Temporal and Species Variation in Cold Hardiness Among Invasive Rhizophagous Weevils (Coleoptera: Curculionidae) in a Northern Hardwood Forest

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ABSTRACT A complex of invasive rhizophagous weevils has established in North American northern hardwood forests. Little is known regarding the overwintering biology of these weevils and thus how cold hardiness and weather affect population dynamics. Field data from winter 2006–2007 showed a decline in larval abundance but an increase in larval weight of the surviving individuals. During winter 2008–2009, we examined several aspects of overwintering biology of *Phyllobius oblongus* (L.), *Polydrusus sericeus* (Schaller), and *Barypeithes pellucidus* (Boheman). Larvae were collected in the Upper Peninsula of Michigan and transported in bulk field soil to the University of Notre Dame, South Bend, IN, for laboratory assays. Supercooling points (SCPs) of *P. oblongus* and *B. pellucidus* larvae not in contact with ice were highest in October and lowest in March, but SCPs of larvae that were in contact with ice did not differ among sampling dates. Larval cold tolerance increased over the winter, with 11% of *P. oblongus* and 40% of *P. sericeus* surviving 24 h at −12°C. Few *B. pellucidus* or *P. oblongus* survived 30 d at temperatures of −3.3°C or lower. Body water content increased from January to March in *P. sericeus* and *B. pellucidus*. Larval hemolymph from all species showed some thermal hysteresis and hexagonal crystal formation, indicative of low levels of antifreeze proteins or glycolipids. These subterranean-dwelling larvae are buffered from ambient winter temperatures, but our data also suggest low levels of freeze avoidance. We discuss how these overwintering strategies may affect adult population dynamics.

KEY WORDS *Barypeithes pellucidus*, lower lethal temperature, *Phyllobius oblongus*, *Polydrusus sericeus*, supercooling point

Insects that overwinter in environments subject to subzero temperatures must adapt by becoming either freeze tolerant (able to survive freezing of their body fluids) or freeze avoiding (do not freeze). Both strategies involve complex physiological processes and adaptations, so comprehensive understanding of their cold tolerance, acclimation, or both requires the investigation of multiple variables to determine the mechanisms of subzero tolerance (Zachariassen 1985, Duman et al. 1991, Clark and Worland 2008, Doucet et al. 2009, Denlinger and Lee 2010).

Knowledge of arthropod overwintering physiology can facilitate more effective pest management and assist in management programs. For example, in temperate latitudes, winter survival rates can have dramatic impacts on insect population dynamics (Bale 2002, 2010; Régniere and Bentz 2007; Cárcamo et al. 2009). Knowledge of invasive species' overwintering physiology can facilitate accurate model construction and prediction of potential infestation areas (Kuhar et al. 2000, McCornack et al. 2005).

A complex of exotic weevils (Coleoptera: Curculionidae) has become established in the northern hardwood forests of North America (Coyle et al. 2008). The most common species are *Phyllobius oblongus* (L.), *Polydrusus sericeus* (Schaller), and *Barypeithes pellucidus* (Boheman) (Pinski et al. 2005a). These weevils were introduced to North America from Europe over a century ago and have been established in hardwood forests of the Great Lakes Region for at least 30 yr (Witter and Fields 1977, Coyle et al. 2008). Adults are folivorous, feeding on several woody plant hosts in the forest understory. Adult *P. oblongus* and *P. sericeus* tend to prefer ironwood [*Ostrya virginiana* (Mill.) K. Koch], but basswood (*Tilia Americana* L.), sugar maple (*Acer saccharum* Marsh.), yellow birch (*Betula alleghaniensis* Britton), and raspberry (*Rubus* spp.) are also suitable hosts (Pinski et al. 2005b, Coyle et al. 2010), whereas *B. pellucidus* feeds on several woody host plants, including raspberries and grapes (*Vitis* spp.) (Coyle et al. 2008). Spatial and

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Table 1. Mean monthly maximum and minimum air temperature, mean and absolute minimum soil temperature recorded, and mean snow cover depth from October 2008 through April 2009 at Taylor Lake near Watersmeet, MI

Yr	Mo	Air temp (°C)[a]		Soil temp (°C)[b]				Snow depth (cm)[a]
				5-cm depth		15-cm depth		
		Max	Min.	Mean min.	Absolute min.	Mean min.	Absolute min.	
2008	Oct.	12.2	−2.3	6.3	1.1	7.3	1.1	5.1
2008	Nov.	5.7	3.8	1.8	−0.6	1.9	−0.6	34.3
2008	Dec.	−12.9	−18.9	−0.5	−0.6	−0.3	−0.6	71.6
2009	Jan.	−13.4	−20.6	−0.6	−1.1	−0.6	−1.1	48.5
2009	Feb.	−10.3	−17.5	−0.6	−0.6	−0.6	−0.6	48.0
2009	Mar.	−6.6	−15.3	−0.6	−0.6	−0.6	−0.6	4.3
2009	April	−2.4	−11.2	1.1	−0.6	0.5	−0.6	55.4

[a] Air temperature and snow depth acquired from NOAA station 20-8680-1.
[b] Soil temperature acquired from NOAA station 20-1486-2 (http://www7.ncdc.noaa.gov/IPS/coop/coop.html).

temporal abundance of the rhizophagous larvae can vary greatly, and densities of ≈1,000 larvae m^{-2} have been recorded previously (Coyle et al. 2008). Larval feeding by this complex may account for up to 15% of fine root turnover (Coyle et al. 2008). Larvae overwinter in the soil, but there are no published reports detailing their overwintering biology or thermal tolerance limits.

