Pergamon

Journal of Insect Physiology 44 (1998) 1173–1180

Journal of Insect Physiology

Long-term reduction of cold hardiness following ingestion of ice-nucleating bacteria in the Colorado potato beetle, *Leptinotarsa decemlineata*

Jon P. Costanzo[†] [a,*], Tricia L. Humphreys[‡] [a], Richard E. Lee Jr.[†] [a], Jason B. Moore[†] [a], Marcia R. Lee[‡] [a], Jeffrey A. Wyman [b]

[a] *Department of Zoology[†] and Department of Microbiology[‡], Miami University, Oxford, OH 45056, USA*
[b] *Department of Entomology, University of Wisconsin-Madison, Madison, WI 53706, USA*

Received 19 February 1998; accepted 13 April 1998

Abstract

We investigated the effect of ingestion of ice-nucleating bacteria on the supercooling capacity and cold hardiness of the Colorado potato beetle (*Leptinotarsa decemlineata* Say), a freeze-intolerant species that overwinters as adults in shallow, terrestrial burrows. Ingestion of ice-nucleating bacteria (*Enterobacter agglomerans, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas syringae*), fed on slices of potato tuber, caused an abrupt decrease in supercooling capacity. No change occurred in the supercooling capacity of beetles fed *Escherichia coli*, as this species lacks ice-nucleating activity. Ingestion rates showed that tubers treated with different species were equally palatable. During diapause induction beetles evacuated food from their guts, but nevertheless retained sufficient ice-nucleating bacteria to diminish supercooling. Beetles fed *P. fluorescens* and *P. putida* exhibited reduced supercooling even after an 8-wk exposure to simulated winter conditions. Furthermore, *P. fluorescens* was isolated 10-wk post-ingestion from diapausing beetles. Our data suggest that ingested bacteria may be retained by insects during entry into diapause and that the cold hardiness of candidate crop pests, such as *L. decemlineata*, may be reduced by feeding them ice-nucleating bacteria prior to winter diapause. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Cold hardiness; Ice-nucleating bacteria; Insect overwintering; Microbial control

1. Introduction

Many insects survive exposure to subzero winter temperatures by virtue of their capacity to supercool, a phenomenon in which a solution remains in a metastable, unfrozen state at temperatures at or below its equilibrium freezing point. Freeze-intolerant species that naturally experience subzero temperatures generally rely on supercooling for survival. The supercooling capacity of insects may be influenced by geographic origin, season, thermal acclimation, life stage, feeding status, endogenous nucleators, and cryoprotectants (Somme, 1982; Lee et al., 1993; Duman et al., 1995). Generally, the capacity to supercool is more than adequate to ensure that freezing of the animal will not occur in its usual winter microenvironment.

A growing literature has promulgated the concept of using ice-nucleating microorganisms to manipulate supercooling capacity, and hence cold hardiness, of candidate insect pests. The Colorado potato beetle (*Leptinotarsa decemlineata* Say), a chrysomelid that overwinters as adults within shallow, terrestrial burrows, is an excellent model for the practical development of this approach. *L. decemlineata*, which has a devastating impact on world-wide agroeconomy (Casagrande, 1987), does not tolerate freezing but nevertheless overwinters in sites where subzero temperatures occur (Mail and Salt, 1933; Minder, 1962; Kung et al., 1992; Milner et al., 1992). Thus, insects inoculated with a potent ice nucleator exhibit diminished supercooling capacity and, presumably, increased winter mortality. Our previous work suggests that exposure of *L. decemlineata* and other insect pests to various ice-nucleating agents,

* Corresponding author. Tel: + 1-513-529-3173; Fax: + 1-513-529-6900; E-mail: costanzo@msmail.muohio.edu

0022-1910/98/$19.00 © 1998 Elsevier Science Ltd. All rights reserved.
PII: S0022-1910(98)00082-1

including certain strains of bacteria, effectively promotes this outcome (Lee et al., 1992a, b, 1994).

One of the challenges of using ice-nucleating microorganisms for biological control of insects is to develop an effective means for exposing pests to the nucleating agent. In the case of *L. decemlineata*, one strategy is to treat adults in late summer, shortly before they burrow into the soil and enter diapause. This strategy requires that the ice-nucleating microorganism must be retained through diapause induction and into winter, when soil temperatures decrease sufficiently to promote freezing of the beetles (Mail and Salt, 1933; Minder, 1962; Milner et al., 1992; Boiteau and Coleman, 1996). Conceivably, these agents may be lost or degraded during diapause induction, which represents a major physiological transition from an active feeding stage to one characterized by aphagia, altered water balance, depressed metabolism, and gut evacuation in preparation for winter. Furthermore, the ice-nucleating agent must retain its potency until ambient temperatures fall into the effective range. Some studies have reported a premature loss of nucleating activity of ice-nucleating microbes associated with heat exposure (Lee et al., 1994; Fields et al., 1995).

