

The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in *Sitophilus granarius* and *Cryptolestes ferrugineus* (Coleoptera)

Paul G. Fields ^{a,*}, Francis Fleurat-Lessard ^b, Lucien Lavenseau ^c, Gérard Febvay ^d,
Lionel Peypelut ^{b,c}, Guy Bonnot ^d

^a Cereal Research Centre, 195 Dafoe Road, Winnipeg, Manitoba R3T 2M9, Canada

^b Centre de Recherche de Bordeaux, INRA, B.P. 81, F-33883 Villenave d'Ornon Cedex, France

^c Département de Physiologie des Invertébrés, URA CNRS 1138 Neuroendocrinologie, Université de Bordeaux I, F-33405 Talence Cedex, France

^d INSA, Laboratoire de Biologie Appliquée 406, UA INRA 203, 20 av. A. Einstein, F-69621 Villeurbanne Cedex, France

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Abstract

Canadian and French laboratory strains of *Sitophilus granarius* (L.) and *Cryptolestes ferrugineus* (Stephens) were cold acclimated by placing adults at 15, 10 and 5°C successively for 2 wk at each temperature before deacclimating them for 1 wk at 30°C. Unacclimated *S. granarius* had an LT₅₀ (lethal time for 50% of the population) of 12 days at 0°C compared with 40 days after the full cold acclimation. At -10°C, unacclimated *C. ferrugineus* had an LT₅₀ of 1.4 days compared with 24 days after the full acclimation. Cold acclimation was lost within a week after returning insects to 30°C. Trehalose, as well as the amino acids proline, asparagine, glutamic acid and lysine were higher in cold acclimated insects for both species. For *S. granarius*, glutamine was higher in cold acclimated insects and isoleucine, ethanolamine and phosphoethanolamine, a precursor of phospholipids, were lower in cold acclimated insects. For *C. ferrugineus*, alanine, aspartic acid, threonine, valine, isoleucine, leucine, phenylalanine and phosphoethanolamine were higher in cold acclimated insects. For both species tyrosine was lower in cold acclimated insects. There were small but significant differences between Canadian and French strains of *S. granarius*, with the Canadian strain being more cold hardy and having higher levels of trehalose. There were small but significant differences between male and female *S. granarius*, with males being more cold hardy and having higher levels of proline, asparagine and glutamic acid. In conclusion, high levels of trehalose and proline were correlated with cold tolerance, as seen in several other insects. However, correlation does not prove that these compounds are responsible for cold tolerance, and we outline further tests that could demonstrate a causal relationship between trehalose and proline and cold tolerance. Crown copyright © 1998 Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

One of the keys to insects' success as a group is their ability to survive a wide range of environmental conditions. Cold is the major environmental obstacle that insects must overcome to survive in temperate and arctic climates. There are two general solutions to this problem: long distance migration, as is the case for the mon-

arch butterfly (*Danaus plexippus* (L.)) in North America, or overwintering with elevated levels of cold tolerance, as is the case for most temperate and arctic species (Danks, 1987). Even tropical insects and those that migrate to warmer climates during the winter have the ability to increase cold tolerance in response to low temperatures (Denlinger, 1991; Larsen and Lee, 1994).

Insects can be divided into two general groups for cold hardiness: freeze-intolerant, the most common, or freeze-tolerant (Storey and Storey, 1988; Baust and Rojas, 1985; Lee, 1991). Freeze-intolerant species cannot survive freezing, and overwintering stages usually

* Corresponding author. Tel.: +1-204-983-1468; Fax: +1-204-983-4604; E-mail: pfields@em.agr.ca

have supercooling points (SCP) 10–40°C lower than summer stages (Sømme, 1982). Freeze-tolerant species usually have SCP above – 10°C throughout the year, and these high SCPs are maintained during the winter through the production of ice nucleators (Storey and Storey, 1988). Regardless of an insect's capacity to survive freezing, most cold acclimated insects have increased levels of low molecular weight polyols, sugars or amino acids; glycerol, sorbitol, trehalose, sucrose, proline and alanine being the most common ones (Storey and Storey, 1988; Lee and Denlinger, 1991).

There are several theories explaining why organisms die at low temperatures (Yancey et al., 1982; Storey and Storey, 1988; Lee and Denlinger, 1991). These theories are classed according to damage that occurs before freezing or after freezing. Non-freezing injury or chilling injury is thought to occur because of detrimental changes in membrane viscosity, enzyme activity or ionic concentrations. Injury caused by freezing is thought to be due to physical damage to cells, or the osmotic shock as salt concentrations increase in the unfrozen cytoplasm as liquid water is lost to ice crystals during freezing.

Stored product insect pests cover a broad taxonomic range (Coleoptera: Anobiidae, Bostrichidae, Bruchidae, Cucujidae, Curculionidae, Dermestidae, Histeridae, Ptinidae and Tenebrionidae; Lepidoptera: Gelechiidae, Pyralidae and Tineidae; Psocoptera: Liposcelidae) and are thought to be tropical in origin, but now have a cosmopolitan distribution due to international commerce. In an effort to avoid the use of insecticides, low temperature is used extensively either to slow the development of pest populations or to control populations (Fields, 1992). There are more than 40 papers describing the cold tolerance of these species. Most stored product insects have SCPs from – 10 to – 20°C. None of these species have been found to be freeze-tolerant, and most species die at temperatures well above the SCP. Most stored product insects survive low temperatures in quiescence; only a few (Dermestidae and Pyralidae) have a diapause stage (Nair and Desai, 1973; Bell et al., 1983). Almost all increase their cold tolerance by two- to 10-fold when acclimated at low (15–5°C) temperatures (Fields, 1992). Despite the widespread interest in the capacity of stored product insects to survive low temperatures, we know very little of the physiological mechanisms of cold tolerance in these species. The goal of this study was to examine the changes in polyols, sugars and free amino acids in two species that are relatively cold-tolerant (Fields, 1992), *Sitophilus granarius* (L.), the granary weevil, and *Cryptolestes ferrugineus* (Stephens), the rusty grain beetle, during cold acclimation and deacclimation.

