor (1989) observed higher mortality in smaller larvae (e.g., 1st and 2nd instars) during the winter months, and mountain pine beetle instars have been found to exhibit stage-specific development rates (Bentz et al. 1991), we found no significant differences among instars in their capacity to cold-harden. This is in contrast to a study with the weevil *Hypera punctata* (F.) where a positive correlation between supercooling points and larval body weight was found, suggesting that larger instars have less capacity to cold-harden (Watanabe and Tanaka 1997). Although we did not measure size of larvae within an instar, adult Douglas fir beetles, *Dendroctonus pseudotsugae* Hopkins, and mountain pine beetles emerging from bolts kept at cool, as compared with warm, temperatures were larger (Atkins 1967, Amman and Cole 1983). The Douglas fir beetles reared at the colder temperatures also had proportionately greater lipid content.

Although others have observed differences in cold-hardening capacity between aspects (Gehrken and Zachariassen 1978), supercooling points of mountain pine beetle larvae collected from the north and south aspects of tree boles were not significantly different. Minimum phloem temperatures within the lodgepole pine from which larvae were collected also were not significantly different between the aspects. Southern exposures of tree boles had significantly warmer temperatures, but this had no consistent effect on supercooling capacity of the larvae. Minimum phloem temperatures, which typically occur around sunrise, appear to have more of an effect on larval cold-hardening than daily maximum temperatures. Supercooling points of larvae may indeed be influenced by changing temperatures within a 24-h period, although this effect was not observed in our monthly samples. Previous laboratory research with other insects has shown that short-term exposure to high temperatures may decrease the capacity to supercool (Lee et al. 1987). If this physiological phenomena occurs in the mountain pine beetle, winters with periodic warming trends could result in lower survival rates than winters with consistent cold temperatures, especially so on southern bole aspects.

Daily maximum, minimum, and average phloem temperatures were significantly different among the sites both years (Table 2). Accumulated temperature units, both above and below a 0 and 5°C threshold, were also significantly different among all sites (Table 3). These differences imply that larvae from at least some geographic sites were exposed to different microhabitat temperature regimes. Observed differences in temperature regimes among the sites may be contributing to the differences observed in larval supercooling points among the sites, and could also imply differential mortality. The most striking differences were observed between the Cedar and Beaver and Ranch sites in 1994–1995. In the middle of winter, larval supercooling points at Cedar were as much as 6°C higher than supercooling points at either Beaver or Ranch (Fig. 4C). The Cedar site, which was the only site composed of all ponderosa pine hosts, was located at the highest elevation (2,622 m), lowest latitude (37°C), and had the fewest accumulated cooling units. Cold units accumulated at the Cedar site were only a 3rd of the cold units accumulated in lodgepole pine at the other sites monitored that year (Fig. 4B). The Cedar site also had the highest mean supercooling points at all sample times except in April (Fig. 2A). Because of a high metabolic cost (Danks 1978), maintaining elevated levels of cold-hardiness (low supercooling points) is not advantageous at times when it is not required. Differences observed between the Cedar and Ranch and Beaver sites indicate that mountain pine beetle larvae may have the capacity to metabolize only amounts of cryoprotectants needed, given a particular thermal history. The thresholds required to trigger this metabolism, as well as other cold-hardening mechanisms, is unclear. Although supercooling points of eggs were not analyzed, we observed viable eggs in ponderosa pine at the Cedar site on all sample dates, whereas viable eggs were found no later than October in any samples from lodgepole pine. Observed differences in cold-hardening capacity between the Cedar and both Ranch and Beaver sites