To overcome these limitations, we are investigating methods for establishing living ice-nucleating microorganisms in the insect gut or on the cuticle to achieve reliable, persistent effects on diminishing supercooling capacity. Our primary objective in the present study was to determine whether the feeding of various ice-nucleating bacteria to *L. decemlineata* in late summer could influence the supercooling capacity of beetles after diapause induction and after an extended period of winter diapause. We also wished to determine the effects of our novel feeding protocol on beetle body mass and hydration state.

2. Material and methods

2.1. Experimental animals

Adult Colorado potato beetles were collected in mid September 1994 from cultivated potato fields at Hancock Agricultural Research Station, Waushara Co., central Wisconsin. Most beetles were second-generation progeny produced (in 1994) in response to unusually warm temperatures and low rainfall. They were collected by baiting recently-harvested potato (*Solanum*) fields with spoiled tubers, and shipped under refrigeration, via overnight carrier, to Miami University. Upon arrival, groups of ~ 50 beetles each were placed in ventilated plastic cups containing 400 g medium-grained silica sand which had been autoclaved, dried in a 65°C oven, and moistened with sterilized, deionized water (25 mg water/g dry sand).

2.2. Preparation of the ice-nucleating bacteria

Cultures were prepared of four species of ice-nucleating bacteria: *Enterobacter agglomerans* BBI (insect origin; Lee et al., 1991) *Pseudomonas putida* F-31 and *P. fluorescens* F-12 (frog origin; Lee et al., 1995), and *P. syringae* cit7 (plant origin; provided courtesy of S. Lindow, Berkeley, CA), as well as *Escherichia coli* ATCC 35421, a species that lacks ice-nucleating activity (Orser et al., 1985). Cultures were removed from storage (− 70°C) and grown aerobically on NAG (nutrient agar with glycerol, 2.5% v/v) at 20°C for 48 h, a regimen that induces expression of ice-nucleating activity.

Ice-nucleating activity of bacterial cultures was assayed by a freezing-droplet method (Vali, 1971), as modified by Lindow (1983), using standardized cell suspensions (10^8 cells/ml sterilized, deionized water) prepared by interpolation from a standard curve relating cell density (as measured with a hemocytometer) to absorbance at 550 nm. Nucleating activity of each suspension was assessed from the distribution of crystallization temperatures (T_c) determined for a group of 40, 10 μl droplets, which were applied to an aluminum pan that floated on the surface of a refrigerated ethanol bath. The T_c of each droplet was recorded as the pan was cooled (0.3°C/min) from 0 to − 6°C. From these data we determined the $T_{c\ max}$ (freezing of the first droplet), as well as the $T_{c\ 50}$ and $T_{c\ 90}$ (freezing of the first 50% and 90% of the droplets, respectively).

In addition to cultured live bacteria, we also fed beetles a suspension (1.0 mg/ml sterilized, deionized water) of a commercial preparation of UV-radiated (killed), lyophilized *P. syringae* (2.02 × 10^4 ice-nucleating sites/g; $T_{c\ max} > -2$°C; Genencor International, Inc., San Francisco, CA).

2.3. Feeding experiments

In preparation for the feeding experiments, beetles were held without food for 24 h at ~ 23°C (12:12, L:D). Beetles were randomly assigned to groups (n = 10 beetles/group) and each group was placed in a sterilized, 60 × 15 mm petri dish containing one slice (40 mm diameter × 5 mm thick) of potato tuber, whose upper surface was evenly coated with 0.3 ml of the prescribed bacterial suspension. Beetles were permitted to feed for 21 h, removed from the feeding dishes and combined with animals from 27 replicate dishes, and then randomly assigned to groups (n = 50 beetles/group) which were placed in freshly-prepared cups containing moistened, autoclaved sand. This procedure was performed for six treatment groups representing each of the six bacterial suspensions.

At the end of the feeding period, the consumed portions of tuber, which appeared as cavities in an otherwise smooth surface, were recorded by shading comparable

areas on a paper template and quantified using image analysis software (ImagePro Plus 3.0, Media Cybernetics, Silver Springs, MD). These data, compiled for a sample of 10 tuber slices representing each treatment group, were expressed as the percent of total surface area consumed.

2.4. Sampling design and diapause induction regimen

Beetles were assayed for supercooling capacity, body mass, body water content, and the presence of bacteria in the gut at three time points: 1) ~ 1.5 h after removing beetles from food; 2) at the conclusion of a 12 d diapause induction regimen (exposure to 15°C; 10:14, L:D); and 3) after diapausing beetles had been maintained under simulated winter conditions (exposure to 5°C; DD) for 8 wks (i.e., 10 wks after feeding). Sterilized, deionized water was periodically added to the sand in the holding cups to maintain the desired substrate moisture level, but the beetles were otherwise undisturbed. Many of the beetles in each treatment group died during the experiment, thus relatively few beetles were available for testing in the final sampling for some treatment groups. Only beetles deemed healthy on the basis of color and responsiveness to handling were used in experiments.