Previous work by Pinski et al. (2005b) estimated that <18% of artificially introduced *P. sericeus* eggs emerged as adults the following year, suggesting that overwintering may be a major source of mortality for these weevil species in the field. Our objective was to investigate aspects of the overwintering biology of these invasive weevils. We first measured larval abundance and body weight under natural conditions. We then evaluated larval lower lethal temperatures, whether they are freeze tolerant or freeze avoiding, and potential adaptations for cold tolerance. Specifically, we compared supercooling points (SCPs), cold tolerance, antifreeze, body weight, and water content among species, and we evaluated temporal variation throughout the winter within species.

Materials and Methods

Study Site. All larvae were sampled near Taylor Lake in the Ottawa National Forest (Gogebic Co., MI) in a northern hardwood forest (46° 14.4′ N, 89° 2.9′ W). The dominant tree species in this area is sugar maple, with basswood and ironwood also present (Pinski et al. 2005a). Raspberry, leatherwood (*Dirca palustris* L.), and gooseberry (*Ribes* spp.) are common understory shrubs. The soil is sandy loam to a depth of 30 cm, below which it becomes very clayey and rocky. Soil temperature was recorded at 5 and 15 cm below ground level by using HOBO data loggers (Onset Computer Corp., Bourne, MA), and air temperatures and snowfall amounts were obtained from a National Oceanic and Atmospheric Administration (NOAA)-operated weather station on the Lac Vieux Desert tribal community center (Table 1), located ≈15 km from the study site.

Larval Abundance and Weight. Insect mortality is typically highest during the larval period (Carey 2001). To determine whether this was the case for these weevils, we sampled larvae five times between 26 October 2006 and 19 April 2007. Fifteen soil cores (5.08 cm in diameter) were collected on each sampling date. Each core was divided into 0–15- and 15–30-cm-depth samples, and each sample was placed in a Ziploc bag and transported to the University of Wisconsin–Madison laboratory in a cooler. Larvae were hand-picked from the samples. To determine larval weight, a separate group of larvae was collected on each date by digging three to five soil pits to a 30-cm depth and bringing bulk soil to the laboratory in coolers. Larvae (n = 23–51 per sample date) were hand-picked from the soil, dried at 60°C for 48 h, and then weighed to the nearest 0.001 mg. Larvae were not identified to species. Because we were interested in the response of the larval community as a whole, and the larvae all seem to inhabit a similar ecological niche, our interpretation of the data would probably not be altered by identifying the larvae to species.

Insects for Bioassays. Larvae were collected between 1000 and 1200 hours on 20 October 2008, 20 January 2009, and 31 March 2009. Larvae were collected by filling 55-liter coolers with bulk soil from the top 30 cm of the forest floor. Large roots and rocks were removed when possible. Care was taken to minimize the amount of time during which soil was exposed to ambient temperature, and soil was not collected during precipitation events. Coolers filled with soil were transported in an unheated, covered truck bed to the University of Notre Dame in South Bend, IN, within 12 h of collection. Larvae were removed from the soil within 24 h of collection by hand sorting and held in a petri dish on ice until processed.

SCPs. SCPs identify the temperature at which an insect freezes. Before SCP determination the live weight of each larva was measured to the nearest 0.001 mg. SCP was measured by attaching a thermocouple wire to the outside of the body of a larva by using petroleum jelly. The larva was then placed into a 1.5-ml microcentrifuge tube, and the thermocouple wire was held in contact with the larva by wedging a piece of foam into the top of the tube. The tube was lowered into one of 16 glass tubes suspended in an ethanol bath, with foam placed on top of the glass tubes to provide additional insulation. The ethanol bath temperature was maintained at 0°C until the

insects equilibrated with it, and the bath was then cooled at a rate of 0.2°C min^{-1} until larval freezing. Freezing was indicated by an increase in temperature resulting from an exotherm (latent heat of crystallization) as recorded with Isothermex version 3.2 computer software (Columbus Instruments, Columbus, OH). The lowest temperature before the exotherm was recorded as the SCP (Lee and Denlinger 1991). Once all insects in a run (11–15 larvae per run, $n = 2$ runs in October and $n = 3$ runs in January and March) had frozen, the cooling was halted and the insects were warmed to 0°C. Insects were removed from the tubes, allowed to warm to 4°C by 3 h postwarming, and assessed for survivorship by gentle prodding. Any movement from a larva was considered to indicate evidence of survival, suggesting that the larva might be freeze tolerant.

SCP determination also was conducted with larvae in contact with ice to determine the possible effect of inoculative freezing across the cuticle, caused by external contact with ice. Larvae were weighed, attached to thermocouple wires, and placed into 1.5-ml microcentrifuge tubes as described above. Microcentrifuge tubes had 25 μl of double-distilled water frozen in the bottom and were kept on ice until being placed into the cooling bath. Initial temperature and cooling rate of the bath, SCP determination (6–12 larvae per run, $n = 2$ runs in October and $n = 4$ runs in January and March), and survivorship assessment were the same as for dry SCPs.

Cold Survivorship. We tested larval survival over short periods (24 h) of exposure at different temperatures on each sampling period, and over a long exposure period (30 d) in larvae collected in early February 2009. This served two purposes: 1) to identify whether the larvae could survive temperatures below their SCPs, indicating freeze tolerance if they could and suggesting freeze avoidance if they could not; and 2) to indicate approximate lower lethal temperatures. Larvae were collected and weighed as described above and held in a petri dish on ice at 0°C before use in assays. Larvae ($n = 72$–75) were kept at 0°C, −3.3°C, −7.9°C (all sampling periods), −12°C, and −18°C (January and March only) on ice for 24 h in plastic containers in an ethanol (EtOH) bath. Larvae held at temperatures below 0°C were cooled at a rate of 0.2°C min^{-1} until they had reached the desired temperature, at which point they were held constant for 24 h. Survivorship was assessed by gentle prodding after larvae warmed to 0°C and then to 4°C by 3 h postwarming. Larvae were boiled (to denature proteins, prevent decomposition, and preserve appearance; Huber 1998), identified to species, dried for 48 h at 70°C, and reweighed.

