

## ORIGINAL PAPER

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## Influence of soil hydric parameters on the winter cold hardiness of a burrowing beetle, *Leptinotarsa decemlineata* (Say)

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**Abstract** This investigation examined the influence of soil moisture and associated parameters on the cold hardiness of the Colorado potato beetle (*Leptinotarsa decemlineata* Say), a temperate-zone species that overwinters in terrestrial burrows. The body mass and water content of adult beetles kept in sand at 4 °C varied over a 16-week period of diapause according to the substratum's moisture content. Changes in body water content, in turn, influenced the crystallization temperature (range -3.3 to -18.4 °C;  $n = 417$ ), indicating that environmental moisture indirectly determined supercooling capacity, a measure of physiological cold hardiness. Beetles held in dry sand readily tolerated a 24-h exposure to temperatures ranging from 0° to -5 °C, but those chilled in sand containing as little as 1.7% water (dry mass) had elevated mortality. Thus, burrowing in dry soils not only promotes supercooling via its effect on water balance, but may also inhibit inoculative freezing. Mortality of beetles exposed to -5 °C for 24 h was lower in substrates composed of sand, clay and/or peat (36–52%) than in pure silica sand (78%) having an identical water content (17.0% dry mass). In addition to moisture, the texture, structure, water potential, and other physico-chemical attributes of soil may strongly influence the cold hardiness and overwintering survival of burrowing insects.

**Key words** Cold hardiness · Inoculative freezing · Insect overwintering · Microenvironment · Moisture relations

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**Abbreviations**  $T_c$  crystallization temperature ·  $FP_{eq}$  equilibrium freezing/melting point ·  $T_b$  body temperature · *BWC* body water content

### Introduction

Most cold-hardy insects survive exposure to low winter temperatures by avoiding the freezing of their body fluids (Sømme 1982; Zachariassen 1985; Lee et al. 1993). These species exploit an inherent capacity to supercool, a phenomenon in which a solution remains in a metastable, unfrozen state at temperatures at or below its  $FP_{eq}$ . The lower limit of supercooling is identified by the  $T_c$ , the  $T_b$  at which freezing is initiated during a cooling trial. In insects,  $T_c$  is influenced by geographic origin, season, thermal acclimation, life stage, feeding status, endogenous nucleators, and cryoprotectants (Sømme 1982; Lee 1991). Assessments of cold hardiness for a given taxon or population that can survive extensive supercooling commonly are based on  $T_c$  measurements made in the laboratory. However, the ecological relevance of such data is often limited because insects in nature are exposed to various biotic and physical factors which enhance or constrain supercooling capacity (Bale 1987).

Environmental moisture is a crucial factor influencing the winter survival of insects that overwinter terrestrially (Danks 1991). Owing to the high specific heat of water, moisture in the soil moderates the thermal environment of burrowing insects by buffering against rapid changes and extremes of air temperature. Environmental moisture also influences organismal water balance (Hadley 1994), a potentially important determinant of physiological cold hardiness (Ring and Danks 1994). The ability to survive chilling episodes may strongly depend on complex interactions between environmental moisture and low temperature (Bale 1991; Lee 1991).

Several tenets of cryobiological theory suggest that winter survival of cold-hardy insects is intimately asso-

ciated with water balance. For example, a reduction in *BWC* enhances cold hardiness by depressing hemolymph  $FP_{eq}$  and, thus, the probability of spontaneous freezing (Salt 1966). Also, the partial dehydration exhibited by various invertebrates, in preparation for winter, increases the relative fraction of "bound" water in cells that is unfreezable (Cloudsley-Thompson 1970; Zachariassen 1991; Ring and Danks 1994).

Insect cold hardiness is also influenced by direct interaction with environmental water, most significantly via contact with external ice crystals, which may seed the freezing of body fluids. Such inoculative freezing is adaptive in species that tolerate freezing because it ensures that ice crystallization occurs at relatively high temperatures (Duman et al. 1995). The biophysical mechanisms governing the penetration of environmental ice into body tissues, first studied by Salt (1966), remain poorly understood. In particular, the role of the hydric microenvironment on the susceptibility to inoculative freezing has received little study (Layne et al. 1990; Gehrken et al. 1991; Forge and MacGuidwin 1992).

We studied the influence of soil moisture on water balance and cold hardiness of the Colorado potato beetle (*Leptinotarsa decemlineata* Say), a temperate-region chrysomelid that overwinters within shallow burrows, typically in sandy soil. Diapause in adult *L. decemlineata*, which is triggered in late summer by decreasing photoperiod, temperature, and food availability (de Wilde et al. 1959; Tauber et al. 1988), reportedly induces a modest cold hardening via partial dehydration and the elimination of endogenous gut nucleators (Fink 1925; Mail and Salt 1933; Ushatinskaya 1978). Beetles are protected from extreme cold by the insulating effect of snow, organic detritus, and the soil in which they hibernate (Mail and Salt 1933; Ushatinskaya 1978). Indeed, the minimum winter temperature experienced by *L. decemlineata* is only  $-3$  to  $-4$  °C (Mail and Salt 1933; Minder 1962; Milner et al. 1992), substantially higher than mean  $T_c$  values reported for winter beetles, ca.  $-7$  to  $-8$  °C (Salt 1933; Lee et al. 1994). Although the risk of freezing may thus appear negligible, winter mortality typically is high, e.g., 80% (Minder 1962; Lashomb et al. 1984; Milner et al. 1992). Our study tested the hypothesis that winter survival of *L. decemlineata* is influenced by thermal and hydric characteristics of the overwintering microenvironment.