2.5. Measurement of supercooling capacity

At each sampling, 20 beetles (or fewer, in some cases) were selected for study from among the cups in each treatment group. Beetles were gently sieved from the sand and placed individually in a plastic 1.8 ml microcentrifuge tube. The tube was then plugged with plastic foam which anchored the sensing junction of a 36-gauge thermocouple against the beetle's ventral surface, and prevented egress. The thermocouple was connected to a multichannel data logger (OM500 or RD3752, Omega Electronics, Stamford, CT) which plotted beetle temperature at 5 s intervals. Each tube was inserted into a glass test tube suspended in a refrigerated bath initially set at 0°C. After attaining thermoequilibrium, beetles were cooled at 0.25°C/min until each had begun to freeze, as heralded by the appearance of an exotherm (i.e., release of the latent heat of crystallization). The resultant T_c was determined from the plotted temperature record. At the conclusion of the supercooling trials, we determined beetle fresh mass and, after beetles had been thoroughly dried in a 65°C oven, dry mass and the water content. Water content was calculated from the mass lost during drying.

2.6. Recovery of ice-nucleating bacteria from beetle guts

At each sampling, additional beetles in each treatment group (when available) were assayed for the presence of ice-nucleating bacteria and other, incidental bacteria in their guts. The guts of these beetles were aseptically removed, placed on agar, and incised to release their contents and expose the inner wall. Guts from beetles fed ice-nucleating bacteria were plated on NAG, whereas those from beetles fed *E. coli* were plated on eosin methylene blue (EMB) agar. Inoculated plates were inverted, incubated in the dark at 20°C and visually inspected 7 d later for the presence of bacterial colonies, both inoculated and incidental. An API 20E biochemical test strip (bioMerieux Vitek, Inc., Hazelwood, MO) was used to identify putative *E. agglomerans* and *E. coli* colonies, whereas an oxidase test was used to confirm colonies morphologically resembling *P. fluorescens* and *P. putida*. Oxidase-positive colonies were subjected to the following additional tests: gelatinase, lecithinase, lipase, glucose, pyocyanin, fluorescein, and L-arginine dihydrolase. Suspected *P. syringae* isolates (from beetles fed live *P. syringae*) were confirmed using the oxidase test, L-arginine dihydrolase, and King's medium B (KB; King et al., 1954; Lindow et al., 1983). We determined the nucleating activity of all gut isolates by assaying an aqueous suspension prepared of each plate's primary streak, standardized for cell density by adjusting turbidity to 0.5 MacFarland standard (Lennette et al., 1985), using the droplet-freezing procedure.

3. Results

3.1. Nucleating activity of ice-nucleating microorganisms

Droplet-freezing assays revealed that suspensions of ice-nucleating bacteria applied to tuber slices exhibited potent nucleating activity, whereas the control suspension containing *E. coli* did not (Table 1). Suspensions

Table 1
Ice-nucleating activity of bacterial suspensions fed to Colorado potato beetles

	$T_{c\ max}$	$T_{c\ 50}$	$T_{c\ 90}$
Enterobacter agglomerans	− 2.5	− 3.3	− 3.5
Pseudomonas fluorescens	− 3.5	− 4.0	− 4.6
P. putida	− 3.1	− 4.1	− 4.9
P. syringae	− 3.0	− 3.4	− 4.5
Escherichia coli (control)	< − 6.0		
sterilized water	< − 6.0		

Indicated are the crystallization temperature (°C) of the first droplet to freeze ($T_{c\ max}$), and the crystallization temperatures associated with the freezing of 50% ($T_{c\ 50}$) and 90% ($T_{c\ 90}$) of the droplet population (n = 40) representing each organism. Concentration of bacterial suspensions was 10^8 cells/ml.

prepared from all species of ice-nucleating bacteria were highly potent, as half the droplets froze before cooling to − 3 to − 4°C. Generally, *E. agglomerans* was the most active agent and *P. putida* was the least (Table 1).

3.2. Feeding responses of Colorado potato beetles

Beetles fed voraciously on the treated tuber slices, concentrating on the periphery and upper, exposed surface. We assumed that the total area of the tuber's surface removed by feeding beetles was indicative of the amount of tuber they consumed. Accordingly, there were no differences (ANOVA: $F_{5,54} = 1.2$, $P > 0.34$; Table 2) in tuber consumption among the different treatment groups, suggesting that all species of bacteria were equally palatable. Based on the amount of bacteria applied to each bait, the calculated ingestion rate was $\sim 1.2 \times 10^6$ bacterial cells per beetle (Table 2). Ingestion rates were not limited by the quantity of food provided, because ≥ 55% of the offering remained at the conclusion of the feeding period. During and after feeding, frass was abundant within the dishes, indicating that some food had completely passed through the gut.

3.3. Supercooling capacity of beetles and recovery of bacteria from beetle guts

Beetles ingesting tuber treated with *E. coli* exhibited T_c values comparable to those of unfed control beetles (~ − 7°C), whereas beetles recently fed ice-nucleating bacteria supercooled much less (ANOVA: $F_{18,352} = 25.8$, $P < 0.0001$; Table 3). Persistence of this effect was determined for beetles tested at the onset of diapause (12 d post-ingestion) and for diapausing beetles tested after 8 wks of exposure to simulated winter conditions (10 wk post-ingestion), by comparing mean T_c for treated beetles with that for the unfed group, as well as that representing the *E. coli* control group at each sample interval. The effectiveness of ingested bacteria in elevating T_c was lost in beetles fed suspensions of *E. agglomerans* and both live and killed *P. syringae* during diapause induction. However, beetles fed *P. fluorescens* or *P. putida* exhibited diminished supercooling capacity, even after remaining in diapause for 8 wks (Table 3).