To determine survivorship after exposure to low temperatures for longer periods, a separate cohort of larvae was collected on 3 February 2009 as described previously, and transported to the University of Wisconsin–Madison forest entomology laboratory. Larvae were divided into six groups ($n = 69$–77) and placed into Erlenmeyer flasks containing ice or soil on the bottom. Two flasks, one of ice and one of soil, were kept at either 0, −3.3, or −7.9°C for 30 d. Larval cooling rate for temperatures below 0°C, postassay survivorship assessment, and insect preservation were the same as for the 24-h assays.

Live Body Mass and Water Content. To avoid freezing in winter some insects dehydrate, which concentrates antifreezes and decreases the volume of freezable water (Holmstrup et al. 2002, Elnitsky et al. 2008, Sformo et al. 2010). Consequently, we determined body water content in the larval weevils. In October, larvae ($n = 76$) were weighed live to the nearest 0.001 mg, dried for 48 h at 70°C, and reweighed. Larvae from the January ($n = 97$) and March ($n = 125$) collection dates were weighed live to the nearest 0.001 mg, dipped in boiling water, stored in 70% EtOH for 1 wk, identified to species, dried for 48 h at 70°C, and reweighed. Percentage of body water was calculated by dividing the dry weight by the live weight of each insect. All larvae used in SCP measurements also were used for determination of live weight and body water content. Species determination of the larvae collected in October was impossible due to inadvertent drying before identification.

Thermal Hysteresis and Crystal Morphology. Antifreeze proteins (AFPs) are common adaptations of insects and are especially common in beetles (Duman 2001, Duman et al. 2010). Antifreeze proteins lower the nonequilibrium freezing point of water by a noncolligative mechanism, but they have only minimal effects on the melting point. Consequently, they produce a difference between the freezing point and the melting points, termed thermal hysteresis, that indicates their presence in a sample. At very low AFP concentrations where they do not produce measurable thermal hysteresis, AFPs affect ice crystal morphology and inhibit recrystallization of ice; consequently, these parameters (ice crystal morphology, recrystallization inhibition, or both) also can be used to assess the presence of AFPs.

Thermal hysteresis was determined in larvae collected in October after larvae were acclimated at 0°C for 24 h. Two groups of larvae were sampled in October: *P. sericeus* ($n = 10$), and a combination of *P. oblongus* and *B. pellucidus* (total $n = 30$). We sampled only *P. sericeus* ($n = 2$) in January. *P. oblongus* and *B. pellucidus* larvae were indistinguishable based on size alone, but we were able to determine *P. sericeus* based on their larger size. Larvae were destroyed by sampling and could not be identified afterwards, hence the pooled sample of multiple species in October. Larvae were blotted dry using a Kimwipe (Kimberly-Clark, Dallas, TX), and a pulled glass micropipette was used to pierce the cuticle and remove a small ($\leq$0.25 μl) amount of hemolymph. In all groups, hemolymph was pooled before examination. A micrometer syringe then delivered between 25 and 100 nl of hemolymph into heavy mineral oil located in the sample well of a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) (Chakrabartty and Hew 1991). The sample was frozen by cooling to −40°C and then slowly warmed until a single ice crystal formed, and then slowly cooled. The melting and freezing points of

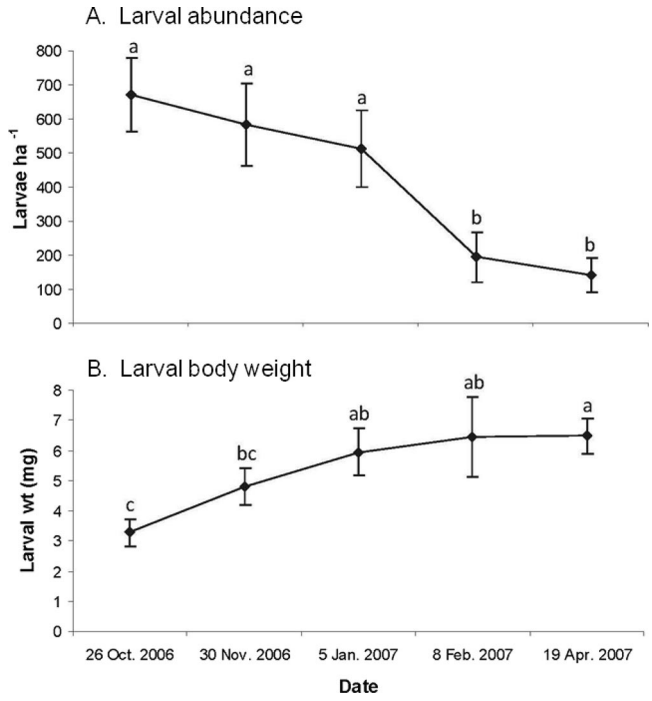

Fig. 1. Larval abundance (A) and body weight (B) of a species complex of invasive rhizophagous weevil larvae collected during winter 2006–2007 near Taylor Lake, Gogebic Co., MI. Means sharing a letter are not significantly different at $\alpha = 0.05$. Monthly mean air and soil temperatures during this time period were comparable to, and not colder than, those listed in Table 1.

the ice crystal, and its growth morphology, were determined at 300× magnification. Crystal growth morphology is a sensitive assay for the presence of AFPs (Griffith and Yaish 2004). At very low concentrations, AFPs do not produce measurable thermal hysteresis but still perturb crystal growth, typically resulting in hexagonal growth morphology. In the absence of AFPs, crystals grow as round disks.