2. Materials and methods

2.1. Cold acclimation and deacclimation

Cryptolestes ferrugineus were originally collected from farms in Manitoba, Canada in 1991. The French strain of *S. granarius* was collected from farms in 1975, and the Canadian strain had been in the laboratory for more than 10 yr. Insects were maintained at 30°C, 60% RH on soft winter wheat cv Soissons; *C. ferrugineus* cultures also had 10% broken wheat and 5% wheat germ. Insects were held in the dark during the rearing and experimental conditions except during manipulations. For all experiments, adult insects were 1–4 wk old at the beginning of the acclimation. To cold acclimate the insects, jars (250 ml with filter paper lids) containing insects (700 *C. ferrugineus* or 500 *S. granarius*) and wheat (16% moisture content, wet mass basis, 110 g of wheat per jar) were placed successively at 15 ± 1°C, 10 ± 1°C, 5 ± 1°C for two wk at each temperature. To deacclimate the insects, the jars were placed at 30 ± 1°C. Throughout the experiment jars were held in closed containers over a saturated NaCl salt solution to maintain a relative humidity of 75% (Winston and Bates, 1960) which maintains the wheat at approximately 16% moisture content over the range of acclimation temperatures (Pixton and Warburton, 1971).

Subsamples of insects were taken at the beginning of the acclimation (30°C), at the end of each 2-wk acclimation period (15, 10 and 5°C) and 1, 2, 5 and 7 days after being replaced at 30°C. For each subsample the cold tolerance, trehalose, proline and moisture content levels of the insects were measured. The number of insects that died during acclimation/deacclimation was noted for each subsample. To determine if there was differential mortality between the sexes, live and dead *S. granarius* were sexed separately after cold exposure. To measure the cold tolerance, insects were placed 100 to a vial (40 ml) with 20 g of wheat and held at either 0 ± 1°C (*S. granarius*) or – 10 ± 1 (*C. ferrugineus*) for 1–63 days. The test temperature was lower for *C. ferrugineus* than *S. granarius* because it is a more cold hardy species (Fields, 1992) and would require several months at 0°C to achieve complete mortality. The SCP for *C. ferrugineus* is between – 17 and – 20°C, and for *S. granarius* it is between – 14 and – 16°C; hence, all mortality observed would be due to chilling injury. There were six to nine durations per species. The lethal time for 50 and 90% of the population were estimated using probit analysis (Finney, 1971). The moisture content of the insects was estimated by weighing 30 *S. granarius*, or 30 groups of 10 *C. ferrugineus* live, drying for 48 h at 40°C under vacuum and reweighing the dried insects.

The experiment was repeated a second time with sampling at the end of the cold acclimation, and 1 and 7 days

after being replaced at 30°C. For the second experiment, the Canadian strain of *S. granarius* was not used, and there were not enough insects to estimate cold tolerance of *C. ferrugineus*. The free amino acids were measured using an automatic amino acid analyser and the levels of trehalose and proline were assessed using gas chromatography.

2.2. Trehalose and proline

Insects (individually for *S. granarius* or in groups of 10 for *C. ferrugineus*) were weighed, placed in 1.5 mL conical polypropylene centrifuge tubes (Eppendorf) and ground by hand in 200 μL of 70% ethanol. There were 13–15 replicates per species per treatment. After the addition of 50 μL of pyridine and mixing, the samples were centrifuged at 6000g at 4°C for 20 min. 200 μL of the supernatant was placed in a glass gas chromatography vial (Wheaton), 20 μL of 2 g L⁻¹ solution of erythritol (the internal standard, retention time 10.7 min; Gillyboeuf et al., 1994) added and the vial placed on a hot plate (approximately 40°C) to dry. Once dry, samples were stored at 4°C. Samples were taken from the refrigerator, reheated for 30 min on the hot plate to prevent water condensation, and silylated by adding in succession: 28 μL of hexamethyldisilazane, 8 μL of pyridine, and 4 μL of trifluoroacetic acid. The vials were sealed and the samples held at room temperature for 18–72 h before injecting 0.5 μL into the gas chromatograph. The gas chromatograph (Chrompack CP 9000) had a capillary column (CP Sil 5, 25 m long, internal diameter 0.25 mm with a 0.12 μm thick film), with a flame ionization detector. The oven temperature rose from 100 to 114°C at 2°C min⁻¹ and from 114 to 280°C it rose 8°C min⁻¹. The areas under the peaks were integrated electronically. Standard curves were made for glycerol, proline, sorbitol and trehalose (retention times 5.3, 5.9, 17.6, and 26.2 min, respectively) using 0.02, 0.25, 0.5, 1.0, 2.0 g L⁻¹ concentrations. The detection limits were approximately 4.6, 12, 2.9, 1.6 nmol mg⁻¹ insect fresh weight, respectively. The identity of the gas chromatography peaks of trehalose and proline was confirmed using gas chromatography/mass spectrophotometry (G. Bourgeois, CESAMO, Université de Bordeaux).

2.3. Free amino acids

To measure the free amino acid concentrations of whole insects, a second experiment was conducted with the French strain of *S. granarius* and with *C. ferrugineus*. In this experiment, the insects were sampled at the end of the cold acclimation, and 1 and 7 days after being replaced at 30°C. The levels of trehalose and proline were measured using gas chromatography again for these samples (both species) as well as the cold tolerance for the French strain of *S. granarius*. Immediately at the

end of each acclimation/deacclimation period, insects were weighed and *S. granarius* were sexed. For each treatment there were 4–5 replicates. There was one insect/replicate for *S. granarius* or 10 insects/replicate for *C. ferrugineus*. *Sitophilus granarius* were sexed before the chemical analysis. To extract free amino acids, each sample was ground by hand in 250 μL of trichloroacetic acid (5% w/v) with 25 (*S. granarius*) or 12.5 (*C. ferrugineus*) nmole of glucosaminic acid added as an internal standard. The homogenate was centrifuged at 9500g for 10 min at 4°C to eliminate cellular fragments and proteins. After having removed trichloroacetic acid and lipids by three extractions with 250 μL of chloroform, the sample was dried by evaporation, centrifuged under vacuum at room temperature (Speedvac) and taken up with 120 μL (*S. granarius*) or 70 μL (*C. ferrugineus*) of 0.05 M lithium citrate buffer pH 2.2. The sample was then submitted to ion exchange liquid chromatography on an automatic amino acid analyser (Beckman 6300), in which amino acids were detected by ninhydrin reaction, identified by their retention time and wavelength ratio, and quantified by their absorption at 570 nm (440 nm for proline).