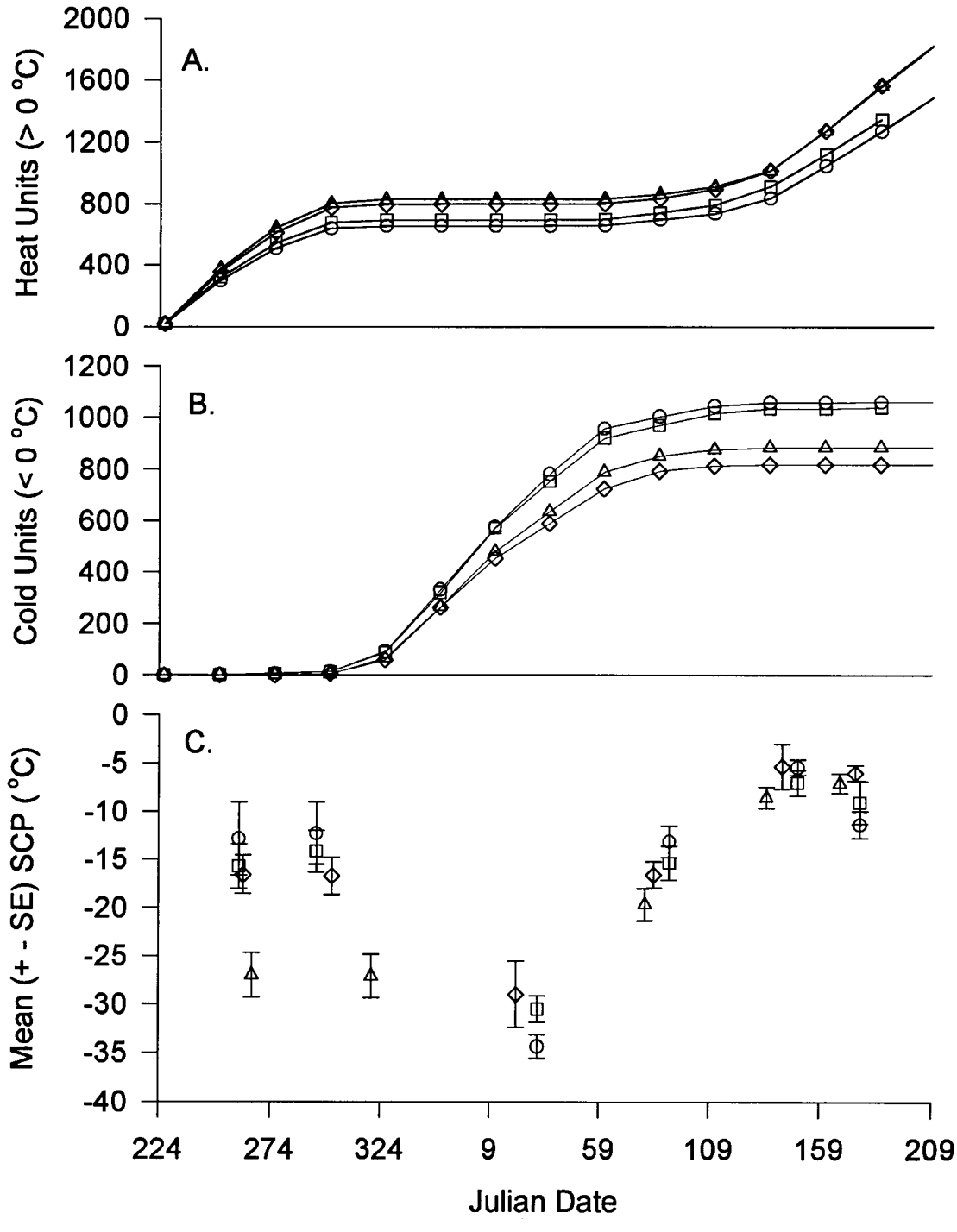

Fig. 3. (A) Accumulated heat units (>0°C) and (B) accumulated cold units (<0°C) at 4 sites in 1992–1993 and (C) associated mean supercooling points (SCP) (°C) and standard errors. Sites: ○, Ranch; □, Galena; △, Logan; ◇, Moose.

may also be influenced, at least in part, by selective pressures on larvae because of tree host nutrition and other host-specific factors which differ between ponderosa and lodgepole pines.