## Materials and methods

Adult Colorado potato beetles were collected in late August and early September 1993 from cultivated potato fields at Hancock Agricultural Research Station, Waushara Co., central Wisconsin. Beetles ( $\approx 6500$ ) were shipped under refrigeration, via overnight carrier, to Miami University and were sorted into groups of 50 beetles. Each group was kept in a ventilated plastic cup containing 400 g silica sand moistened with deionized water (water content = 2.5%, dry mass).

Beetles were induced into diapause by exposing them to 15 °C, 12:12 (L:D) for 7 days, and thereafter to 4 °C, 0:24, for  $\geq 18$  days

before use in the experiments. Water was added periodically to the sand as required, but the beetles were otherwise undisturbed. Only beetles deemed healthy on the basis of color and behavior were used in experiments.

## Experimental substrates

The primary experimental substratum, a medium-grained silica sand (Sakrete Co.), was chosen to mimic soils predominating in the beetles' native habitat. Nominally dry, damp, moist, and wet substrates were prepared by reconstituting aliquots of oven-dried (65 °C) sand with deionized water to 0, 1.7, 2.5, and 17.0% dry mass, respectively. These moisture levels represented 0, 9, 14, and 92%, respectively, of the mean ( $\pm 1$  SE;  $n = 4$  replicates) saturation water content,  $18.5 \pm 0.7\%$  (dry mass), determined from the mass of deionized water samples held against gravity. Experiments involving heterogeneous substrates (see below) used composites of sand, clay and peat. Mean particle density, dry bulk density (ratio of soil mass to unit volume) and porosity (index of relative pore volume) were measured on oven-dried aliquots of each substrate (Troeh and Palmer 1967). Thermocouple psychrometric techniques (Riggle and Slack 1980) were used to measure water potential, on five replicate samples, with stainless steel psychrometers (Wescor, PST-55) and a dew-point microvoltmeter (Wescor, HR-33T) that was coupled to a software-driven data-interface module (ADInstruments, MacLab/8). Psychrometers, calibrated at 23 °C using appropriate NaCl solutions, provided reliable readings at water potentials  $\leq -50$  kPa. Data were used to construct water retention curves depicting the relationships between substratum water content and water potential.

## Effects of substrate moisture on water balance and supercooling capacity

Approximately 750 beetles were randomly assigned in equal numbers to one of three treatment groups (dry, moist, and wet substrate), with each treatment group further divided into seven sample subgroups ( $n \approx 35$  beetles each). Beetles in each subgroup were housed communally within ventilated plastic cups containing 400 g prepared sand and exposed to 4 °C, DD for up to 16 weeks. At intervals of 2–4 weeks, 20 beetles were selected for study from one cup within each treatment group. Beetles were gently sieved from the sand and individually placed inside a polyethylene 1.8-ml microcentrifuge tube. The tube's opening was plugged with plastic foam, which anchored the sensing junction of a 36-gauge thermocouple against the beetle's ventral surface and prevented egress. Each preparation was placed in a glass test tube suspended in a refrigerated bath and, after attaining thermoequilibrium at 0 °C, was progressively cooled at  $0.25$  °C  $\text{min}^{-1}$ . The onset of freezing was verified by the appearance of an exotherm (i.e., release of the heat of crystallization), and the resultant  $T_c$  was determined for each beetle from records of  $T_b$  plotted at 5-s intervals by a multi-channel data logger (Omega, OM500 or RD3752). At the conclusion of the supercooling trials, the *BWC* of each beetle, expressed as a percentage of dry body mass, was determined from the mass lost during oven drying at 65 °C.

The effects of prolonged exposure to various substrate moisture levels on the supercooling capacity and various physiological parameters of diapausing beetles were analyzed using standard two-factor ANOVA models (substratum moisture  $\times$  incubation time); mean separations were conducted using Fisher's protected least significant differences (PLSD) calculated at  $P = 0.05$ . Because significant interaction ( $P < 0.001$ ) occurred between the main factors for all parameters except dry body mass, additional ANOVA's were employed separately to evaluate the effects of substratum moisture and incubation time on fresh body mass, *BWC*, and  $T_c$ . Regressions of beetle  $T_c$  on fresh body mass and *BWC* were conducted using the method of least-squares.

### Effects of substrate moisture and temperature on cold hardiness

A factorial experiment, consisting of 20 different treatments, was used to investigate interactive effects of substrate moisture (dry, damp, moist or wet sand) and low temperature (0, -2, -3, -4, or -5 °C) on cold hardiness. Mortality was determined in ten replicate tests ( $n = 10$  beetles each) for each treatment; thus, the results of this experiment were based on responses of 2000 beetles.

Groups of ten beetles were selected at random and chilled inside  $20 \times 200$  mm glass tubes containing  $\approx 50$  cm<sup>3</sup> of the prescribed substratum. Beetles were positioned such that each was isolated from other beetles and the tube wall. They were habituated to the substrates for 24 h in a refrigerator (4 °C, DD), after which each tube was instrumented with a 30-gauge thermocouple (inserted into the substratum's center) and suspended in a refrigerated ethanol bath (Forma Scientific). After all tubes reached thermoequilibrium at 0 °C, the bath was programmed to further cool and ultimately maintain beetles at the target  $T_b$ . Freezing of the substrates was initiated by adding small ice crystals to each tube; the resulting exotherm confirmed that all beetles were exposed to frozen substrate during the ensuing, 24-h incubation. Exotherms lasted 5–25 min, depending on each substrate's water content, although cooling rates were generally comparable ( $\approx 0.15$  °C min<sup>-1</sup>).