3. Results

3.1. Cold tolerance

Slowly cooling *S. granarius* and *C. ferrugineus* adults from 30 to 5°C increased their cold tolerance (Figs 1 and 2). Unacclimated *S. granarius* (French strain) had an LT₅₀ of 12 days at 0°C (9–15 days, 95% fiducial limits) and an LT₉₀ of 22 days (17–37 days). After the full cold acclimation schedule *S. granarius* had an LT₅₀ of 40 days (36–44 days) and an LT₉₀ of 68 days (59–85 days). At -10°C, unacclimated *C. ferrugineus* had an LT₅₀ of 1.4 days (1.0–1.7 days) and an LT₉₀ of 2.7 days (2.1–3.8 days). After the full acclimation schedule *C. ferrugineus* had an LT₅₀ of 24 days (16–35 days) and an LT₉₀ of 42 days (30–120 days). Returning insects to 30°C caused a loss of cold acclimation within a week.

The fiducial limits between the LT₅₀ of the Canadian and French strains of *S. granarius* (Fig. 1) overlapped in all but one treatment (final sampling at 30°C). However, the LT₅₀ of the Canadian strain was consistently longer than the French strain, demonstrating a significant difference when the experiment is taken as a whole (Wilcoxon Signed Rank Test, $n = 7$, $P = 0.016$). Similar trends were not seen with the LT₉₀ (Wilcoxon Signed Rank Test, $n = 7$, $P = 0.98$).

The second experiment with the French strain of *S. granarius* gave levels of cold tolerance similar to the first experiment. After the full cold acclimation this strain had an LT₅₀ of 34 days (26–52 days) and an LT₉₀ of 64 days (45–221 days). After 1 day at 30°C, *S. grana-*

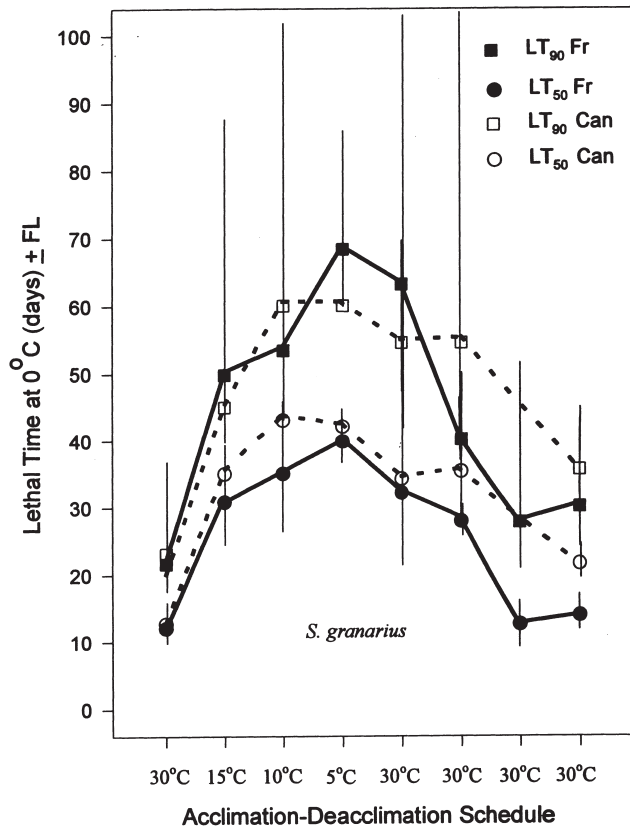


Fig. 1. The changes in survival at 0°C as measured by the lethal time for 50% (LT_{50}) and 90% (LT_{90}) \pm 95% fiducial limits (FL) of the population of adult *S. granarius* French and Canadian strains in relation to cold acclimation and deacclimation. Insects were successively exposed to 15, 10, and 5°C for 2 wk at each temperature to cold acclimate them, and returned to 30°C for 1, 2, 5 and 7 days before being placed at 0°C to test for cold survival.

rius had an LT_{50} of 30 days (no estimate of the fiducial limits as $g = 4.7$, g , or index of significance for potency estimation should be less than 1, Finney, 1971) and an LT_{90} of 50 days. After 7 days at 30°C, *S. granarius* had an LT_{50} of 19 days (13–24 days) and an LT_{90} of 28 days (23–60 days).

3.2. Trehalose and proline

For *C. ferrugineus* during the acclimation and deacclimation, trehalose (Fig. 3) and proline (Fig. 4) levels followed the same trends as were observed for the cold tolerance (trehalose; $r^2 = 0.40$, $n = 7$, $P = 0.053$, proline; $r^2 = 0.68$, $n = 7$, $P = 0.022$), increasing during acclimation and decreasing during deacclimation. The situation is more complex for *S. granarius*. There was an initial drop in trehalose after 2 wk at 15°C, after which there was a consistent rise in trehalose until insects were returned to 30°C. Similar trends were seen for proline (Fig. 4). Consequentially, there was no linear correlation between cold tolerance and trehalose or proline. However, the highest levels of trehalose and proline were

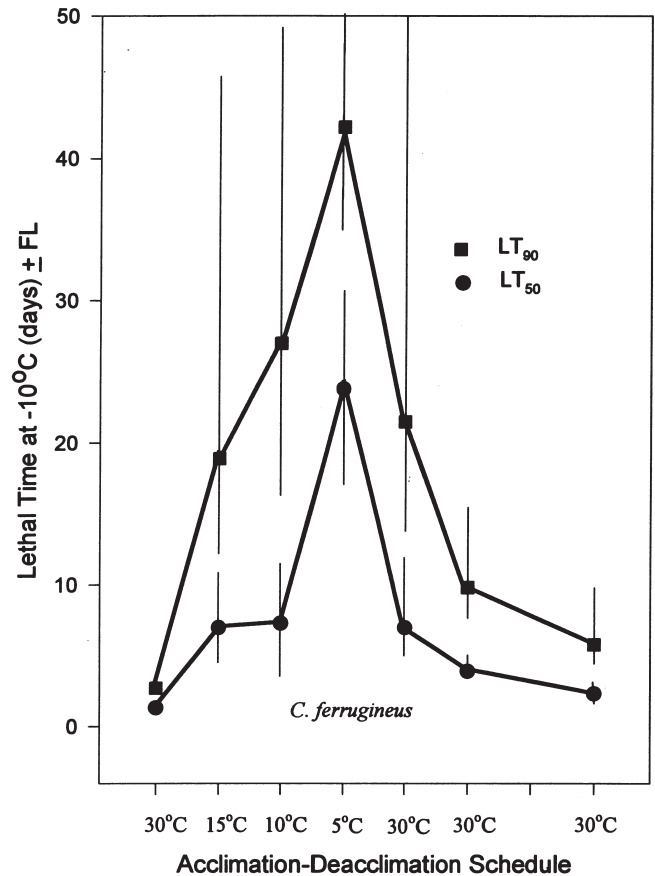


Fig. 2. The changes in survival at -10°C as measured by the lethal time for 50% (LT_{50}) and 90% (LT_{90}) \pm 95% fiducial limits of the population of adult *C. ferrugineus* in relation to cold acclimation and deacclimation. Cold acclimation and deacclimation as in Fig. 1.

found in the most cold-tolerant insects. Once cold acclimated, the Canadian strain of *S. granarius* had higher levels of trehalose than the French strain and both strains of *S. granarius* had more trehalose than *C. ferrugineus*. There was no difference in proline levels between the species once acclimated. In both species, glycerol and sorbitol were below detection limits.