Observed seasonality in the cold-hardening response suggests that mountain pine beetle larvae respond to fluctuating temperatures in the microhabit (Figs. 1 and 2), as described for several scolytid bark

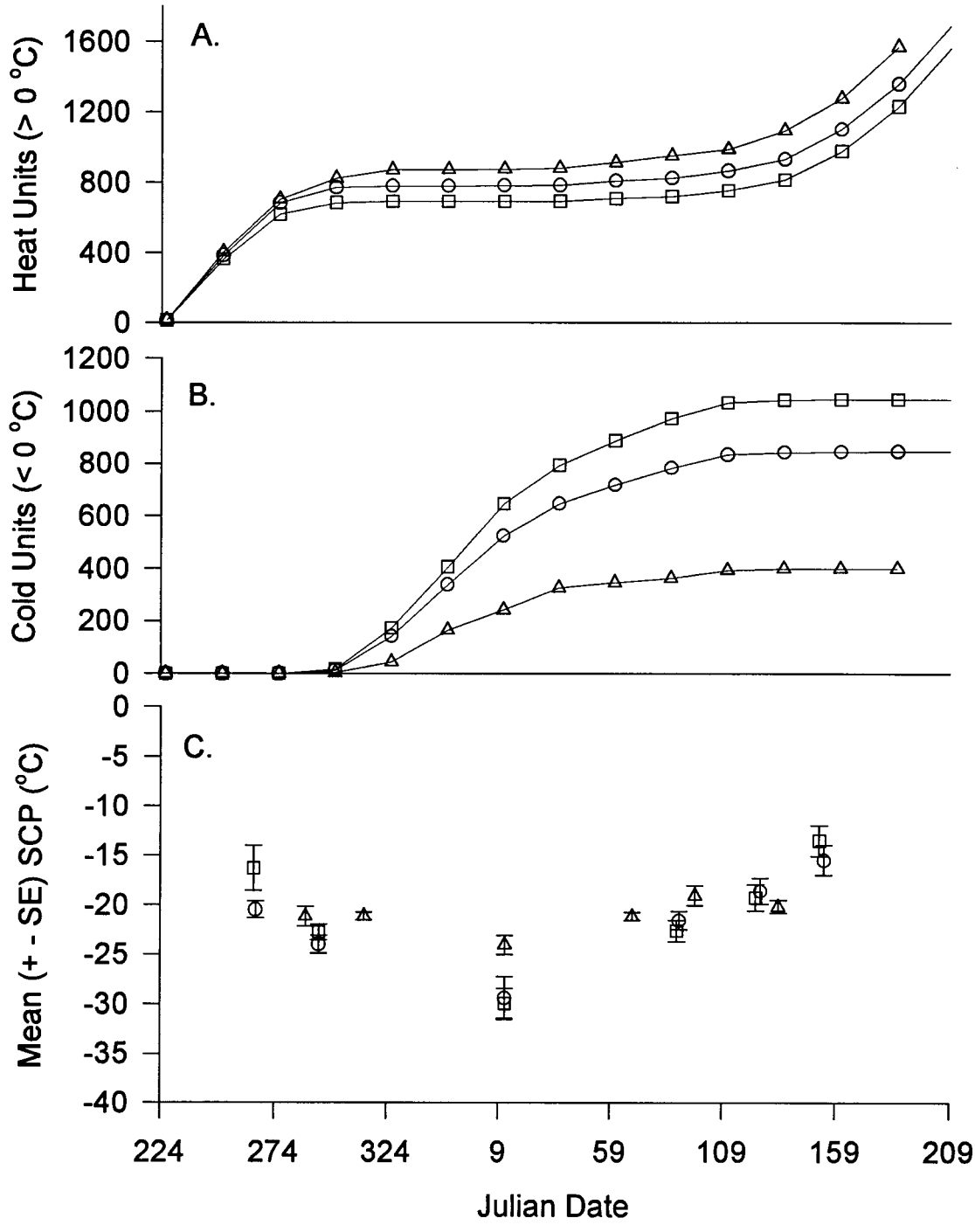

Fig. 4. (A) Accumulated heat units (>0°C) and (B) accumulated cold units (<0°C) at 3 sites in 1994–1995 and (C) associated mean supercooling points (SCP) (°C) and standard errors. Sites: ○, Ranch; □, Beaver; △, Cedar.