Statistical Analyses. Larval abundance and weight were analyzed using a repeated measures analysis of variance (ANOVA) (PROC MIXED, version 8.1, SAS Institute, Cary, NC) with sampling date as a dependant variable. Means were compared using the least-square means procedure.

To achieve normality, SCP data were log transformed. The absolute values of those data were analyzed using a three-way ANOVA (PROC GLM, version 8.1, SAS Institute) with weevil species, sampling date, and SCP type (larvae with or without contact with ice) as dependant variables, and their interactions. Means were compared using the Tukey test.

The proportions of surviving *B. pellucidus*, *P. oblongus*, and *P. sericeus* larvae in the 24-h trials were analyzed among temperatures within a species by using Barnard's exact test (Barnard 1945) (R version 2.11.2010-05-06, Free Software Foundation, Boston, MA), which is more powerful than the commonly used Fisher exact test (Mehta and Senchaudhuri 2003). In the 30-d trial, only *B. pellucidus* and *P. oblongus* were obtained in sufficient numbers for analysis. Preliminary analyses of the 30-d trial of cold survivorship indicated no difference between soil and ice as media, so these data were pooled for the final analysis (PROC ANOVA, version 8.1, SAS Institute). Data were analyzed by species, and all means were separated using the least significant difference (LSD) test at an $\alpha = 0.05$ significance level.

Body weight and body water content correlate with SCP in some insect species (Lundheim and Zachariassen 1993, Zachariassen et al. 2010); therefore, linear regressions (PROC REG, version 8.1, SAS Institute) were conducted to examine relationships between larval live weight and body water content, and larval live weight and SCP. Data also were analyzed as a two-way ANOVA (PROC GLM, version 8.1, SAS Institute) to examine the effects of species and collection date on larval live weight and body water content. Means were compared using the Tukey test. Because larvae from the October sampling period were not identified to species, these comparisons were only made between the January and March sampling dates.

Results

Larval Abundance and Weight Patterns. Larval abundance declined significantly over the 2006–2007 winter ($F = 6.96$; df = 4, 116; $P < 0.0001$; Fig. 1A). Concurrently, mean weight of surviving larvae from this field site increased ($F = 9.15$; df = 4, 174; $P < 0.0001$; Fig. 1B).

Table 2. Statistical values for SCPs of rhizophagous weevil larvae collected near Taylor Lake, Gogebic Co., MI, in October 2008, January 2009, and March 2009

Source	*F*	df	*P*
Species	4.02	2, 193	0.0197
Date	6.01	2, 193	0.0030
Species × date	3.81	4, 193	0.0054
Type	93.20	1, 193	<0.0001
Species × type	3.47	2, 193	0.0333
Date × type	6.64	2, 193	0.0017
Species × date × type	2.81	4, 193	0.0058

Species tested were *P. oblongus*, *P. sericeus*, and *B. pellucidus;* dates were October, January, or April, and type was either SCP conducted on dry larvae or larvae in contact with ice.

SCPs. SCPs varied among species, sampling dates, SCP type (larvae in contact with ice, or not, when the SCP is determined), and their interactions (Table 2). SCPs of *B. pellucidus* larvae not in contact with ice were higher in October than in January or April (Fig. 2A). *P. oblongus* larvae not in contact with ice exhibited decreasing SCPs throughout the winter (Fig. 2A). *P. sericeus* larval SCPs did not differ over the winter for larvae not in contact with ice.

SCPs of larvae in contact with ice did not differ over time in any species tested, or among species. However the SCPs determined with larvae in contact with ice (the more relevant SCP for these soil-dwelling larvae) were significantly higher than those taken with the larvae not in contact with ice, indicating that larvae were susceptible to inoculation from ice in their environment. The lowest individual SCP recorded was −18.4°C, from a *P. oblongus* larva not in contact with ice in March. No larvae survived after being frozen in any of the SCP tests.

Cold Survivorship. *B. pellucidus* survival was highest at 0°C in each month tested, although in March it was not significantly different from that at −3.5°C (Fig. 3). Survival at −3.5°C was nearly twice as high in March as it was in October or January. No larvae survived at −7.9°C in October, whereas some did in January and March. No *B. pellucidus* survived at −12 or −18°C. Survivorship of *P. oblongus* in October was generally lower than in January or March at each temperature tested (Fig. 3). Few larvae survived below −3.5°C, and none survived at −18°C. Most *P. sericeus* larvae survived at 0°C. Survival in October and March was generally lower than in January (Fig. 3). Extremely small sample sizes in March ($n = 3$ at 0°C, $n = 1$ at both −3.5 and −7.9°C) most likely led to the lack of significance among *P. sericeus* survival at these temperatures.

Among larvae collected on 3 February 2008, both *B. pellucidus* ($F = 18.60$; df = 2, 3; $P = 0.0204$) and *P. oblongus* ($F = 30.77$; df = 2, 5; $P = 0.0100$) survival was higher at 0°C than −3.3 and −7.9°C after 30 d (Fig. 4). No larvae survived 30 d at −7.9°C.

Live Body Weight and Water Content. Larval weight of *B. pellucidus* (all $P > 0.1955$) and *P. sericeus* (all $P > 0.0861$) did not correlate with SCP under any conditions. However, larval weight of *P. oblongus* was positively correlated with SCP in January, both for larvae that were ($F = 17.43$; df = 1, 3; $P = 0.0250$; $R^2 = 0.2614$) and were not ($F = 11.35$; df = 1, 15; $P = 0.0042$; $R^2 = 0.3927$) in contact with ice.