The second experiment resulted in levels of trehalose and proline with trends similar to the first experiment. The French strain of *S. granarius* after the full acclimation schedule had 45 ± 3 nmol trehalose mg^{-1} (mean \pm SEM) and 58 ± 3 nmol proline mg^{-1} after 1 day at 30°C; 27 ± 2 nmol trehalose mg^{-1} and 43 ± 2 nmol proline mg^{-1} and after 7 days at 30°C; 44 ± 2 nmol trehalose mg^{-1} , 44 ± 2 nmol proline mg^{-1} . For *C. ferrugineus* after the full acclimation schedule there were: 31 ± 1 nmol trehalose mg^{-1} and 49 ± 2 nmol proline mg^{-1} , after 1 day at 30°C; 30 ± 1 nmol trehalose mg^{-1} and 40 ± 1 nmol proline mg^{-1} and after 7 days 22 ± 1 nmol trehalose mg^{-1} and 35 ± 1 nmol proline mg^{-1} .

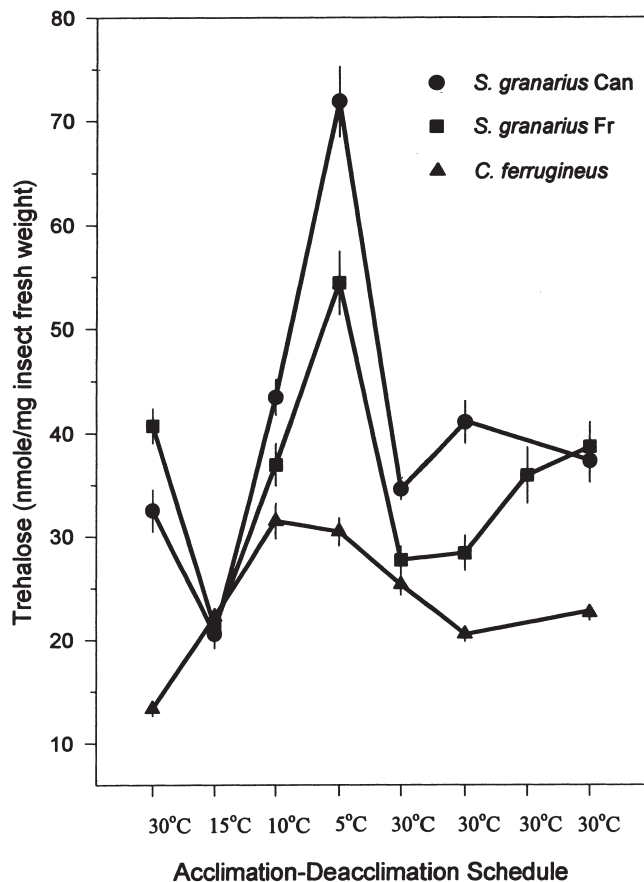


Fig. 3. The changes in trehalose (nmole mg^{-1} whole insect fresh weight, mean \pm SEM) of *S. granarius* French and Canadian strains, and *C. ferrugineus*. Cold acclimation and deacclimation as in Fig. 1.

3.3. Free amino acids

There were several differences in free amino acids between cold acclimated insects and insects that had lost their cold acclimation by holding them for 1 or 7 days at 30°C (Tables 1 and 2). In both species, proline, the most abundant amino acid, was reduced during deacclimation (Fig. 4). There was a good agreement between the two methods for determining proline, the intercept was not significantly different from 0 ($P = 0.22$) and the slope of the line was close to 1 (proline by gas chromatography = 0.79 ± 0.17 (proline by liquid chromatography) + 11 ± 8 , $r^2 = 0.75$, $P = 0.003$). Three other amino acids, asparagine, glutamic acid and lysine, also decreased with deacclimation and tyrosine increased with deacclimation in both *C. ferrugineus* and *S. granarius*.

With *C. ferrugineus* seven more amino acids decreased with deacclimation, alanine, aspartic acid, threonine, valine, isoleucine, leucine and phenylalanine, as well as phosphoethanolamine, a phospholipid precursor, while beta-alanine increased during deacclimation. For *S. granarius*, there were also several significant differences during deacclimation. Glutamine decreased

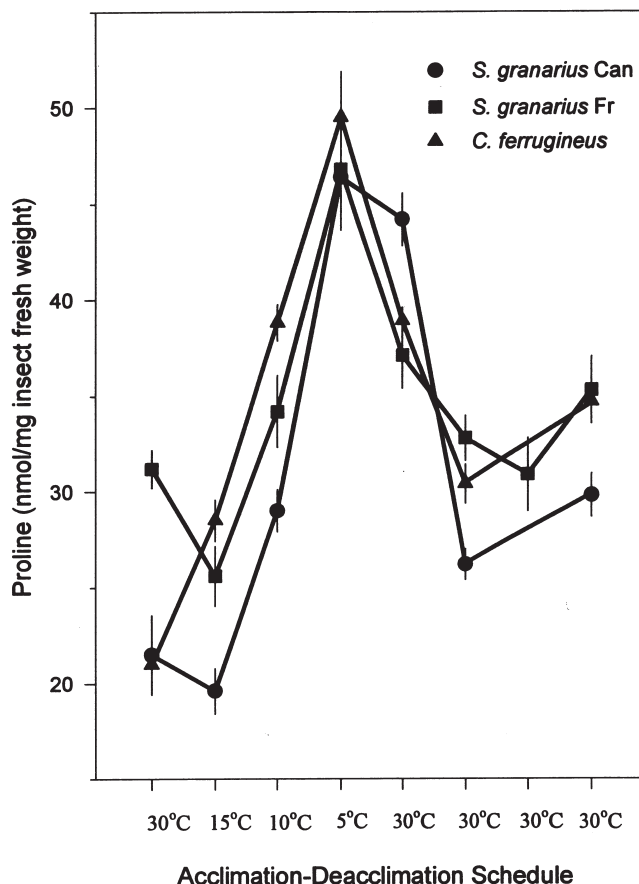


Fig. 4. The changes in proline (nmole mg^{-1} whole insect fresh weight, mean \pm SEM) of *S. granarius* French and Canadian strains, and *C. ferrugineus* as measured by gas chromatography. Cold acclimation and deacclimation as in Fig. 1.

consistently with deacclimation and phosphoethanolamine, isoleucine and ethanolamine increased consistently with deacclimation (Table 2). However, many of the amino acids did not show a consistent trend of decline or increase with deacclimation. For example, glycine was greatest after 1 day at 30°C with the cold acclimated insects and insects that had been held at 30°C for 7 days had less glycine.