Survival data for beetles incubated in experimental substrates were analyzed using two-factor ANOVA's (temperature  $\times$  water content); mean separations were conducted using Fisher's PLSD ( $P < 0.05$ ). Because significant interaction occurred between the main factors ( $P < 0.001$ ), additional ANOVA's were used separately to evaluate the influences of substrate temperature and water content on beetle survival.

### Time-course of mortality in wet sand

The viability of beetles exposed to wet sand at -5 °C was studied over a 24-h period. Test tubes containing sand (water content, 17.0%) and beetles were prepared, habituated for 24 h at 4 °C, equilibrated at 0 °C in the cold bath, cooled, and inoculated with ice as previously described. Four replicate tubes, each containing 12 beetles, were removed from the bath at time zero (coincident with the ice inoculation of the substrate), at 0.5 h (when the tubes first attained the target minimum temperature, -5 °C), and at subsequent 2-, 4-, 8-, 12-, and 24-h intervals during incubation at -5 °C. Mean mortality was compared among sample groups using ANOVA and separated using Fisher's PLSD ( $P < 0.05$ ) on angularly-transformed percentage values.

### Viability in heterogeneous substrates

This experiment determined the mortality of beetles chilled at -5 °C for 24 h in frozen substrates composed of sand, clay, and/or peat. We used mixtures of the silica sand described previously, volcanic clay (domestic cat litter) pulverized using a mortar and pestle, and horticultural peat passed through a 2-mm<sup>2</sup> sieve. All materials were oven-dried, thoroughly mixed in the prescribed proportions, and rehydrated with deionized water (water content, 17.0%) shortly before use. Five replicate tubes, each containing 12 beetles, were prepared from each of the four experimental substrates: sand (100%), sand-clay (90/10%), sand-peat (90/10%), and sand-clay-peat (90/5/5%). The tubes containing beetles were habituated for 24 h at 4 °C, equilibrated at 0 °C in the cold bath, cooled, and inoculated with ice as previously described. Mean mortality was compared among the treatment groups using ANOVA and separated using Fisher's PLSD ( $P < 0.05$ ) using angularly-transformed percentage values.

### Viability assay

Following the prescribed incubation, tubes containing beetles were removed from the bath and refrigerated (4 °C, DD) for 12 h, after which the beetles were recovered from the substratum, placed in

ventilated plastic cups containing 50–75 g moist sand (water content, 2.5%), and refrigerated for an additional 12 h. To assess their viability, beetles were aroused from diapause by holding them in an environmental cabinet (15 °C, 12:12 L:D) for 24 h, and then observed for 10–20 min during passive warming to room temperature ( $\approx 23$  °C). Only beetles exhibiting spontaneous locomotion or movement of the antennae and legs in response to mandibular stimulation (Kung et al. 1992) were scored "viable." This assay was developed from pilot experiments in which beetles (previously incubated at -5 °C for 40 h in dry or wet sand) were scored successively at 5, 24, 72, 96, 120, and 144 h following their transfer to the 15 °C cabinet. A consistent mortality estimate was first attained at 24 h.

### Hydric characteristics of natural substrates

Samples of the soil in which *L. decemlineata* overwinters were collected at Hancock Agricultural Research Station and at Adams, a nearby privately-owned farm, between December 1993 and April 1994. The sampling site at both locations was a windrow of coniferous trees (chiefly *Pinus* spp.) oriented parallel to the east end of a cultivated potato field. At each sampling, mineral soil originating  $\approx 15$  cm below a 7- to 10-cm stratum of coniferous mulch (i.e., A1 horizon) was collected from 5–6 excavations, spaced  $\approx 2$  m apart, within 2.5 m of the field's edge. Samples weighing  $\approx 0.5$  kg were sealed in moisture-proof containers, shipped via overnight carrier to Miami University, and promptly analyzed in duplicate for water content. Seasonal changes in soil moisture were analyzed by comparing sample means using ANOVA and separated using Fisher's PLSD ( $P < 0.05$ ). Particle size analyses of January samples were conducted by the Soil & Plant Analysis Laboratory, University of Wisconsin-Madison. Saturation water content and water potential were measured on material pooled from samples collected at both field sites in January 1994.