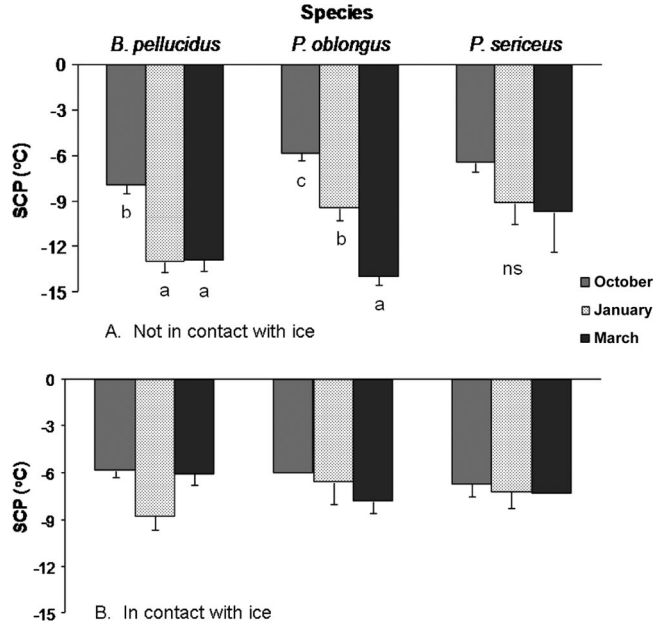

Fig. 2. Larval SCPs of larvae without (A) and with (B) contact with ice in three invasive rhizophagous weevil species during October 2008, January 2009, and March 2009. Within a species, months sharing a letter are not significantly different at $P = 0.05$.

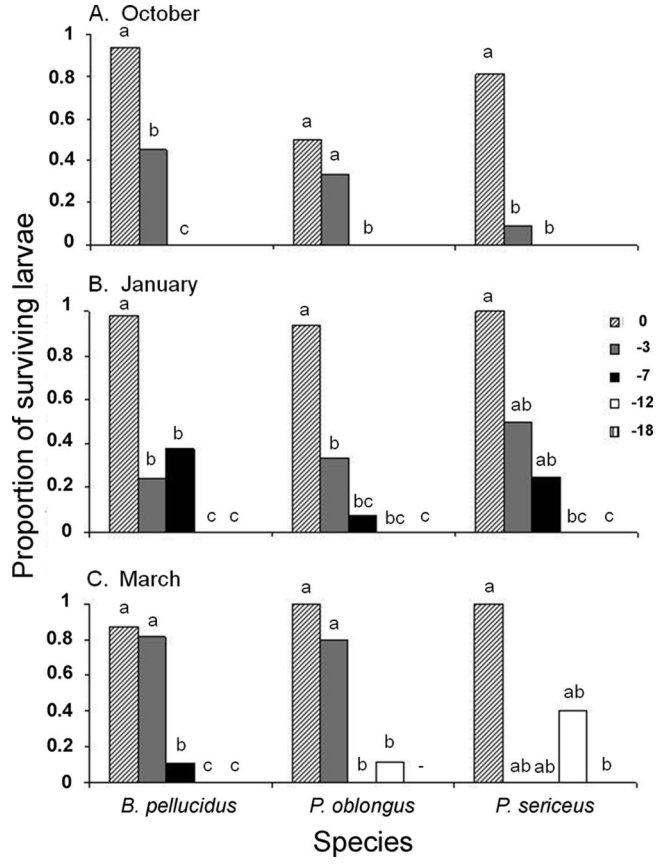

Fig. 3. Larval survival of three invasive rhizophagous weevil species after a 24-h cold treatment collected in October (A), January (B), and March (C). Within a species, temperatures sharing a letter are not significantly different at $P = 0.05$.

In March *P. sericeus* larvae were the largest (13.5 ± 1.1 mg), followed by *P. oblongus* (8.5 ± 0.3 mg) and *B. pellucidus* (3.7 ± 0.1 mg) ($F = 3.23$; df = 2, 214; $P = 0.0416$). Larval live weight of *P. sericeus* and *P. oblongus*, but not *B. pellucidus*, increased between January and March (Fig. 5A). Percentage of body water in late March ranged from 75.3 to 81.0% and was significantly lower in *B. pellucidus* than in *P. sericeus* and *P. oblongus* ($F = 6.16$; df = 2, 214; $P = 0.0025$). Percentage of body water of *B. pellucidus* and *P. sericeus* increased between January and March, but *P. oblongus* body water remained stable (Fig. 5B).

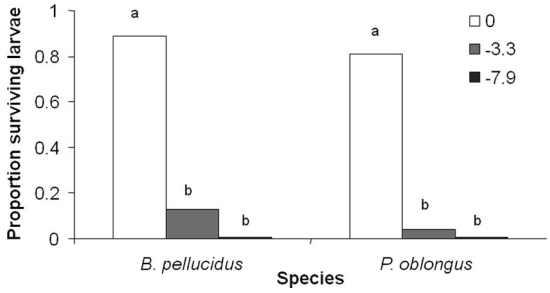

Fig. 4. Larval survival of two invasive rhizophagous weevil species after a 30-d cold treatment. Within a species, temperatures sharing a letter are not significantly different at $P = 0.05$.

There was a strong positive relationship between larval live weight and dry weight, and between larval live weight and body water content, on every sampling date when larvae from all three species were combined (Table 3). Larval live weight of each species was positively correlated with dry weight (Table 3). Larval live weight and percentage of body water content were positively correlated in *B. pellucidus* and *P. sericeus* but not *P. oblongus* (Table 3).