3.4. Gender differences

Male *S. granarius* French strain had significantly higher survival than females in 12 of the 37 samples (z -test, $P \leq 0.05$, one-tailed test, each sample was an acclimation–duration combination with 100 insects, samples with 0 or 100% survival excluded); in the remaining 25 samples there were no differences between males and females. When all the samples were totalled there was 45.0% ($n = 2097$) survival for males and 35.6% ($n = 2294$) survival for females (z -test, $P \leq 0.001$). Similar trends were seen for *S. granarius* Canadian strain, significantly more males survived than females in 4 of 19 samples (z -test, $P \leq 0.05$); in the

Table 1

Concentration of free amino acids in whole adults of *C. ferrugineus* after different treatments: cold acclimated (the insects have been placed successively at 15°C, 10°C and 5°C for 2 wk each) or cold acclimated and then placed at 30°C for 1 or 7 days

Amino acid ^a	Free amino acid concentration (nmole mg ⁻¹ fresh weight)			ANOVA ^b
	Cold acclimated	Cold acclimated + 1 day at 30°C	Cold acclimated + 7 days at 30°C	
PETN	2.28 ± 0.13 a	0.88 ± 0.23 b	0.46 ± 0.01 b	***
ASP	1.12 ± 0.03 a	1.02 ± 0.06 a	0.76 ± 0.01 b	***
THR	5.9 ± 0.4 a	5.0 ± 0.2 ab	3.9 ± 0.5 b	**
SER	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	ns
ASN	0.37 ± 0.05 a	0.25 ± 0.02 b	0.22 ± 0.01 b	*
GLU	6.8 ± 0.1 a	4.5 ± 0.1 b	4.3 ± 0.1 b	***
GLN	5.2 ± 0.9	4.7 ± 0.3	3.0 ± 0.3	ns
PRO	48.1 ± 1.8 a	36.3 ± 2.1 b	29.2 ± 1.5 c	***
GLY	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	ns
ALA	8.6 ± 0.5 a	6.7 ± 0.5 b	6.5 ± 1.1 b	*
VAL	4.1 ± 0.1 a	2.9 ± 0.1 b	2.3 ± 0.2 c	***
CYS	0.004 ± 0.001	0.35 ± 0.28	0.02 ± 0.01	ns
CTA	0.014 ± 0.003	0.017 ± 0.003	0.018 ± 0.004	ns
ILE	1.49 ± 0.06 a	0.72 ± 0.03 b	0.76 ± 0.06 b	***
LEU	0.78 ± 0.04 a	0.57 ± 0.02 b	0.55 ± 0.07 b	**
TYR	0.89 ± 0.03 b	0.99 ± 0.05 ab	1.20 ± 0.11 a	*
PHE	0.49 ± 0.05 a	0.21 ± 0.01 b	0.27 ± 0.02 b	***
β-ALA	0.18 ± 0.02 b	0.20 ± 0.01 b	0.34 ± 0.06 a	*
ETN	0.26 ± 0.02	0.29 ± 0.04	0.32 ± 0.09	ns
ORN	0.14 ± 0.02	0.10 ± 0.02	0.09 ± 0.01	ns
LYS	3.5 ± 0.5 a	1.5 ± 0.1 b	2.0 ± 0.1 b	**
HIS	2.2 ± 0.2	1.7 ± 0.3	2.4 ± 0.2	ns
ARG	3.2 ± 0.1	3.0 ± 0.1	3.1 ± 0.2	ns
Total	97.9 ± 1.6 a	74.2 ± 1.7 b	64.1 ± 4.1 c	***

Note: There were 4–5 replicates and 10 insects in each replicate; data are expressed as mean ± SE.

^aPETN = phosphoethanolamine; CTA = cystathionine; ETN = enthanolamine. Methionine was not determined, its concentration being below the sensitivity of method, ninhydrin-positive non-amino acids PETN and ETN are also included.

^b*P*-values of ANOVA are expressed as: ns = not significant; * = *P* < 0.05; ** = *P* < 0.01; *** = *P* < 0.001. When the treatment effect was significant, the three treatments were further compared with Student–Newman–Keuls test: columns with different letters are significantly different. As proline and alanine have been previously shown to increase with acclimation, one-tailed tests were used for these amino acids.

remaining 15 samples there were no differences between males and females. For all the samples combined there was 50.7% (*n* = 885) survival for males and 37.2% (*n* = 1149) for females (*z*-test, *P* ≤ 0.001).

Males had approximately 6 nmol mg⁻¹ more trehalose than females (cold acclimated, male 49 ± 4, female 42 ± 3; cold acclimated + 1 day at 30°C, male 29 ± 4, female 24 ± 3; cold acclimated 7 days at 30°C, male 48 ± 4, female 41 ± 3 nmol mg⁻¹, mean ± SEM; two-way ANOVA, acclimation *P* = 0.0001, sex *P* = 0.037). For proline, using the automatic amino acid analyser method, males had more proline than females (Table 2), and these differences disappeared when insects lost their cold acclimation. Using the gas chromatography method, we also see differences with males having more proline than females (cold acclimated, male 62 ± 4, female 53 ± 3; cold acclimated + 1 day at 30°C, male 46 ± 2, female 41 ± 2; cold acclimated + 7 days at 30°C, male 46 ± 3, female 41 ± 1 nmol mg⁻¹, mean ± SEM; two-way ANOVA, acclimation *P* = 0.001, sex *P* = 0.006). Asparagine, glutamic acid and several other amino acids were

also higher in males than females. In contrast, isoleucine and lysine were higher in females.