beetles (Hansen et al. 1980, Gehrken 1984, Miller and Werner 1987, Luik and Voolma 1990). A year-to-year fluctuation in cold-hardiness at the same site in response to temperature change was also observed. Phloem temperatures of infested trees at the Ranch site that were <100 m apart were significantly colder in 1992–1993 than 1994–1995. Larvae responded with a mean supercooling point which was −5°C colder in 1992–1993. A considerable amount of variation among supercooling points of individual larvae on any day, as

Table 4. 1992–1993 mean supercooling points for each instar, by site and sample date

Site	Instar	Sept.	Nov.	Jan.	March	May	June
Ranch	1st	−31.1	−6.8	N/A	−9.6	N/A	N/A
		(1, 0.0)	(1, 0.0)		(1, 0.0)		
	2nd	−14.5	−12.9	−35.0	−15.2	−3.3	N/A
		(6, 3.5)	(8, 3.0)	(4, 1.5)	(9, 1.3)	(2, 0.0)	
	3rd	−9.7	−12.0	−33.9	−13.2	−6.6	−12.0
		(9, 0.9)	(4, 2.9)	(11, 0.9)	(13, 1.1)	(7, 0.8)	(11, 0.8)
	4th	N/A	−12.6	−35.0	−7.8	−5.1	−10.6
			(3, 0.9)	(3, 0.4)	(3, 0.5)	(15, 0.5)	(10, 1.2)
Galena	1st	−14.5	N/A	−21.2	N/A	−7.2	N/A
		(2, 5.0)		(1, 0.0)		(1, 0.0)	
	2nd	−15.2	−12.7	−30.8	−14.0	−8.2	N/A
		(7, 2.9)	(7, 1.6)	(15, 1.0)	(9, 1.6)	(3, 1.7)	
	3rd	−17.8	−16.1	−31.0	−16.3	−7.3	−9.1
		(3, 0.7)	(15, 1.8)	(12, 1.1)	(16, 1.0)	(5, 1.2)	(9, 1.7)
	4th	N/A	−11.7	−30.2	−14.9	−5.3	−8.9
			(8, 1.8)	(6, 1.6)	(4, 3.5)	(3, 0.6)	(6, 1.4)
Logan	1st	−27.4	N/A	−13.9	N/A	N/A	N/A
		(2, 3.6)		(1, 0.0)			
	2nd	−27.4	−24.4	N/A	−21.8	−10.0	−8.9
		(7, 2.0)	(4, 2.9)		(7, 0.8)	(1, 0.0)	(1, 0.0)
	3rd	−25.9	−27.8	−32.0	−19.5	−8.9	−6.4
		(6, 2.2)	(16, 1.2)	(1, 0.0)	(16, 1.3)	(10, 0.6)	(2, 1.3)
	4th	−28.1	N/A	N/A	−17.7	−6.5	−7.0
		(2, 2.4)			(6, 1.4)	(3, 0.6)	(7, 0.6)
Moose	1st	N/A	−16.2	−29.9	−14.3	N/A	N/A
			(1, 0.0)	(4, 3.7)	(4, 2.9)		
	2nd	−16.2	−15.6	−27.5	−18.0	−2.0	N/A
		(11, 1.4)	(10, 2.4)	(2, 1.2)	(11, 1.2)	(1, 0.0)	
	3rd	−17.1	−15.9	−30.1	−15.6	−9.1	−6.8
		(6, 1.8)	(22, 1.1)	(6, 3.4)	(10, 1.3)	(1, 0.0)	(3, 0.2)
	4th	−17.1	−20.5	−26.5	−17.1	−5.2	−5.4
		(3, 3.2)	(8, 2.4)	(3, 3.5)	(8, 0.8)	(4, 1.1)	(4, 0.5)

Total sample size = 467. N/A indicates no larvae were sampled at that time. Supercooling points are in °C. Numbers in parentheses beneath means indicate values for (sample size, ± standard error).

has been found with other insects (Block 1982, Watanabe and Tanaka 1997), was also observed.