Thermal Hysteresis and Crystal Morphology. *P. sericeus* larval hemolymph from October had a melting point of −0.59°C, and a freezing point of −0.68°C. Although thermal hysteresis of 0.09°C is small, it is easily within the range where it suggests the presence of low levels of AFPs or antifreeze glycolipids. Hemolymph from the mixed medium-sized larvae, likely *P. oblongus* and *B. pellucidus*, in October had a melting point of −0.29°C, and a freezing point of −0.31°C. This small level of thermal hysteresis (0.02°C) is not indicative of the presence of AFPs. However, hemolymph from the medium-sized larvae exhibited strong hexagonal growth, indicating a low concentration of an-

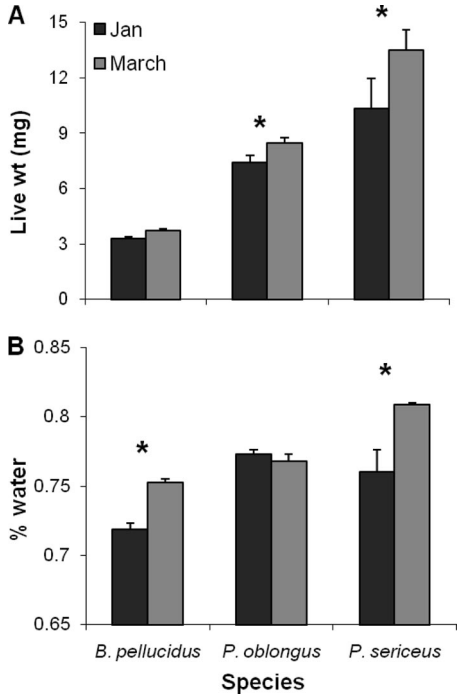

Fig. 5. Larval live weight (A) and body water content (B) of invasive rhizophagous weevil larvae in January and April 2009. Within a species, an asterisk indicates a significant difference between months at $P = 0.05$.

tifreeze. *Polydrusus sericeus* larval hemolymph from January had a melting point of −0.95°C, and a freezing point of −0.97°C, but there was strong hexagonal growth, indicating the presence of low levels of AFPs.

The depressed melting point of *P. sericeus* hemolymph in January relative to October (−0.95 versus −0.59°, respectively, a difference equal to ≈194 mOsm) suggests the production of some polyhydroxy alcohols in winter.

Discussion

Our results provide new information regarding the overwintering biology of three species of invasive rhizophagous weevils in northern hardwood forests. Relative to many other freeze-avoiding coleopterans, SCPs of *P. oblongus*, *P. sericeus*, and *B. pellucidus* were not particularly low, especially when in contact with ice (Duman et al. 2010). However, these larvae seem to be sufficiently protected from freezing given their thermally buffered environment in the soil, typically under a protective snow cover (Table 1). Sampling throughout the 2005 through 2009 winters indicated that >72% of all curculionid larvae (total larvae, 894) were found in the top 15 cm of soil (Coyle 2010). Soil temperatures at 15 cm below the surface were not below −1.1°C (Table 1) during the 2008–2009 winter. Because soil temperatures stayed well above SCPs all winter, SCP alone does not explain overwintering mortality or field emergence patterns.

Some larvae remained unfrozen down to −18°C, but none survived after the initial exotherm; therefore, they do not seem to be freeze tolerant. The lowest mean SCPs of larvae not in contact with ice were −13 to −14°C in January, March, or both for *B. pellucidus* and *P. oblongus*, but there was no significant difference in SCPs with season in *P. sericeus* (Fig. 2). This absence of seasonal change also was found in SCPs of the larval weevil *Ceutorhynchus obstrictus* (Marsham) (Cárcamo et al. 2009), but it differs from the clover leaf weevil, *Hypera punctata* (F.), where SCPs in both fed and starved larvae increased from neonate to fourth instar (Watanabe and Tanaka 1997). If we had sampled neonate SCPs in late August, it is possible that we would have seen a decrease in SCPs between August and October. However, neonates are difficult to collect in the field and attach to thermocouple wires, due to their very small size.

SCPs of larvae in contact with ice, probably the more ecologically relevant situation, were higher than those of larvae not in contact with ice. SCPs ranged

Table 3. Relationship of larval live weight with dry weight, percentage of body water, and SCP

	Live wt. (mg)	*a*	*b*	R^2	*F*	*P*
B. pellucidus	Dry wt (mg)	3.21	0.6058	0.7367	476.58	<0.0001
	% body water	4.68	0.1020	0.0365	7.43	0.0071
	SCP (°C)	1.84	0.0638	0.0151	1.59	0.2029
P. oblongus	Dry wt (mg)	3.65	1.2779	0.8662	227.56	<0.0001
	% body water	16.03	−10.4808	−0.0140	0.52	0.4765
	SCP (°C)	1.99	0.0228	0.0091	0.54	0.4634
P. sericeus	Dry wt (mg)	4.11	1.0844	0.8253	57.67	<0.0001
	% body water	58.22	−33.8794	0.2839	5.76	0.0353
	SCP (°C)	1.86	0.0097	0.0146	0.37	0.5487
Sample date, Oct.	Dry wt (mg)	3.49	−0.0600	0.9438	1242.85	<0.0001
	% body water	23.01	−10.4115	0.0701	5.58	0.0208
	SCP (°C)	−0.01	1.9415	0.0078	0.28	0.6027
Sample date, Jan.	Dry wt (mg)	4.55	−0.7195	0.9321	1139.92	<0.0001
	% body water	41.83	−25.7950	0.2837	37.63	<0.0001
	SCP (°C)	−0.01	2.2453	0.0116	0.85	0.3601
Sample date, March	Dry wt (mg)	4.47	−0.3377	0.9379	1856.58	<0.0001
	% body water	23.02	−12.8322	0.0897	12.11	0.0007
	SCP (°C)	−0.10	2.4555	0.0167	0.70	0.4089