3.5. Water content and acclimation mortality

For *S. granarius*, insect water content dropped during cold acclimation from a high of 54% to 44% (wet mass basis). When returned to 30°C, they gained water to 52% (Fig. 5). The second experiment with the French strain gave similar results, except for the last measure (cold acclimated; 44.9 ± 0.5%, cold acclimated + 1 day at 30°C; 49.3 ± 0.6%, cold acclimated + 7 days at 30°C; 45.7 ± 1.1%). The fresh weight varied little (2.62 ± 0.05–2.30 ± 0.04 mg/adult for the French strain, 3.30 ± 0.08–3.55 ± 0.06 mg/adult for the Canadian strain), and there was no difference in fresh weight between male and females (two-way ANOVA, acclimation *P* = 0.66, sex *P* = 0.66, second experiment, with the French strain).

For *C. ferrugineus*, insect water content remained stable during cold acclimation (Fig. 5). The drop in moisture content after returning insects to 30°C was not

Table 2

Concentration of free amino acids in whole adults of *S. granarius* after different treatments: cold acclimated (the insects have been placed successively at 15°C, 10°C and 5°C for 2 wk each) or cold acclimated and then placed at 30°C for 1 or 7 days

Amino acid ^a	Sex	Free amino acid concentration (nmole mg ⁻¹ fresh weight)			ANOVA ^b	
		Cold acclimated	Cold acclimated + 1 day at 30°C	Cold acclimated + 7 days at 30°C	Treatment effect	Sex effect
PETN	F	5.2 ± 0.7 a	6.2 ± 1.5 a	8.3 ± 0.3 b	***	ns
	M	3.0 ± 0.2	4.4 ± 0.6	8.8 ± 1.0		
ASP	F	0.7 ± 0.1	1.0 ± 0.1	0.8 ± 0.2	ns	***
	M	1.4 ± 0.1	1.5 ± 0.1	1.2 ± 0.2		
THR	F	6.7 ± 1.0	4.9 ± 0.4	6.8 ± 1.1	ns	ns
	M	7.1 ± 1.3	7.0 ± 1.0	8.4 ± 0.8		
SER	F	1.3 ± 0.1	1.3 ± 0.3	1.7 ± 0.2	ns	*
	M	1.9 ± 0.1	1.9 ± 0.1	1.6 ± 0.1		
ASN	F	0.31 ± 0.01 a	0.24 ± 0.02 ab	0.24 ± 0.01 b	*	**
	M	0.34 ± 0.02	0.34 ± 0.02	0.28 ± 0.04		
GLU	F	6.4 ± 0.3 a	5.3 ± 0.3 b	5.0 ± 0.3 b	***	***
	M	8.1 ± 0.2	6.1 ± 0.3	5.9 ± 0.3		
GLN	F	3.5 ± 0.3 a	3.3 ± 0.5 a	2.4 ± 0.4 b	***	***
	M	5.8 ± 0.3	4.6 ± 0.4	3.0 ± 0.4		
PRO	F	53 ± 2 a	45 ± 1 b	41 ± 1 c	***	*
	M	57 ± 2	53 ± 2	41 ± 3		
GLY	F	1.2 ± 0.1 a	1.6 ± 0.1 b	1.4 ± 0.1 a	**	ns
	M	1.3 ± 0.1	2.2 ± 0.3	1.2 ± 0.1		
ALA	F	5.6 ± 0.5 a	3.4 ± 0.2 b	4.5 ± 0.5 ab	*	ns
	M	4.7 ± 0.5	4.5 ± 0.5	5.0 ± 0.5		
VAL	F	2.2 ± 0.2 a	3.2 ± 0.3 b	2.6 ± 0.1 a	***	ns
	M	1.9 ± 0.2	3.2 ± 0.2	2.2 ± 0.2		
CYS	F	0.04 ± 0.01 a	0.05 ± 0.01 b	0.04 ± 0.01 a	*	ns
	M	0.03 ± 0.01	0.06 ± 0.01	0.03 ± 0.01		
CTA	F	0.36 ± 0.06	0.28 ± 0.03	0.37 ± 0.06	ns	ns
	M	0.48 ± 0.09	0.36 ± 0.04	0.30 ± 0.04		
ILE	F	0.9 ± 0.1 a	1.4 ± 0.2 b	1.2 ± 0.1 b	***	*
	M	0.7 ± 0.1	1.2 ± 0.1	1.0 ± 0.1		
LEU	F	0.8 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	ns	ns
	M	0.7 ± 0.1	0.9 ± 0.2	0.9 ± 0.1		
TYR	F	1.2 ± 0.1 a	1.1 ± 0.2 a	1.7 ± 0.1 b	**	ns
	M	1.1 ± 0.1	1.0 ± 0.1	1.4 ± 0.1		
PHE	F	0.4 ± 0.1 a	0.7 ± 0.1 b	0.6 ± 0.1 a	**	ns
	M	0.4 ± 0.1	0.8 ± 0.2	0.5 ± 0.1		
β-ALA	F	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	ns	ns
	M	0.06 ± 0.01	0.09 ± 0.01	0.13 ± 0.07		
ETN	F	2.1 ± 0.6 a	2.0 ± 0.5 a	3.4 ± 1.0 b	*	*
	M	0.9 ± 0.1	0.9 ± 0.3	2.1 ± 0.3		
ORN	F	0.14 ± 0.04	0.11 ± 0.02	0.19 ± 0.05	ns	ns
	M	0.15 ± 0.02	0.10 ± 0.01	0.15 ± 0.2		
LYS	F	3.8 ± 0.2 a	2.0 ± 0.3 b	2.3 ± 0.3 b	***	*
	M	2.9 ± 0.3	1.3 ± 0.1	2.0 ± 0.5		
HIS	F	4.2 ± 0.3	3.5 ± 0.4	3.5 ± 0.9	ns	ns
	M	3.6 ± 0.2	3.1 ± 0.4	2.5 ± 0.1		
ARG	F	3.9 ± 0.2	3.6 ± 0.3	3.6 ± 0.1	ns	ns
	M	3.5 ± 0.1	3.3 ± 0.2	3.6 ± 0.3		
Total	F	104 ± 2 a	91 ± 5 b	93 ± 1 b	**	ns
	M	107 ± 2	102 ± 2	93 ± 5		

Note: There were 5 replicates and 1 insect in each replicate; data are expressed as mean ± SE.

^aPETN = phosphoethanolamine; CTA = cystathionine; ETN = ethanolamine. Methionine was not determined, its concentration being below the sensitivity of method.

^bP-values of ANOVA are expressed as: ns = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. A two-way ANOVA (main effects are treatment and sex) was performed for each amino acid. In all cases the two-way interaction was not significant. When the treatment effect was significant, the three treatments were further compared with Student–Newman–Keuls test: columns with different letters are significantly different. As proline and alanine have been previously shown to increase with acclimation, one-tailed tests were used for these amino acids.