With the exception of *E. coli*, all species of the microorganisms fed to beetles were found in the respective guts of at least some beetles shortly after feeding (Table 4). This was also the case for most treatment groups after beetles were induced into diapause. However, the species ingested was recovered from beetles in only one treatment group (*P. fluorescens*) after having been exposed to simulated winter conditions for 8 wks. All cultures of ice-nucleating bacteria derived from gut isolates exhibited ice-nucleating activity comparable to that of the original suspensions (data not shown).

Along with the target species, cultures of guts from both fed and unfed beetles produced additional aerobic colonies presumed to be bacterial and fungal elements of the normal flora. These species appeared most frequently in cultures from recently-fed beetles (which had been feeding in the field up to several days before use in our experiments), but less commonly in cultures from beetles subjected to diapause induction or prolonged exposure to simulated winter conditions. The relative proportion of ingested target species to other microbes varied among the treatment groups. The ice nucleating strain of *P. fluorescens* was not only recovered in 83% of beetles fed this organism at 10 wks post-ingestion, but was also the predominant aerobic species cultured from the gut. Furthermore, non-nucleating strains of *P. fluorescens* were cultured from beetles in all treatment groups, suggesting that this species is a common constituent of the beetle's normal gut flora.

3.4. Changes in body mass and water content

Feeding and diapause induction strongly influenced beetle body mass (ANOVA: $F_{16,297} = 3.6$, $P < 0.0001$; Table 5). Recently-fed beetles weighed ~ 25 milligrams more than unfed beetles. However, during diapause induction, beetles returned to their original mass. This result, together with the appearance of frass in their holding cups, indicates that beetles eliminated the contents of their guts during this period. With the exception of

Table 2
Consumption of potato tuber slices treated with bacteria, as indicated by the area of the surface removed, by Colorado potato beetles. Mean values shown ± 1 SEM, based on results from $n = 10$ feeding stations per treatment group

	Tuber consumption area (mm^2)	% of offering	Ingested cells ($\times 10^6$) per beetle
E. coli (control)	483 ± 19	39 ± 1	1.2 ± 0.05
Enterobacter agglomerans	487 ± 41	39 ± 3	1.2 ± 0.01
Pseudomonas flourescens	560 ± 24	45 ± 2	1.4 ± 0.06
Pseudomonas putida	516 ± 39	42 ± 2	1.2 ± 0.10
Pseudomonas syringae			
live	496 ± 38	40 ± 3	1.2 ± 0.10
killed	556 ± 22	45 ± 2	—

Table 3
Supercooling capacity, as determined by temperature of crystallization (T_c, °C), of Colorado potato beetles at three time points after ingestion of ice-nucleating bacteria, relative to unfed controls and beetles fed a non-ice-nucleating strain of *E. coli*

Feeding treatment	Recently fed (~ 1.5 h post-ingestion)	Diapause onset (12 d post-ingestion)	Diapause (10 wk post-ingestion)
unfed	− 6.8 ± 0.1 (19)	–	–
E. coli (control)	− 6.9 ± 0.1 (20)	− 7.2 ± 0.2 (20)	− 6.4 ± 0.2 (20)
Enterobacter agglomerans	− 4.7 ± 0.4*† (19)	− 6.3 ± 0.3 (20)	− 6.8 ± 0.4 (20)
Pseudomonas fluorescens	− 4.2 ± 0.1*† (20)	− 4.3 ± 0.3*† (20)	− 3.9 ± 0.3*† (20)
Pseudomonas putida	− 3.9 ± 0.1*† (20)	− 5.0 ± 0.4*† (20)	− 3.4 ± 0.1*† (7)
Pseudomonas syringae			
live	− 4.1 ± 0.2*† (20)	− 6.9 ± 0.1 (20)	− 5.8 ± 0.3 (8)
killed	− 4.4 ± 0.1*† (20)	− 7.4 ± 0.3 (20)	− 6.9 ± 0.3 (20)

Means (shown ± 1 SEM, based on the number of beetles given in parentheses) identified with an asterisk differed significantly from mean for unfed group, whereas those identified with a dagger differed significantly from mean for the *E. coli* group within the same column (ANOVA, Bonferroni Multiple Comparisons; $P < 0.05$).

Table 4
Recovery of previously-ingested, living ice-nucleating bacteria from the culture of guts of Colorado potato beetle

Feeding treatment	Recently fed (~ 1.5 h post-ingestion)	Diapause onset (12 d post-ingestion)	Diapause (10 wk post-ingestion)
E. coli (control)	2/10	0/10	0/4
Enterobacter agglomerans	8/10	0/10	0/7
Pseudomonas fluorescens	9/10	4/10	5/6
Pseudomonas putida	10/10	2/10	0/4
Pseudomonas syringae (live)	4/10	1/10	0/1

For each sample, the number of beetles testing positive for the bacteria is shown to the left of the virgule, the number of beetles tested is given to the right.