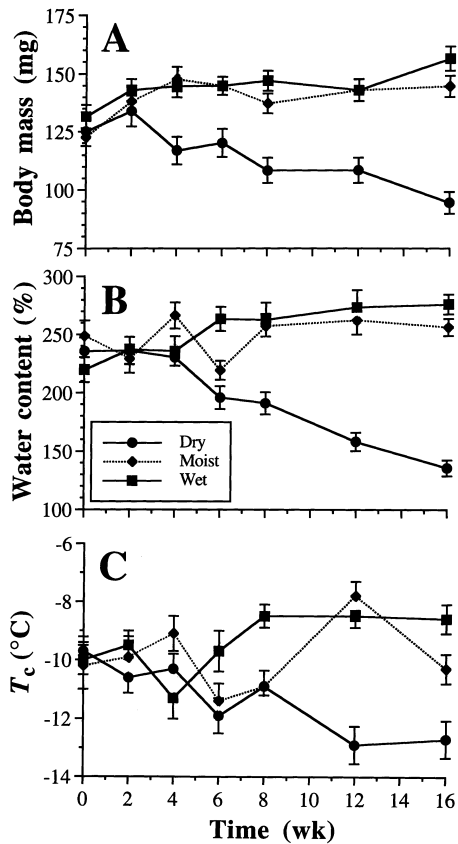
## Results

### Effects of substrate moisture on water balance and supercooling capacity

Beetles kept in dry sand progressively lost mass over the 16-week experiment ( $P < 0.001$ ), whereas a significant mass gain occurred in beetles exposed to moist ( $P = 0.002$ ) and wet ( $P < 0.028$ ) sand (Fig. 1A). Net changes in the fresh body mass of beetles after 16 weeks, relative to the corresponding initial (time zero) samples, were -24.5%, +18.1%, and +19.0%, in the dry, moist, and wet substrate groups, respectively. Mean values for fresh body mass were comparable among treatment groups at the outset of the experiment ( $P > 0.32$ ), but had diverged significantly within the first 4 weeks ( $P < 0.001$ ).

Changes in beetle *BWC* generally paralleled trends in the fresh body mass of beetles kept in dry ( $P < 0.001$ ), moist ( $P = 0.020$ ), and wet ( $P = 0.007$ ) sand (Fig. 1B). Net changes in *BWC* of beetles kept in dry and wet substrates were -42.4% and +25.5%, respectively; however, the *BWC* of beetles kept in moist sand was not statistically distinguishable between time zero and 16-week samples ( $P > 0.63$ ). Although mean *BWC* of beetles initially was comparable among the 3 treatment groups ( $P > 0.26$ ), significant differences had developed by 6 weeks ( $P < 0.001$ ).

The mean ( $\pm$  SE)  $T_c$  was initially comparable ( $P = 0.854$ ) among the dry ( $-9.7 \pm 0.6$  °C), moist



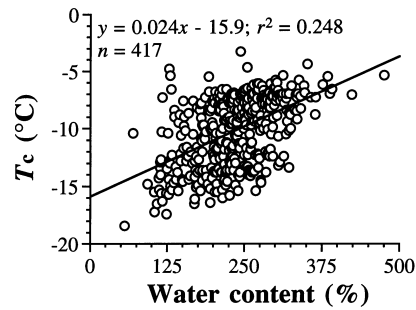
**Fig. 1A–C** Changes in body mass (A), body water content (B), and crystallization temperature (C) of Colorado potato beetles (*Leptinotarsa decemlineata*) during diapause at 4 °C, DD, in dry, moist, and wet sand (water content: 0, 2.5, and 17.0% dry mass, respectively). Means ( $\pm 1$  SE) are based on  $n = 19$ –20 beetles

( $-10.2 \pm 0.8$  °C), and wet ( $-10.0 \pm 0.5$  °C) treatment groups. Significant changes occurred in the  $T_c$  of beetles in dry ( $P = 0.010$ ), moist ( $P = 0.001$ ), and wet ( $P = 0.002$ ) substrates during the experiment, such that means differed markedly among the final 16-week samples ( $-12.7 \pm 0.7$  °C,  $-10.3 \pm 0.5$  °C, and  $-8.6 \pm 0.5$  °C, respectively; Fig. 1C). However, only in the dry sand treatment did initial and final sample means differ significantly. Combining data for all treatment groups showed that  $T_c$  (range  $-3.3$  °C to  $-18.4$  °C;  $n = 417$ ) was independent of fresh body mass ( $P > 0.10$ ), but strongly correlated with  $BWC$  ( $P = 0.001$ ; Fig. 2).

Dry body mass was not influenced by substrate moisture ( $P > 0.19$ ) or exposure duration ( $P > 0.33$ ). Mean ( $\pm$  SE) values for beetles incubated in dry, moist, and wet sand were initially  $39 \pm 3$ ,  $37 \pm 2$ , and  $42 \pm 2$  mg, respectively, comparable to the respective final samples,  $41 \pm 2$ ,  $41 \pm 2$ , and  $42 \pm 2$  mg.

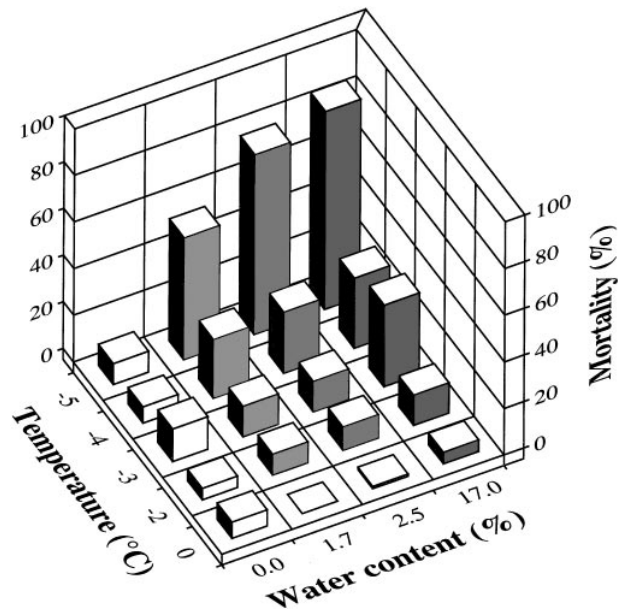
#### Effects of substrate moisture and temperature on viability

Generally, beetle cold hardiness was inversely related to substrate moisture, particularly at lower temperatures