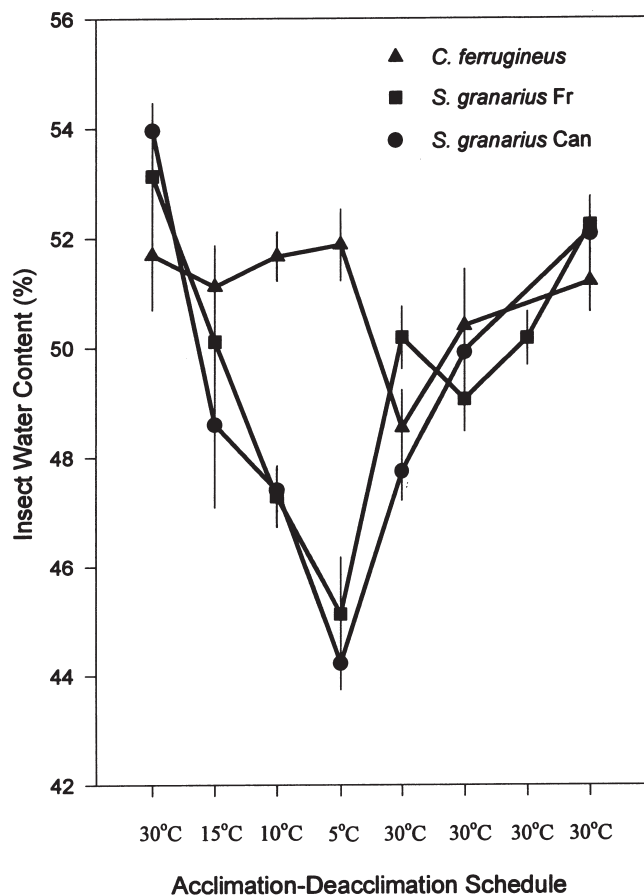


Fig. 5. The changes in water content (% wet mass basis, mean \pm SEM) of *S. granarius* French and Canadian strains, and *C. ferrugineus*. Cold acclimation and deacclimation as in Fig. 1.

seen in the second experiment (cold acclimated; $51.9 \pm 1.0\%$, cold acclimated + 1 day at 30°C ; $52.6 \pm 0.5\%$, cold acclimated + 7 days at 30°C ; $52.0 \pm 0.8\%$). The fresh weight consistently rose from 0.185 ± 0.003 mg/adult at the beginning of the experiment to 0.202 ± 0.003 . There were similar trends in the second experiment.

Mortality during acclimation/deacclimation rose gradually for *S. granarius*, but never exceeded 10% (Fig. 6). Similar mortality occurred during the second experiment. *Cryptolestes ferrugineus* had higher mortalities: more than 60% at the end of the first experiment and just over 40% during the second experiment.

4. Discussion

We saw a similar level of cold tolerance of *S. granarius* as seen in previous studies; LT_{50} of 31 days (24, 39 days fiducial limits) after being acclimated at 15°C for 14 days in this study compared to an LT_{50} of 29 days at 0.5°C with adults that had been acclimated at $29\text{--}18^\circ\text{C}$ for 20 days (Stojanovic, 1965). David et al. (1977)

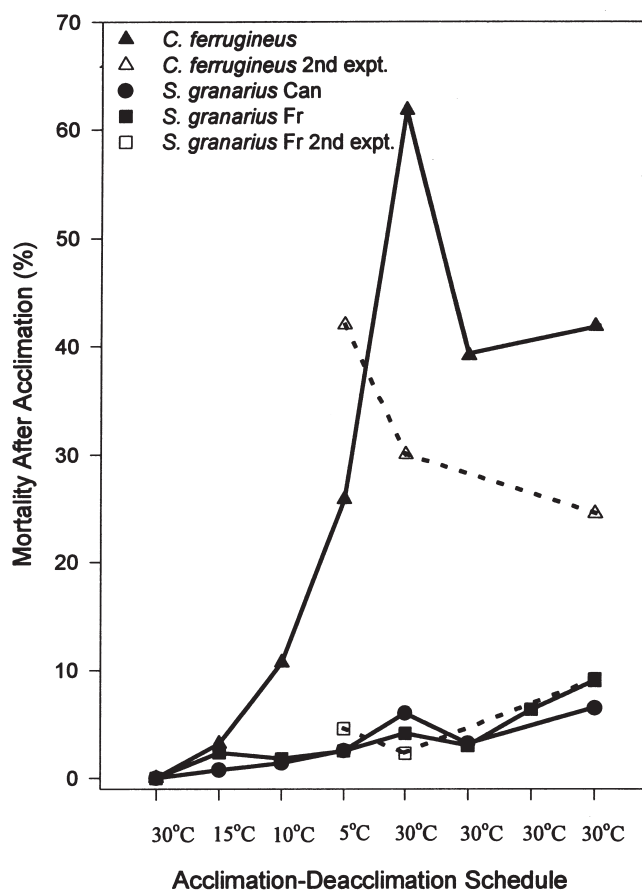


Fig. 6. The mortality of *S. granarius* and *C. ferrugineus* during acclimation and deacclimation, before insects were placed at either 0°C (*S. granarius*) or -10°C (*C. ferrugineus*). Cold acclimation and deacclimation as in Fig. 1.

showed that *S. granarius* can cold acclimate when held at $21\text{--}10^\circ\text{C}$ for several days, although it is difficult to compare these data with our results as they used higher temperatures to test cold tolerance, and they did not estimate the LT_{50} . As the SCP of *S. granarius* is approximately -15°C (Fields, 1992), and exposure temperatures did not go below 0°C , the mortality seen in these tests was due to chilling injury.

The mortality seen in the tests with *C. ferrugineus* at -10°C were due to non-freezing mortality because the SCP of *C. ferrugineus* is between -17 and -21°C (Fields, 1992). Previous studies have shown that *C. ferrugineus* can increase in cold tolerance by 8–40-fold (Smith, 1970; Fields, 1990) when cold acclimated. The percent survival obtained in our tests was lower than insects cold acclimated in granaries. By February, insects placed in granaries in Canada had an LT_{50} of 40 days (34, 54 days) at -10°C (Fields, 1990), compared to an LT_{50} of 24 days for the insects acclimated in the laboratory in this study. This could be due to the differences in the cold acclimation program, where field insects were cold acclimated for 12 wk to a low of $-$

2.2°C, whereas the laboratory insects were cold acclimated for 6 wk to a low of 5°C.