Table 5
Body mass (mg) of Colorado potato beetles at three time points after ingestion of ice-nucleating bacteria, relative to controls ingesting nothing or non-ice-nucleating strain of *E. coli*

Feeding treatment	Recently fed (~ 1.5 h post-ingestion)	Diapause onset (12 d post-ingestion)	Diapause (10 wk post-ingestion)
unfed	103 ± 5 (19)	–	–
E. coli (control)	128 ± 5* (20)	109 ± 4 (20)	111 ± 8 (9)
Enterobacter agglomerans	126 ± 5* (20)	119 ± 5 (20)	123 ± 8 (10)
Pseudomonas fluorescens	121 ± 8 (20)	110 ± 3 (20)	118 ± 9 (10)
Pseudomonas putida	118 ± 8 (20)	109 ± 4 (20)	— (0)
Pseudomonas syringae			
live	134 ± 5* (20)	110 ± 5 (20)	— (0)
killed	129 ± 6* (20)	108 ± 4 (20)	148 ± 8* (10)

Means (shown ± 1 SEM, based on the number of beetles given in parentheses) identified with an asterisk differed significantly from mean for unfed group (ANOVA, Dunnett Multiple Comparisons Test; $P < 0.05$).

one treatment group (beetles fed killed *P. syringae*), the body mass of beetles fed ice-nucleating bacteria, after an 8-wk exposure to simulated winter conditions, was comparable to that of recently-collected, unfed beetles (Table 5).

Mean values for body water content of beetles in different treatment groups differed significantly (ANOVA: $F_{16,297} = 2.0$, $P < 0.015$); however, this outcome was solely due to results for one group. Beetles fed killed *P. syringae* and tested 8 wks after exposure to simulated winter conditions had an unusually low water content (Table 6). Otherwise, body water content was unaffected by feeding, induction into diapause, or exposure to simulated winter conditions (Table 6).

Table 6
Mean body water content (mg water/mg dry mass) of Colorado potato beetles at three time points after ingestion of ice-nucleating bacteria, relative to controls ingesting nothing or non-ice-nucleating strain of *E. coli*

Feeding treatment	Recently fed (~ 1.5 h post-ingestion)	Diapause onset (12 d post-ingestion)	Diapause (10 wk post-ingestion)
unfed	437 ± 23 (19)	–	–
E. coli (control)	403 ± 19 (20)	405 ± 21 (20)	386 ± 26 (9)
Enterobacter agglomerans	430 ± 23 (20)	445 ± 16 (20)	450 ± 51 (10)
Pseudomonas fluorescens	460 ± 20 (20)	432 ± 19 (20)	414 ± 31 (10)
Pseudomonas putida	448 ± 25 (20)	451 ± 25 (20)	— (0)
Pseudomonas syringae			
live	461 ± 26 (20)	454 ± 28 (20)	— (0)
killed	424 ± 14 (20)	367 ± 28 (20)	323 ± 25* (10)

Means (shown ± 1 SEM, based on the number of beetles given in parentheses) identified with an asterisk differed significantly from mean for unfed group (ANOVA, Dunnett Multiple Comparisons Test; $P < 0.05$).

4. Discussion

Results of the present study bolster earlier findings (Lee et al., 1992a, b, 1994) that inoculating an insect with ice-nucleating microbes can cause a marked decrease in its capacity to supercool. The present study is unique in its approach to introducing living ice-nucleating bacteria to the target insect. *L. decemlineata* readily consumed various species of bacteria, which had the desired effect in limiting the beetles' supercooling capacity, for at least 10 wks post-ingestion. Supercooling capacity of fed beetles was reduced by up to 3.5°C, relative to unfed beetles sampled prior to diapause (mean T_c, − 6.8°C; Table 3). Given that supercooling may be markedly lower in fully-acclimatized, overwintering beetles (e.g., T_c, − 11.7°C; Boiteau and Coleman, 1996), the effect of feeding ice-nucleating microbes may be even more pronounced under field conditions. Importantly, the T_c's of beetles fed *P. fluorescens* or *P. putida* were sufficiently raised that temperatures encountered by beetles during winter (Milner et al., 1992; Boiteau and Coleman, 1996) may prove lethal.

Other workers have been successful in manipulating the gut flora of insects. Wood lice (*Porcellio scaber*) fed *P. fluorescens* purged the food bolus within 5 h, but nevertheless retained enough bacteria that they appeared in the frass for up to 6 d post-ingestion (Clegg et al., 1994). Chapco and Kelln (1994) found that the gut of a grasshopper (*Melanoplus sanguinipes*) retained ingested bacteria for > 3 wks. Queensland fruit flies (*Bactrocera tryoni*) fed two species of bacteria normally present in the gut were retained for 55 d (Murphy et al., 1994). A major finding of the present study was that ingested bacteria apparently were retained (as evidenced by the elevated T_c values for the beetles and the isolation of bacteria from some of the isolated guts) even after the gut was purged in preparation for winter diapause, a mechanism that, according to dogma, rids the body of ice nucleators that would otherwise constrain supercooling capacity. Although this result may be a consequence of ingesting such large numbers of bacteria, our recent work (unpublished data) indicates that *L. decemlineata* retain their normal gut flora during winter diapause.