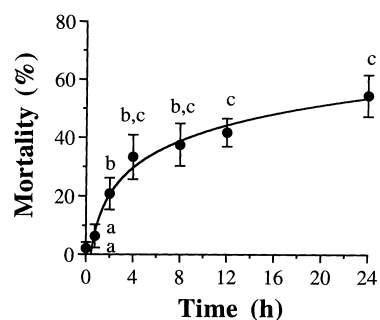


**Fig. 2** Correlation of body water content ( $BWC$ ; % dry mass) and crystallization temperature ( $T_c$ ) of Colorado potato beetles (*Leptinotarsa decemlineata*) during diapause at 4 °C, DD, in dry, moist, and wet sand (water content: 0, 2.5, and 17.0% dry mass, respectively)

(Fig. 3). Although exposure to temperatures ranging from 0 to  $-5$  °C had little effect on the viability of beetles incubated in dry sand ( $P > 0.44$ ), mortality was strongly temperature dependent for beetles incubated in damp ( $P < 0.001$ ), moist ( $P < 0.001$ ), and wet ( $P < 0.001$ ) sand. For example, whereas no differences in mortality occurred among the treatment groups at 0 °C (range 1–7%;  $P > 0.07$ ) or  $-2$  °C (range 5–13%;  $P > 0.26$ ), substrate water content strongly influenced beetle mortality at  $-3$  °C ( $P = 0.045$ ),  $-4$  °C ( $P = 0.001$ ), and  $-5$  °C ( $P < 0.001$ ). Notably, the mortality of beetles exposed to  $-5$  °C was 3.5-fold higher in damp versus dry sand, although increasing water content had no added effect (Fig. 3).



**Fig. 3** Mortality of Colorado potato beetles (*Leptinotarsa decemlineata*) chilled for 24 h in sand containing various amounts of moisture. The height of each column represents a mean based on  $n = 200$  beetles; SE (not shown) range 1.0–7.6%



**Fig. 4** Time-course of mortality of Colorado potato beetles (*Leptinotarsa decemlineata*) exposed to  $-5^{\circ}\text{C}$  in wet sand (water content: 17.0% dry mass). Means ( $\pm 1$  SE;  $n = 12$  beetles) identified by different letters were statistically distinguishable ( $P < 0.05$ )

#### Time-course of mortality in wet sand

Mortality of beetles chilled in wet sand was strongly time dependent ( $P < 0.001$ ). Few dead beetles were recovered at time zero, when the substratum was inoculated, or at 30 min, when the substratum first attained the target temperature,  $-5^{\circ}\text{C}$  (Fig. 4). Subsequently, mortality increased rapidly, albeit at a diminishing rate, reaching a mean ( $\pm$  SE) of  $54.2 \pm 7.2\%$  by 24 h (Fig. 4).

#### Viability of beetles in heterogeneous substrates

Mortality was strongly influenced by the substratum's composition, ranging from 78% in sand to 36% in sand-clay-peat ( $P = 0.005$ ; Table 1). Physical and hydric characteristics of the composite substrates differed from pure sand (Table 1). Generally, beetle mortality increased with increasing substrate water potential, particle density, and bulk density, but was inversely correlated with saturation water content (Table 1).

**Table 1** Mortality of Colorado potato beetles (*Leptinotarsa decemlineata*) exposed to  $-5^{\circ}\text{C}$  for 24 h, relative to physical parameters of four experimental substrates (water content: 17.0%).

	Sand	Sand-Clay	Sand-Peat	Sand-Clay-Peat
Beetle mortality (%)	$78 \pm 7^a$	$52 \pm 6^b$	$46 \pm 8^b$	$36 \pm 6^b$
Composition (%)				
sand	100	90	90	90
clay	0	10	0	5
peat	0	0	10	5
Saturation content (%)				
gravimetric	18.5	32.0	39.5	34.2
volumetric	30.6	48.5	48.3	49.0
Water potential (kPa)	$\gg -50$	-50	-110	-55
Particle density ( $\text{g cm}^{-3}$ )	2.68	2.60	2.36	1.95
Bulk density ( $\text{g cm}^{-3}$ )	1.65	1.51	1.22	1.43
Porosity (%)	39	42	40	41

#### Hydric characteristics of natural substrates

Substrates used by overwintering *L. decemlineata* at the Hancock and Adams sites were classified as loamy sand (sand, 85%; silt, 5%; clay, 10%) and sand (sand, 91%; silt, 1%; clay, 8%), respectively. Both soils contained little organic matter (1.3% and 0.6%, respectively). Particle density ( $2.43 \text{ g cm}^{-3}$ ) and bulk density ( $1.58 \text{ g cm}^{-3}$ ) of these soils were most similar to the sand-clay experimental substratum (Table 1), although their porosity (35%) was relatively lower, perhaps reflecting a smaller average particle size.

Water contents of soil in which *L. decemlineata* overwinter varied significantly during December-April at Hancock ( $P < 0.001$ ) and Adams ( $P < 0.001$ ), with maximum levels of 9–10% attained in mid-winter (Table 2). These field values represented about one-third of the saturation water content (33.2% of dry mass), but nevertheless were associated with high water potentials ( $> -50 \text{ kPa}$ ).

#### Discussion

Some field studies suggest that soil moisture indirectly reduces the winter survival of *L. decemlineata* (Minder 1962; Ushatinskaya 1978). For example, moisture-retaining (e.g., clay) soils restrict beetles to shallow depths where they are exposed to extreme cold (Minder 1962), whereas well-drained, porous soils permit beetles to burrow deeper where more moderate temperatures prevail (Lashomb et al. 1984; Milner et al. 1992). Winter mortality is sometimes associated with excessive moisture even in soils that do not freeze (Ushatinskaya 1978), perhaps because moisture promotes the proliferation of the parasitic fungus, *Beauveria bassiana* (Weber and Ferro 1993). Our data reveal that soil moisture is deleterious to overwintering *L. decemlineata* owing also to direct effects on physiological cold hardiness.