The twofold increase in trehalose concentrations in *S. granarius* and *C. ferrugineus* during cold acclimation was similar to the two- to fourfold increases in trehalose of many other insects (Rains and Dimock, 1978; Shimada and Riihimaa, 1990; Grubor-Lajsic et al., 1992; Goto et al., 1993; Kostal and Simek, 1995). The maximum level of trehalose accumulated during cold acclimation is very similar between species: 73 nmol mg⁻¹ in *Eurosta solidaginis* (Fitch) (Storey et al., 1981), 37 nmol mg⁻¹ fresh weight in *Chymomyza costata* (Shimada and Riihimaa, 1990), 47 nmol mg⁻¹ in *Ostrinia nubilalis* (Hubn.) (Grubor-Lajsic et al., 1992), 64 nmol mg⁻¹ in *Enosima leucotaeniella* (Ragonot) (Goto et al., 1993), 32 nmol mg⁻¹ in *Delia radicum* L. (Kostal and Simek, 1995), 26 nmole mg⁻¹ in *C. ferrugineus* and 61 nmol mg⁻¹ in *S. granarius* (this study). Unlike many insects (Storey and Storey, 1988; Lee and Denlinger, 1991), *C. ferrugineus* and *S. granarius* did not accumulate either glycerol or sorbitol during cold acclimation. They were similar to *E. leucotaeniella* (Goto et al., 1993), *C. costata* (Shimada and Riihimaa, 1990) and *Popilius disjunctus* Illiger (Rains and Dimock, 1978), species that accumulate trehalose or proline but not glycerol or sorbitol during cold acclimation.

Total free amino acids concentrations often increase 1.5–2.0-fold during insect cold acclimation (Mansingh, 1967; Rains and Dimock, 1978; Morgan and Chippendale, 1983; Hanzal and Jegorov, 1991). Different amino acids increase during cold acclimation depending upon the species: alanine, arginine, proline, lysine, glutamine, histidine, threonine and glycine (Mansingh, 1967), alanine, proline, serine (Morgan and Chippendale, 1983), proline and alanine (Storey, 1983) alanine, lysine, glycine, phenylalanine, glutamine (Hanzal and Jegorov, 1991) (listed in order of percent increase during acclimation), with proline and alanine being the most common. In *C. ferrugineus* and *S. granarius* we also saw an increase in proline, as well as several other amino acids. Alanine only increased in *C. ferrugineus*.

Although *S. granarius* lost up to 10% water during acclimation, this would not be enough to account for the rise in trehalose or proline. As there is no free water in stored grain, the only water available to insects is the water absorbed in the food and water produced by metabolism. As feeding is greatly reduced at 15°C, and completely stopped at 8°C on whole grain (Granovsky and Mills, 1982), the insects are not able to replenish their water during cold acclimation. Once *S. granarius* was returned to 30°C and able to feed, the moisture content returned to preacclimation levels. We do not know why *C. ferrugineus* did not also show a similar drop in moisture content during acclimation.

The progressive mortality of *C. ferrugineus* during the acclimation/deacclimation period complicates the

interpretation of the data. It is possible that the mortality was responsible for the progressive increase in fresh weights as there is an overlap in the weight distributions in the first and last samples. Heavier individuals could be more fit, and have greater survival during the acclimation period. However, the hypothesis that individuals with low cold tolerance, trehalose and proline levels died selectively during acclimation and is responsible for the changes seen during acclimation can be rejected because the increases in cold tolerance, trehalose and proline levels go beyond the initial distributions.

There has been extensive work showing that proline and trehalose stabilize artificial membranes under low temperature stress (Crowe et al., 1983; Rudolph and Goins, 1991) are useful in artificially increasing cold tolerance for cryopreservation of plants (Xin and Li, 1993; Helliot et al., 1995) and occur at high levels in cold acclimated plants (Koster and Lynch, 1992; Nishida and Murata, 1995). However, a common proof that these substances act as cryoprotectants in insects is that there is a positive correlation between cold tolerance and proline and trehalose concentrations (Lee, 1991). A more rigorous proof would be to artificially raise trehalose concentrations in non-cold-acclimated insects and demonstrate there is a corresponding rise in cold tolerance. Manipulating concentrations of the wide number of proposed cryoprotectants in vivo would not be simple. Injection of putative cryoprotectants, either into the gut or into the haemolymph, is relatively simple, and Sømme (1968) demonstrated that injecting glycerol into unacclimated *Ephesia kuehniella* (Zeller) increased their survival at -10°C. However, the levels would not remain high for very long, as most of these compounds are closely regulated (Chen, 1985; Friedman, 1985). Blocking key enzymes that are responsible for the catabolism and anabolism of trehalose or important free amino acids would be another approach. Validoxylamine A is a potent and specific blocker of trehalase, and when injected into *Periplaneta americana* (L.) it causes a threefold increase in haemolymph trehalose concentrations (Kono et al., 1994). Manipulation of neuropeptides or their secondary messengers may be another mechanism to change trehalose levels in vivo (Holman et al., 1990).

Small differences in cold tolerance between sexes have been observed in *T. castaneum*, *C. pusillus* (Schönherr), *C. ferrugineus* and *P. interpunctella* (Hübner) (Fields, 1992). Contrary to our results, Granovsky and Mills (1982) demonstrated that female *S. granarius* subjected to a simulated winter with a minimum temperature of 4.4°C had greater survival than males.

In addition to their putative role as a cryoprotectant, amino acids should be viewed in a physiological and metabolic context (Chen, 1985; Liadouze et al., 1995). Insects are unusual in that they accumulate large amounts of free amino acids, especially within the hae-

molymp (Chen, 1985). The free amino acid composition reflects the balance between the intake (deriving mainly from food but also, directly or indirectly, from metabolic biosynthesis), utilization (protein biosynthesis for anabolism, metabolic and energetic uses) and excretion. Many of these processes are affected by low temperatures, for example intake and excretion are greatly reduced below 15°C. There are differences in the free amino acids between sexes with ovogenesis being a major sink for amino acids (Chen, 1985). This may be the reason that aspartate, serine, asparagine, glutamate, glutamine and proline, all non-essential amino acids that are highly metabolizable and linked to the energetic pathways, are lower in females compared to males. Conversely, essential amino acids, such as isoleucine and lysine, were significantly higher in females. These observations are in agreement with the hypothesis of a greater perturbation of anabolism in females, which seem to allocate a greater part of their limited resources to energetic purposes.

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