We observed no cold-induced mortality in the small sample of larvae cooled to temperatures just above the measured supercooling point, suggesting a selection for lower supercooling points than the minimum tem-

Table 5. 1994–1995 mean supercooling points for each instar, by site and sample date

Site	Instar	Sept.	Oct.	Nov.	Jan.	March	April	May	June
Ranch	1st	N/A	−21.8	N/A	−22.9	N/A	−22.5	−24.1	−19.7
			(4, 0.6)		(1, 0.0)		(2, 3.1)	(1, 0.0)	(1, 0.0)
	2nd	N/A	−22.9	N/A	−28.4	N/A	−23.3	−19.3	−14.5
			(3, 0.8)		(9, 1.7)		(6, 0.5)	(14, 1.3)	(4, 1.9)
	3rd	−13.1	−20.4	N/A	−28.8	N/A	−22.7	−17.4	−14.3
		(2, 4.6)	(5, 1.3)		(14, 1.4)		(22, 0.8)	(19, 1.3)	(11, 1.7)
	4th	−16.8	−23.5	N/A	−32.2	N/A	−22.6	−20.8	−12.7
		(14, 1.2)	(20, 0.4)		(17, 0.8)		(22, 1.0)	(21, 0.9)	(22, 0.9)
Beaver	1st	N/A	−21.7	N/A	−26.1	N/A	−24.2	N/A	N/A
			(3, 0.7)		(1, 0.0)		(1, 0.0)		
	2nd	N/A	−23.0	N/A	−25.5	N/A	−20.0	−20.0	−20.7
			(7, 0.5)		(2, 12.4)		(9, 0.6)	(5, 1.3)	(2, 1.5)
	3rd	−20.5	−22.4	N/A	−30.1	N/A	−21.6	−18.2	−14.2
		(10, 0.4)	(7, 0.2)		(21, 1.2)		(29, 0.6)	(25, 1.1)	(10, 1.8)
	4th	N/A	−25.2	N/A	−29.1	N/A	−22.3	−18.6	−15.5
			(21, 0.7)		(15, 1.7)		(15, 1.0)	(26, 0.9)	(27, 0.8)
Cedar	1st	N/A	−22.4	−20.8	−23.2	−21.4	−19.7	−20.4	N/A
			(3, 0.9)	(11, 0.4)	(4, 1.60)	(38, 0.3)	(14, 1.2)	(11, 0.6)	
	2nd	N/A	−20.2	−20.8	−23.6	−20.6	−18.5	−20.1	N/A
			(4, 0.6)	(14, 0.5)	(21, 0.6)	(23, 0.5)	(30, 0.8)	(44, 0.3)	
	3rd	N/A	−21.1	−21.6	−22.9	−21.5	−19.6	−19.9	N/A
			(2, 0.2)	(12, 0.4)	(2, 0.4)	(6, 0.6)	(8, 0.4)	(6, 0.5)	
	4th	N/A	N/A	−21.5	−26.9	−21.7	−19.6	−21.4	N/A
				(9, 0.4)	(5, 1.9)	(4, 0.4)	(7, 0.5)	(1, 0.0)	

Total sample size = 750. N/N indicates no larvae were sampled at that time. Supercooling points are in °C. Numbers in parentheses beneath means indicate values for (sample size, ± standard error).

Table 6. Results of NNOVN used to determine factor effects on supercooling points in 1992–1993 and 1994–1995

Source	1992–1993			1994–1995		
	F	df	*P*	*F*	df	*P*
Site	2.80	3, 107	0.05	3.99	2, 89	0.05
Month	35.51	5, 107	0.001	50.87	7, 89	0.001
Aspect	0.18	1, 107	0.488	1.84	1, 89	0.098
Instar	0.11	3, 107	0.839	0.94	3, 89	0.172
Site × Month	3.36	15, 107	0.01	—		

— interaction not analyzed because of missing cells.

perature occurring under the bark. However, additional research is needed in this area to ascertain if larvae can withstand long periods at these temperatures. Although the average supercooling point on a particular day was typically below the minimum phloem temperature, many individual supercooling points were often warmer than the minimum phloem temperature, especially in the spring, suggesting that mortality could be occurring (Figs. 1 and 2). Additionally, supercooling points in the spring were often higher than those in the fall at the same site. This could be due in part to the fact that maximum phloem temperatures in the spring were generally above lower developmental thresholds for 3rd and 4th instars (see Bentz et al. 1991), perhaps triggering a feeding response and concomitant decreased cold-hardening in the spring. Spring, therefore, may be the most susceptible time for cold-induced mortality in this insect.