Means ( $\pm$  SE) are based on  $n = 60$  beetles; values identified by similar letters were statistically indistinguishable ( $P < 0.05$ )

**Table 2** Water content (% dry mass) of soil samples from overwintering sites of the Colorado potato beetle (*Leptinotarsa decemlineata*) at Hancock and Adams field sites, central Wisconsin,

	December	January	February	April	<i>P</i>
Hancock	5.3 ± 0.6 <sup>a</sup>	8.7 ± 0.3 <sup>b</sup>	8.0 ± 0.3 <sup>b</sup>	7.4 ± 0.6 <sup>b</sup>	< 0.001
Adams	6.2 ± 0.2 <sup>a</sup>	5.2 ± 0.2 <sup>a</sup>	9.7 ± 1.1 <sup>b</sup>	9.5 ± 1.1 <sup>b</sup>	< 0.001

during winter 1993–94. Means (± SE) are based on 5–6 samples, with each sample tested in duplicate; values identified by similar letters were statistically indistinguishable ( $P < 0.05$ )

### Influence of soil moisture on beetle cold hardiness

Insects of the Temperate Zone often exhibit a partial dehydration during diapause induction, followed by static regulation of *BWC* during winter (Cloudsley-Thompson 1970; Sømme 1982; Zachariassen 1991; Ring and Danks 1994). Similarly, *L. decemlineata* voids 30–60% of its initial water prior to diapause and maintains a relatively low and constant *BWC* during winter, e.g., 120–130% of dry mass (Fink 1925; Ushatinskaya 1978). Insects that survive exposure to subfreezing temperatures by supercooling (such as *L. decemlineata*) better resist water loss than do species that tolerate freezing (Lundheim and Zachariassen 1993). Nevertheless, significant water exchange via cuticular and respiratory transpiration, excretion, and hygroscopic absorption of water into the cuticle can occur during diapause, even in species that do not tolerate freezing (Hadley 1994; Ring and Danks 1994). Our study of *L. decemlineata* suggests that such changes in water balance may influence this species' cold hardiness.

In the present study, substrate moisture strongly influenced the hydration state of *L. decemlineata*. Net changes in *BWC* of beetles exposed to dry and wet sand were –42% and +26%, respectively, whereas *BWC* of beetles exposed to (intermediate) moist sand was unchanged. Given the water contents of native soils, which were similar to those measured previously (Milner et al. 1992), *L. decemlineata* at our field sites apparently remained in neutral water balance during the winter of record. However, it seems clear that water balance in this species is subject to local and temporal variation in microenvironmental moisture.

Our data suggest that the hydration state of insects can influence physiological cold hardiness through its effect on supercooling capacity. Notably, a 42% loss of body water in *L. decemlineata* kept in dry sand was associated with a marked decrease in  $T_c$  (from –9.7° to –12.7 °C), whereas the  $T_c$  of beetles exposed to wet sand generally increased (Fig. 1). The direct relationship between *BWC* and  $T_c$  (Fig. 2) likely reflects the influence of solute concentration on  $FP_{eq}$  (Salt 1966; Zachariassen 1991). The baseline  $T_c$  of our *L. decemlineata*,  $\approx -10$  °C, was lower than that ( $-7.5 \pm 0.3$  °C,  $n = 46$ ) previously reported for this population by Lee et al. (1994), perhaps because the sand in which our beetles were held was substantially drier (water content 2.5% versus 7.0%).

The inverse relationship between *BWC* and supercooling capacity (Fig. 2) suggests that the risk of spon-

taneous ice nucleation may be greater in heavier (larger?) beetles. However, our data do not exhibit the usual relationship between supercooling capacity and fresh body mass (Block and Young 1979; Sømme 1982; Lee 1991). In fact, regression analysis of the initial (untreated) samples in each treatment group showed that  $T_c$  and fresh body mass were *inversely* correlated ( $n = 59$ ;  $r^2 = 0.06$ ;  $P = 0.037$ ). Regression of *BWC* against dry body mass revealed that this finding can be explained by the relatively lower *BWC* maintained by larger beetles ( $r^2 = 0.77$ ;  $P < 0.001$ ). It is unclear whether this trend simply reflects ontogenetic or size variation in fluid homeostasis (Hadley 1994), or if during diapause larger beetles adaptively dehydrate more extensively than smaller ones.

### Substrate moisture and diapause energetics

Exposure of *L. decemlineata* to wet soils reduces the depth of torpor and increases the consumption of energy substrates during winter (Minder 1962). Fat reserves, which initially account for 38–40% of dry mass, decrease during winter by 19–28% in (damp) loamy soil as compared to only 15–17% in (drier) sandy soil, with the enhanced lipid catabolism in the former paralleled by increases in basal  $O_2$  consumption and the activities of regulatory enzymes (Fink 1925; Ushatinskaya 1978). Such a metabolic increase generally reduces dry body mass (Lundheim and Zachariassen 1993). Because dry body mass remained constant over the 16-week experiment, exposure to very dry or wet substrates was not particularly stressful to beetles in the present study.