All relationships were linear in the form $y = ax + b$. Data were analyzed using simple linear regression (PROC REG, SAS Institute).

between −6 and −9°C over the winter and did not vary between October and March. This indicated that inoculative freezing initiated by external ice across the cuticle limited supercooling. This is consistent with the very low hemolymph AFP activity (thermal hysteresis), and the small decrease in hemolymph melting points between October and midwinter, suggesting only small accumulations of polyhydroxy alcohols such as glycerol. Only *P. oblongus* larvae in January showed positive relationships between SCP and larval weight or body water content. Data from *P. oblongus* concur with other soil dwelling larvae, such as *H. punctata*, which showed a strong positive relationship between SCP and larval body weight (Watanabe and Tanaka 1997).

Although the SCPs of these weevils, especially those of larvae in contact with ice, are not especially low, this modest level of supercooling is probably sufficient to prevent death from freezing, based on the soil temperatures they experience (Table 1). However, it seems that freezing is generally not the most critical factor limiting low temperature tolerance in these weevils, because they cannot tolerate long periods (30 d) of temperatures well above their SCPs (Fig. 4). Larvae may be able to survive short periods below 0°C, but survivorship declines significantly after only 24 h at −3.5 or −7.9°C (Fig. 3). Therefore, significant mortality seems to result from effects of cold other than freezing, such as membrane malfunction, osmotic stress, dehydration, or metabolic disruption related to cold-induced changes in enzyme activity (Storey and Storey 1988).

Our data are similar to *Otiorhynchus sulcatus* (F.), another invasive rhizophagous weevil. *O. sulcatus* larval survival rates at 2, −3, and −6°C after 30 d were very similar to survival rates we observed in *B. pellucidus* and *P. oblongus* during our 30 d trial. For example, after 30 d at −3°C, *O. sulcatus* larval survivorship was 10% (Stenseth 1987), whereas *B. pellucidus* survivorship was 13% and *P. oblongus* was 4% (Fig. 4). Snowfall as a soil insulator probably plays an important role in determining overwintering success of rhizophagous weevils. As shown in Table 1, soil temperatures at 5 cm depth were not lower than −1.1°C throughout winter 2008–2009, although the minimum air temperature during this period was nearly −21°C.

Increasing larval body weight throughout winter could be due to higher survival of larger species, higher water content, or growth of individuals. To examine the first possibility, we divided larvae from January and March collections into three size classes, each corresponding to one weevil species based on our laboratory data. These were ≤5, 5 ≤ 10, and >10 mg in January and ≤6, 6 ≤ 10, and >10 mg in April. The distributions were 55, 24, and 21% in January and 51, 27, and 22% in March. This small change does not support disproportionate interspecific mortality. Second, the proportion of body water may have increased, resulting in greater body weights. This may be the case, but not in all species. *B. pellucidus*, the most common species belowground, did not increase in size from January to March (Fig. 5). However, *P. oblongus*, the most abundant species on foliage, increased in size, but not body water content, indicating an increase in body mass. The third species we examined, *P. sericeus*, increased in both body mass and body water content. Thus, the most parsimonious explanation is that larvae may be feeding and growing during the winter, and this is further supported by our observations that some larvae from all sample periods seemed to have material in their guts.

We observed a 79% decrease in larval survival from October to April (Fig. 1), indicating that these weevil species suffer significant mortality during winter. Although some winters are considerably colder, and have less snow cover than that of 2008–2009, temperature is not the only factor contributing to mortality. Factors such as soil moisture, physical properties, pathogens, and vegetation all can influence larval overwintering biology and adult emergence (Ricca et al. 1996, Yamazaki et al. 2003, Hou et al. 2009).

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References Cited

Bale, J. S. 2002. Insects and low temperatures: from molecular biology to distributions and abundance. Philos. Trans. R. Soc. Lond. B 357: 849–862.

Bale, J. S. 2010. Implications of cold tolerance for pest management. Chap. 14, pp. 342–373. *In* D. L. Denlinger and R. E. Lee, Jr. (eds.), Low temperature biology of insects. Cambridge University Press, New York.

Barnard, G. A. 1945. A new test for 2 × 2 tables. Nature 156: 177.

Cárcamo, H. A., C. E. Herle, J. Otani, and S. M. McGinn. 2009. Cold hardiness and overwintering survival of the cabbage seedpod weevil, *Ceutorhynchus obstrictus*. Entomol. Exp. Appl. 133: 223–231.

Carey, J. R. 2001. Insect biodemography. Annu. Rev. Entomol. 46: 79–110.

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

Clark, M. S., and M. R. Worland. 2008. How insects survive the cold: molecular mechanisms—a review. J. Comp. Physiol. B 178: 917–933.

Coyle, D. R. 2010. Ecology and impact of a suite of invasive weevils (Coleoptera: Curculionidae) in a northern hardwood forest. Ph.D. dissertation, University of Wisconsin–Madison.

Coyle, D. R., W. J. Mattson, and K. F. Raffa. 2008. Invasive root feeding insects in natural forest ecosystems of North

America. Chapter 8, pp. 134–151. *In* S. N. Johnson and P. J. Murray (eds.), Root feeders: an ecosystem perspective. CABI Press, Oxfordshire, United Kingdom.

Coyle, D. R., W. J. Mattson, and K. F. Raffa. 2010. Laboratory performance of two polyphagous invasive weevils on the predominant woody plant species of a northern hardwood community. Environ. Entomol. 39: 1242–1248.