It is well documented that individuals experience greater cold-hardening capabilities when exposed to cold temperatures (Baust and Miller 1970). However, the temperature at which acclimation for cold-hardening begins appears to be species-specific and mediated by climate. Acclimation for some insects is more effective at 3–5°C (Baust and Miller 1970), whereas in others polyol production was enhanced at subzero temperatures (−5 to −10°C) (Young and Block 1980). An increase in the rate of temperature acclimation below 0°C was correlated with a decrease in supercooling points, although we are unable to ascertain from our field study the appropriate threshold trigger for accumulation of cryoprotectants. Insects also respond to changes in the duration and temperature of daily cycles (Beck 1991). Observed maximum cold-hardiness occurred after the peak of daily cold units yet before the maximum accumulation of cold units (Figs. 3 and 4). Cold-hardening then decreased with a decrease in the rate of accumulation of cold units (below 0°C), whereas heat units remained relatively constant during this period. The rate of change in daily thermal units most likely play an important role in mountain pine beetle cold-hardening, and a better understanding of time lags and the decay effects of temperature history on cold-hardiness is needed.

Considering the diversity and complexity of the cold-hardening process, it is unlikely that any single environmental cue is responsible for the complex biochemical strategies that occur (Baust 1982). However, for bark beetles, which spend the majority of time under the bark of host trees, temperature is the most reliable environmental cue for cold-hardening. Glycerol was found to be the predominant cryoprotectant in larval mountain pine beetle from central Idaho, whereas levels of sorbitol and dulcitol were negligible. Results on seasonality of this compound in larval mountain pine beetle and its direct relation to temperature are inconclusive given our results. However, glycerol content in adult and larval spruce beetles, *Dendroctonus rufipennis* Kirby, from Alaska (Miller and Werner 1987) and 4th-instar mountain pine beetle (Sømme 1964) from California appear to be regulated by temperature, as does the ethylene glycol concentration in *Ips acuminatus* Gyllenhal larvae (Gehrken 1984). Other mechanisms most likely also play a role, including other cryoprotectants and thermal hysterisis provided by high molecular weight particles such as proteins and glycoproteins.

We have investigated only 1 of the processes that may be responsible for cold-hardening and associated mortality in mountain pine beetle larval stages: freezing and the production of glycerol which plays a role in this process. Thermal history experienced by larvae beneath the bark of pine trees, as well as the daily change in thermal cycles are both important in the cold-hardening process for this insect. Larval instars were found to be freeze intolerant, with seasonal changes in supercooling capacity associated with phloem temperatures. All instars were found to overwinter in both lodgepole and ponderosa pine. Regional populations of mountain pine beetles can differ significantly in cold-hardiness, apparently because of local weather patterns. An important question that remains to be answered is if populations of mountain pine beetle at southern latitudes have adapted to the local climate, or if they are capable of surviving temperature regimes experienced by populations at more northern latitudes and colder sites. The amount of cold-hardening that occurs in mountain pine beetle populations appears to be dictated by temperature regimes at each particular site. It is unclear, however, if all populations in the intermountain region have the same capacity to cold-harden, and only do so if temperatures dictate it. This sensitivity of mountain pine beetles to local microclimate emphasizes their importance as biological indicators of regional climate change. Observed variation among individual supercooling points within an instar, flexibility in overwintering life stage, and the ability of larvae to respond seasonally to changing temperature conditions within the microhabitat represent important patterns of phenotypic plasticity which contribute to the success of this important outbreak species.

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