Retention of bacteria within the gut after feeding varied with the particular species of bacteria ingested. For example, *E. coli* was detected in cultures prepared from only 20% of recently fed beetles. Similarly, Bakula (1969) could not establish *E. coli* in the gut of *Drosophila melanogaster* while the normal flora was still present, perhaps because *E. coli* is not a common constituent of the gut flora of insects. Given its origin as a (pathogenic) plant epiphyte, the poor retention of *P. syringae* was perhaps not surprising. On the other hand, *E. agglomerans*, which was originally isolated from the gut of a beetle (Lee et al., 1991), was not detected by either cultural recovery or its influence on supercooling capacity beyond the diapause induction regimen. Both *P. putida* and *P. fluorescens*, which were isolated from the gut of a freeze-tolerant frog (Lee et al., 1995), were detected by one or both of these methods even after beetles had been exposed to simulated winter conditions for 8 wks. Both species commonly occur in the insect gut (Tanada and Kaya, 1993; Mead et al., 1988).

Cultural recovery proved to be a more conservative indicator of the presence of ice-nucleating bacteria. For example, beetles fed either *P. fluorescens* or *P. putida* apparently retained enough of these bacteria to diminish their supercooling capacity 10 wks later (Table 3); however, we successfully recovered ingested microbes from only the former (Table 4). To overcome this limitation we are presently developing two alternative methods of detection. In one approach, a plasmid carrying a genetic marker is introduced into the bacterial strains to facilitate cultural detection. We are also developing a method for detection of the ice-nucleating gene using polymerase chain reaction (PCR). These methods will permit qualitative and quantitative analysis of retention and proliferation of ingested bacterial strains.

Diapause in adult *L. decemlineata* is triggered in late summer by decreases in photophase, ambient temperature, and food availability (de Wilde et al., 1959; Tauber et al., 1988). During entry into diapause, many insects enhance their supercooling capacity by reducing body mass and water content, as well as eliminating material from the gut (Cloudsley-Thompson, 1970; Somme, 1982; Zachariassen, 1991; Ring and Danks, 1994). In the present study, beetles preparing for diapause eliminated food from their guts, as indicated by the return of body mass to the pre-feeding level and the appearance of frass in their cages. However, our beetles did not exhibit the pronounced decrease in body mass and water content accompanying diapause induction in *L. decemlineata* under field conditions (Fink, 1925; Ushatinskaya, 1978), but rather maintained their hydration state irrespective of their feeding and activity status. Possibly some environmental cue, such as a decrease in substrate water potential, may promote these physiological responses under field conditions (Costanzo et al., 1997). Alternatively, the unusual responses of these beetles may suggest that they had emerged relatively late in summer or, perhaps less likely, were second-generation adults. Notably, the dry body mass of these beetles (generally, < 30 mg; Fig. 1) was lower than that of adults collected at the same site the previous year (average, ~ 40 mg; Costanzo et al., 1997). This explanation is consistent with our finding that many beetles died or became moribund during exposure to simulated winter conditions and a previous report that late-emerging adults typically incur relatively high overwintering mortality (Voss and Ferro, 1992).

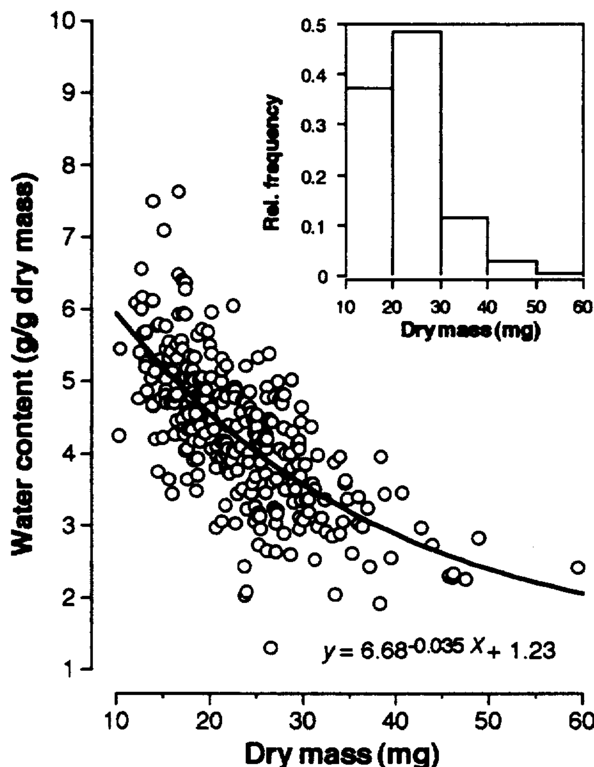

Fig. 1. Relative frequency histogram of body mass (inset), and the relationship between body mass and body water content, for second-generation adult Colorado potato beetles (*L. decemlineata*) used in the study. Curve was fitted to one-phase exponential decay model using nonlinear regression (r^2 = 0.486, df = 287, absolute sum of squares = 152.6).