### Viability of beetles in frozen substrates

Although prolonged exposure to environmental moisture clearly influenced physiological cold hardiness through its effect on water balance, our data also suggest that acute exposure to a frozen substratum may have even more profound consequences for winter survival. Beetles chilled in dry sand readily survived exposure to temperatures as low as –5 °C (Fig. 3), presumably by supercooling. (Note also that in the first experiment only 1 of the 140 beetles in the moist sand exhibited a  $T_c \geq -5$  °C.) In contrast, environmental moisture interacted strongly with temperature, as mortality increased markedly in the presence of even very little water (e.g.,

1.7%). Kung et al. (1992) reported a similar interaction, which may partly reconcile the apparent discrepancy between limits of supercooling determined for *L. decemlineata* in the laboratory (e.g.,  $T_c = -7$  to  $-10$  °C) and the high mortality occurring at more moderate field temperatures (e.g.,  $-3$  to  $-4$  °C).

Unfortunately, our experimental design did not permit us to determine whether beetles chilled in frozen substrates were actually frozen, although mortality under these conditions was likely due to inoculative freezing of body fluids. Some mortality may have been caused by factors other than inoculative freezing, such as excessive desiccation, metabolic perturbation associated with environmental hypoxia, and damage due to compression within the expanding soil matrix. The likelihood and severity of these perils partially depend on the amount of ice that forms in the substratum and, ultimately, ambient temperature and moisture. The pattern of mortality shown in Fig. 4 may indicate that more than one factor is involved.

Hydric relations with the microenvironment may strongly influence the susceptibility of an insect to inoculative freezing (Salt 1936; Salt 1963; Layne et al. 1990; Lundheim and Zachariassen 1993). Our study suggests that soil texture may be a particularly important factor. Notably, the water content of the sand-clay (90/10%) mixture could be increased at least ten fold over that of pure sand without elevating beetle mortality (Table 1; Fig. 3), a result that conceivably reflects variation in water potential which is reduced in colloidal and organic soils owing to their greater adsorption of water (Hillel 1971; Fig. 5). Low environmental water potential increases the survival of nematodes (*Meloidogyne hapla*) in frozen soil, partly by reducing the volume of ice within pores (Forge and MacGuidwin 1992). The importance of water potential is less clear in the present study because this parameter varied little among the experimental substrates (i.e., all were  $> -110$  kPa; Table 1). Nevertheless, the arrangement and interaction of particles in soil is influenced by various physico-chemical attributes (e.g., the mass and volume ratios of integral solids, water, and air) that may also affect the cold hardness of burrowing insects. Factors that reduce ice content and inhibit contact with ice in the soil matrix may be particularly crucial to winter survival. For example, relative to sand, composite substrates were lighter (lower average particle density and bulk density) and capable of holding more moisture, even though their pore space was comparable (Table 1). Higher viability of beetles in composite substrates may relate to the relatively larger fractions of (presumably unfrozen) hygroscopic water in such materials. Also, substrates composed of fine clay and organic particles may have lower ice contents if their much smaller pores harbor isolated pockets of supercooled water. Conceivably such pores may hamper direct physical contact between ice and body tissues, as well as the diffusion of vapor through the soil matrix (Hillel 1971). The latter effect would inhibit delayed ice inoculation, a process by

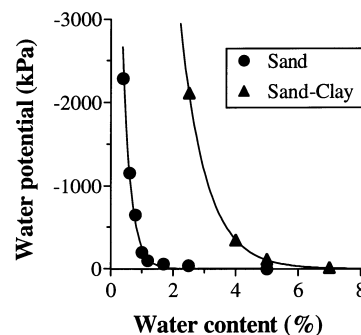


Fig. 5 Comparison of water retention curves for pure sand and sand-clay (90/10%) experimental substrates

which saturated vapor emanates from the insect cuticle and condenses on adjacent ice crystals, thus causing ice to propagate toward the vapor's source (Salt 1963).

The distribution of *L. decemlineata* is limited to regions where sustainable populations can predictably avoid freezing within their hibernacula (Mail and Salt 1933; Ushatinskaya 1978). In northern North America, adults typically overwinter in well-drained soils near agricultural fields, often bordering hedgerows or wooded habitats (Kung et al. 1992; Milner et al. 1992; Weber and Ferro 1993). Field studies indicate that successful overwintering depends on burrow depth (typically 8–13 cm), soil temperature, and soil moisture level (Fink 1925; Minder 1962; Ushatinskaya 1978; Lashomb et al. 1984; Weber and Ferro 1993). Our study suggests that burrowing in porous, dry soils not only promotes supercooling via its effect on water balance, but may also inhibit inoculative freezing. Ultimately, the cold hardness of *L. decemlineata*, and likely other species of burrowing insects, is governed by interaction between environmental moisture and low temperature, as well as the physico-chemical attributes of soil that influence this interaction.