Denlinger, D. L., and R. E. Lee, Jr. 2010. Low temperature biology of insects. Cambridge University Press, New York.

Doucet, D., V. K. Walker, and W. Qin. 2009. The bugs that came in from the cold: molecular adaptations to low temperature in insects. Cell. Mol. Life Sci. 66: 1401–1418.

Duman, J. G. 2001. Antifreeze and ice nucleator proteins in terrestrial arthropods. Annu. Rev. Physiol. 63: 327–357.

Duman, J. G., D. W. Wu, L. Xu, D. Tursman, and T. M. Olsen. 1991. Adaptations of insects to subzero temperatures. Q. Rev. Biol. 66: 387–410.

Duman, J. G., K. R. Walters, T. Sformo, M. A. Carrasco, P. K. Nickell, Xia Lin, and B. M. Barnes. 2010. Antifreeze and ice nucleator proteins. Chapter 3, pp. 59–90. *In* D. L. Denlinger and R. E. Lee, Jr. (eds.), Low temperature biology of insects. Cambridge University Press, New York.

Elnitsky, M. A., S.A.L. Hayward, J. P. Rinehart, D. L. Denlinger, and R. E. Lee, Jr. 2008. Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica.* J. Exp. Biol. 211: 524–530.

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

Holmstrup, M., M. Bayley, and H. Ramlov. 2002. Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable arctic invertebrates. Proc. Natl. Acad. Sci. U.S.A. 99: 5716–5720.

Hou, M. L., Y. Q. Han, and W. Lin. 2009. Influence of soil moisture on supercooling capacity and associated physiological parameters of overwintering larvae of rice stem borer. Entomol. Sci. 12: 155–161.

Huber, J. T. 1998. The importance of voucher specimens, with practical guidelines for preserving specimens of the major invertebrate phyla for identification. J. Nat. Hist. 32: 367–385.

Kuhar, T. P., R. R. Youngman, and C. A. Yaub. 2000. Alfalfa weevil (Coleoptera: Curculionidae) population dynamics and mortality factors in Virginia. Environ. Entomol. 29: 1295–1304.

Lee, R. L., Jr., and D. M. Denlinger. 1991. Insects at low temperatures. Springer, New York.

Lundheim, R., and K. E. Zachariassen. 1993. Water balance of over-wintering beetles in relation to strategies for cold tolerance. J. Comp. Physiol. B 163: 1–4.

McCornack, B. P., M. A. Carrillo, R. C. Venette, and D. W. Ragsdale. 2005. Physiological constraints on the overwintering potential of the soybean aphid (Homoptera: Aphididae). Environ. Entomol. 34: 235–240.

Mehta, C. R., and P. Senchaudhuri. 2003. Conditional versus unconditional exact tests for comparing two binomials. (http://www.cytel.com/Papers/twobinomials.pdf).

Pinski, R. A., W. J. Mattson, and K. F. Raffa. 2005a. Composition and seasonal phenology of a nonindigenous root-feeding weevil (Coleoptera: Curculionidae) complex in northern hardwood forests in the Great Lakes Region. Environ. Entomol. 34: 298–307.

Pinski, R. A., W. J. Mattson, and K. F. Raffa. 2005b. Host breadth and ovipositional behavior of adult *Polydrusus sericeus* and *Phyllobius oblongus* (Coleoptera: Curculionidae), nonindigenous inhabitants of northern hardwood forests. Environ. Entomol. 34: 148–157.

Régniere, J., and B. Bentz. 2007. Modeling cold tolerance in the mountain pine beetle, *Dendroctonus ponderosae.* J. Insect Physiol. 53: 559–572.

Ricca, M. A., F. W. Weckerly, and R. D. Semlitch. 1996. Effects of soil moisture and temperature on overwintering biology of *Curculio* larvae (Coleoptera: Curculionidae). Am. Midl. Nat. 136: 203–206.

Sformo, T., K. Walters, K. Jeannet, B. Wowk, G. M. Fahy, B. M. Barnes, and J. G. Duman. 2010. Deep supercooling, vitrification and limited survival to-100 degrees C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. Proc. Natl. Acad. Sci. U.S.A. 213: 502–509.

Stenseth, C. 1987. Cold hardiness in the black vine weevil (*Otiorhynchus sulcatus* (F.)) (Coleoptera: Curculionidae). Nor. J. Agric. Sci. 1: 41–43.

Storey, K. B., and J. M. Storey. 1988. Freeze tolerance: constraining forces, adaptive mechanisms. Can. J. Zool. 66: 1122–1127.

Watanabe, M., and K. Tanaka. 1997. Overwintering status and cold hardiness of *Hypera punctata* (Coleoptera: Curculionidae). Cryobiology 35: 270–276.

Witter, J. A., and R. D. Fields. 1977. Phyllobius oblongus and *Sciaphillus asperatus* associated with sugar maple reproduction in northern Michigan. Environ. Entomol. 6: 150–154.

Yamazaki, K., S. Sugiura, and K. Kawamura. 2003. Ground beetles (Coleoptera: Carabidae) and other insect predators overwintering in arable and fallow fields in central Japan. Appl. Entomol. Zool. 4: 449–459.

Zachariassen, K. E. 1985. Physiology of cold tolerance in insects. Physiol. Rev. 65: 799–832.

Zachariassen, K. E., J. G. Duman, E. Kristiansen, S. Pedersen, and N. Li. 2010. Ice nucleation and antifreeze proteins in animals. *In* S. Graether (ed.), Biochemistry and function of antifreeze proteins. Nova Science Publishers, Hauppauge, NY. (in press).

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