Overall, our results suggest that ingestion of ice-nucleating bacteria prior to winter diapause does not have sustained effects on either body mass or body water content. Anomalous values for body mass and water content were obtained for the group of beetles fed killed, commercial preparation of *P. syringae* and tested 8 wks after exposure to simulated winter conditions. These beetles were heavier and contained less water than beetles in other groups. This outcome may result from the presence of salts and other fermentation by-products introduced during manufacture of this commercial preparation (LaDuca et al., 1995). Alternatively, the sample, drawn from the few remaining individuals in this treatment group, may have been comprised of relatively large beetles, which maintain a relatively lower body water content than smaller beetles (Fig. 1). The low body water content of large beetles more than compensates for the limitation on supercooling capacity imposed by their body size, as larger beetles actually supercool more extensively than smaller ones (Costanzo et al., 1997). Indeed, the greater supercooling capacity reported earlier for beetles from this population (T_c ~ − 10°C, Costanzo et al., 1997), relative to that of the (smaller) beetles in the present study (T_c ~ − 7°C), likely reflects the 1.9-fold higher water content of the latter.

Judging from results of the supercooling trials, ingested ice-nucleating bacteria may have been retained in the guts of *L. decemlineata* throughout diapause induction and during an extended period of simulated winter conditions. The use of these organisms for the control of insect pests requires a fundamental knowledge of bacterial-host interactions (Chapco and Kelln, 1994). Establishment of ice-nucleating bacteria in the gut may be a viable strategy of biological control of certain insects, but requires additional information concerning the long-term establishment of microorganisms in insects.

Acknowledgements

We thank Valerie Bennett for technical assistance, and two anonymous reviewers for providing thoughtful critiques of the manuscript. Grant support for this research was provided by the USDA (96-02349-99003) and the NSF (IBN-95-07437 and IBN-97-28573).

References

Bakula, M., 1969. The persistence of a microbial flora during postembryogenesis of *Drosophila melanogaster*. Journal of Invertebrate Pathology 14, 365–374.

Boiteau, G., Coleman, W., 1996. Cold tolerance in the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). Canadian Entomologist 128, 1087–1099.

Casagrande, R.A., 1987. The Colorado Potato Beetle: 125 years of mismanagement. Bulletin of the Entomological Society of America 33, 142–150.

Chapco, W., Kelln, R.A., 1994. Persistence of ingested bacteria in the grasshopper gut. Journal of Invertebrate Pathology 64, 149–150.

Clegg, C.D., Elsas, J.D., Van Anderson, J.M., Lappin-Scott, H.M., 1994. Assessment of the role of a terrestrial isopod in the survival of a genetically modified pseudomonad and its detection using the polymerase chain reaction. FEMS Microbiology Ecology 15, 161–168.

Cloudsley-Thompson, J.L., 1970. Terrestrial invertebrates. In: Whittow, G.C., (Ed.), Comparative Physiology of Thermoregulation. Vol. 1 Invertebrates and Nonmammalian Vertebrates. Academic Press, New York, NY, pp. 15–77.

Costanzo, J.P., Moore, J.B., Lee, R.E., Kaufman, P.E., Wyman, J.A., 1997. Influence of soil hydric parameters on the winter cold hardiness of a burrowing beetle *Leptinotarsa decemlineata* (Say). Journal of Comparative Physiology B167, 169–176.

De Wilde, J., Duintjer, C.S., Mook, L., 1959. Physiology of diapause in the adult Colorado potato beetle (*Leptinotarsa decemlineata* Say)-I The photoperiod as a controlling factor. Journal of Insect Physiology 3, 75–85.

Duman, J.G., Olsen, T.M., Yeung, K.L., Jerva, F., 1995. The roles of ice nucleators in cold tolerant invertebrates. In: Lee, R.E., Warren, G.J., Gusta, L.V., (Eds.), Biological Ice Nucleation and Its Applications. The American Phytopathological Society, St. Paul, Minnesota, pp. 201–219.

Fields, P.G., Pouleur, S., Richard, C., 1995. The effect of high temperature storage on the capacity of an ice-nucleating-active bacterium and fungus to reduce insect cold-tolerance. Canadian Entomologist 127, 33–40.

Fink, D.E., 1925. Physiological studies on hibernation in the potato beetle *Leptinotarsa decemlineata* Say. Biological Bulletin 6, 381–406.

King, E.O., Ward, M.K., Raney, D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44, 301–307.

Kung, K.-J.S., Milner, M., Wyman, J.A., Feldman, J., Nordheim, E., 1992. Survival of Colorado potato beetle (Coleoptera: Chrysomelidae) after exposure to subzero thermal shocks during diapause. Journal of Economic Entomology 85, 1695–1700.