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## References

- Bale JS (1987) Review: insect cold hardness: freezing and supercooling – an ecophysiological perspective. *J Insect Physiol* 33: 899–908
- Bale JS (1991) Implications of cold hardness for pest management. In: Lee RE, Denlinger DL (eds) *Insects at low temperature*. Chapman and Hall, New York, pp 461–498
- Block W, Young SR (1979) Measurement of supercooling in small arthropods and water droplets. *Cryo-Lett* 1: 85–91
- Cloudsley-Thompson JL (1970) Terrestrial invertebrates. In: Whittow GC (ed) *Comparative physiology of thermoregulation*, vol 1. Invertebrates and nonmammalian vertebrates. Academic Press, New York, pp 15–77
- Danks HV (1991) Winter habitats and ecological adaptations for winter survival. In: Lee RE, Denlinger DL (eds) *Insects at low temperature*. Chapman and Hall, New York, pp 231–259

- Fink DE (1925) Physiological studies on hibernation in the potato beetle, *Leptinotarsa decemlineata* Say. Biol Bull 6: 381–406
- Forge TA, MacGuidwin AE (1992) Effects of water potential and temperature on survival of the nematode *Meloidogyne hapla* frozen soil. Can J Zool 70: 1553–1560
- Gehrken U, Strømme A, Lundheim R, Zachariassen KE (1991) Inoculative freezing in overwintering tenebrionid beetle, *Bolitophagus reticulatus* Panz. J Insect Physiol 37: 683–687
- Hadley NF (1994) Water relations of terrestrial arthropods. Academic Press, San Diego, California, pp 356
- Hillel D (1971) Soil and water: physical principles and processes. In: Kozlowski TT (ed) Physiological ecology: a series of monographs, texts and treatises, Academic Press, New York, pp 288
- Kung K-JS, Milner M, Wyman JA, Feldman J, Nordheim E (1992) Survival of Colorado potato beetle (Coleoptera: Chrysomelidae) after exposure to subzero thermal shocks during diapause. J Econ Entomol 85: 1695–1700
- Lashomb JH, Ng Y-S, Ghidui G, Green E (1984) Description of spring emergence by the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), in New Jersey. Environ Entomol 13: 907–910
- Layne JR, Lee RE Jr, Huang JL (1990) Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*). Can J Zool 68: 506–510
- Lee RE Jr (1991) Principles of insect low temperature tolerance. In: Lee RE, Denlinger DL (eds) Insects at low temperature. Chapman and Hall, New York, pp 17–46
- Lee RE Jr, Lee MR, Strong-Gunderson JM (1993) Insect cold-hardiness and ice nucleating active microorganisms including their potential use for biological control. J Insect Physiol 39: 1–12
- Lee RE Jr, Costanzo JP, Kaufman PE, Lee MR, Wyman JA (1994) Ice-nucleating active bacteria reduce the cold-hardiness of the freeze-intolerant Colorado potato beetle (Coleoptera: Chrysomelidae). J Econ Entomol 87: 377–381
- Lundheim R, Zachariassen KE (1993) Water balance of overwintering beetles in relation to strategies for cold tolerance. J Comp Physiol B 163: 1–4
- Mail GA, Salt RW (1933) Temperature as a possible limiting factor in the northern spread of the Colorado potato beetle. J Econ Entomol 26: 1068–1075
- Milner M, Kung K-JS, Wyman JA, Feldman J, Nordheim E (1992) Enhancing overwintering mortality of Colorado potato beetle (Coleoptera: Chrysomelidae) by manipulating the temperature of its habitat. J Econ Entomol 85: 1701–1708
- Minder IF (1962) Hibernation conditions and survival rate of the Colorado potato beetle in different types of soils. In: Arnoldi KV (ed) Ecology and physiology of diapause in the Colorado beetle. Indian National Scientific Documentation Centre (Translated and published in 1976 for the USDA-ARS and NSF), New Delhi, India, pp 28–58
- Riddle WA (1981) Cold hardiness in the woodland snail, *Anguispira alternata* (Say) (Endodontidae). J Thermal Biol 6: 117–120
- Riggle FR, Slack DC (1980) Rapid determination of soil water characteristic by thermocouple psychrometry. Trans Am Soc Agr Eng 23: 99–103
- Ring RA, Danks HV (1994) Desiccation and cryoprotection: overlapping adaptations. Cryo-Lett 15: 181–190
- Salt RW (1933) Some experiments on the freezing and hardening of the adults of the Colorado potato beetle, *Leptinotarsa decemlineata* Say. M.Sc. thesis, Montana State University, Bozeman, Montana
- Salt RW (1936) Studies on the freezing process in insects. Technical Bulletin of the Minnesota Agricultural Experimental Station 116: 1–41
- Salt RW (1963) Delayed inoculative freezing of insects. Can Entomol 95: 1190–1202
- Salt RW (1966) Factors influencing nucleation in supercooled insects. Can J Zool 44: 117–133
- Sømme L (1982) Supercooling and winter survival in terrestrial arthropods. Comp Biochem Physiol 73A: 519–543
- Tauber MJ, Tauber CA, Obyrcki JJ, Gollands B, Wright RJ (1988) Voltinism and the induction of aestival diapause in the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Ann Entomol Soc Am 81: 714–754
- Troeh FR, Palmer RG (1967) Introductory soil science laboratory manual. Iowa State University Press, Ames, Iowa, pp 95
- Ushatinskaya RS (1978) Seasonal migration of adult *Leptinotarsa decemlineata* (Insecta, Coleoptera) in different types of soil and physiological variations of individuals in hibernating populations. Pedobiologia 18: 120–126
- Weber DC, Ferro DN (1993) Distribution of overwintering Colorado potato beetle in and near Massachusetts potato fields. Entomol Exp Appl 66: 191–196
- Wilde J de, Duintjer CS, Mook L (1959) Physiology of diapause in the adult Colorado potato beetle (*Leptinotarsa decemlineata* Say) – I The photoperiod as a controlling factor. J Insect Physiol 3: 75–85
- Zachariassen KE (1985) Physiology of cold tolerance in insects. Physiol Rev 65: 799–832
- Zachariassen KE (1991) The water relations of overwintering insects. In: Lee RE, Denlinger DL (eds) Insects at low temperature. Chapman and Hall, New York, pp 47–63

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