LaDuca, R.J., Rice, A.F., Ward, P.J., 1995. Applications of biological ice nucleators in spray-ice technology. In: Lee, R.E., Warren, G.J., Gusta, L., (Eds.), Biological Ice Nucleation and Its Applications. American Phytopathology Society, St. Paul, Minnesota, pp. 337–350.

Lee, R.E., Strong-Gunderson, J.M., Lee, M.R., Grove, K.S., Riga, T.J., 1991. Isolation of ice nucleating active bacteria from insects. Journal of Experimental Zoology 257, 124–127.

Lee, M.R., Lee, R.E., Strong-Gunderson, J.M., Minges, S.R., 1992a. Treatment with ice nucleating active fungi and surfactants decrease insect supercooling capacity. Cryobiology 29, 743.

Lee, R.E., Strong-Gunderson, J.M., Lee, M.R., Davidson, E.C., 1992b. Ice-nucleating active bacteria decrease the cold-hardiness of stored grain insects. Journal of Economic Entomology 85, 371–374.

Lee, R.E., Lee, M.R., Strong-Gunderson, J.M., 1993. Insect cold-hardiness and ice nucleating active microorganisms including their potential use for biological control. Journal of Insect Physiology 39, 1–12.

Lee, J.R.E., Costanzo, J.P., Kaufman, P.E., Lee, M.R., Wyman, J.A., 1994. Ice-nucleating active bacteria reduce the cold-hardiness of the freeze-tolerant Colorado potato beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology 87, 377–381.

Lee, M.R., Lee, R.E., Strong-Gunderson, J.M., Minges, S.R., 1995. Isolation of ice nucleating active bacteria from the freeze-tolerant frog *Rana sylvatica*. Cryobiology 32, 358–365.

Lennette, E.H., Balows, A., Hausler, W.J., Shadomy, H.J., 1985. Manual of Clinical Microbiology. America Society for Microbiology, Washington, D.C., pp. 1095.

Lindow, S.E., Arny, D.C., Upper, C.D., 1982. Bacterial ice nucleation: a factor in frost injury to plants. Plant Physiology 70, 1084–1089.

Lindow, S.E., 1983. Methods of preventing frost injury caused by epiphytic ice nucleation active bacteria. Plant Diseases 67, 327–333.

Mail, G.A., Salt, R.W., 1933. Temperature as a possible limiting factor in the northern spread of the Colorado potato beetle. Journal of Economic Entomology 26, 1068–1075.

Mead, L.J., Khachatourians, G.G., Jones, G.A., 1988. Microbial ecology of the gut in laboratory stocks of the migratory grasshopper, *Melanoplus sanguinipes* (Fab.) (Orthoptera: Acrididae). Applied and Environmental Microbiology 54, 1174–1181.

Milner, M., Kung, K.-J.S., Wyman, J.A., Feldman, J., Nordheim, E., 1992. Enhancing overwintering mortality of Colorado potato beetle (Coleoptera: Chrysomelidae) by manipulating the temperature of its habitat. Journal of Economic Entomology 85, 1701–1708.

Minder, I.F., 1962. Hibernation conditions and survival rate of the Colorado potato beetle in different types of soils. In: Arnoldi, K.V., (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.

Murphy, K.M., Teakle, D.S., MacRae, I.C., 1994. Kinetics of colonization of adult Queensland fruit flies (*Bactrocera tryoni*) by dinitrogen-fixing alimentary tract bacteria. Applied and Environmental Microbiology 60, 2508–2517.

Orser, C., Staskawicz, B.J., Panopoulos, N.J., Dahlbeck, D., Lindow, S.E., 1985. Cloning and expression of bacterial ice nucleation genes in *Escherichia coli*. Journal of Bacteriology 164, 359–366.

Ring, R.A., Danks, H.V., 1994. Desiccation and cryoprotection: overlapping adaptations. Cryo-Letters 15, 181–190.

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

Tanada, Y., Kaya, H.K., 1993. Insect Pathology. Academic Press, San Diego, pp 666.

Tauber, M.J., Tauber, C.A., Obrycki, J.J., Gollands, B., Wright, R.J., 1988. Voltinism and the induction of aestival diapause in the Colorado potato beetles, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Annals of the Entomological Society of America 81, 714–754.

Ushatinskaya, R.S., 1978. Seasonal migration of adult *Leptinotarsa decemlineata* (Insecta, Coleoptera) in different types of soil and physiological variations of individuals in hibernating populations. Pedobiologia, Bd 18, 120–126.

Vali, G., 1971. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. Journal of the Atmospheric Sciences 28, 402–409.

Voss, R.H., Ferro, D.N., 1992. Population dynamics of the Colorado potato beetle (Coleoptera: Chrysomelidae) in western Massachusetts. American Potato Journal 69, 473–482.

Zachariassen, K.E., 1991. The water relations of overwintering insects. In: Lee, R.E., Jr., Denlinger, D.L., (Eds.), Insects at Low Temperature. Chapman and Hall, New York, NY, pp. 47–63.