The Journal of Experimental Biology 209, 4102-4114
Published by The Company of Biologists 2006
doi:10.1242/jeb.02484

Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression

A. Tomčala[1,2], M. Tollarová[1,2], J. Overgaard[3], P. Šimek[1] and V. Koštál[1,2,*]

[1]*Biology Centre AS CR, Institute of Entomology, Branišovská 31, 370 05 České Budějovice, Czech Republic,* [2]*Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic and* [3]*Department of Terrestrial Ecology, National Environmental Research Institute, Silkeborg, Denmark*

*Author for correspondence (e-mail: kostal@entu.cas.cz)

Accepted 10 August 2006

Summary

Adults of the insect *Pyrrhocoris apterus* acquire chill tolerance through the process of autumnal acclimatization. Field and laboratory experiments were conducted to separate the triggering effects of low temperatures, desiccation and diapause progression on the physiological characteristics related to chill tolerance with emphasis on the restructuring of glycerophospholipid (GPL) composition. Changes in relative proportions of major molecular species of glycerophosphoethanolamines (GPEtns) and glycerophosphocholines (GPChols) in thoracic muscle and fat body tissues were followed using HPLC coupled to electrospray ionisation mass spectrometry. The increase in relative proportion of 1-palmitoyl-2-linoleyl-*sn*-GPEtn at the expense of 1,2-dilinoleyl-*sn*-GPChol was the most prominent feature of the complex change observed in both tissues during autumnal acclimatization in the field. The relative proportion of total GPEtns increased, while the proportion of total GPChols decreased. The relative proportion of unsaturated fatty acyls slightly decreased. A similar restructuring response was seen during acclimatization in the field and cold acclimation in the laboratory. By contrast, the GPL changes related to desiccation and diapause progression were relatively small, differed qualitatively from the cold-acclimation response, and were accompanied with no increase of chill tolerance. Other features of autumnal acclimatization, i.e. depression of supercooling capacity and accumulation of polyhydric alcohols, were also triggered solely by low temperatures.

Key words: membrane phospholipids, temperature, acclimatization, cold acclimation, desiccation, diapause, cold tolerance, ectotherm, Insecta, Heteroptera.

Introduction

Poikilotherm organisms, from bacteria, through plants to animals, show capacity for remodelling of the lipid composition of their biological membranes in response to changing ambient temperature (Sinensky, 1974; Steponkus, 1990; Cossins, 1994; Hazel, 1995; Hazel, 1997; Wallis and Browse, 2002). Cold acclimation characteristically leads to one or a combination of the following adjustments as summarized by Hazel (Hazel, 1989; Hazel, 1995): (a) increased proportion of unsaturated fatty acids; (b) restructuring of glycerophospholipid (GPL) molecular species; (c) elevated relative proportions of glycerophosphoethanolamines (GPEtns) to glycerophosphocholines (GPChols); (d) reduced proportions of plasmalogens compared to diacyl GPLs; (e) adjustment of cholesterol content. Such changes has been postulated to prevent perturbation of membrane structure and function when the body temperature changes. Direct genetic proof for the role of increased unsaturation in enhancing survival at decreasing ambient temperatures has been obtained in cyanobacteria and plants (Browse et al., 1994; Murata, 1994; Allakhverdiev et al., 1999; Inaba et al., 2003). Also in insects, acclimatory responses to cold has been shown to involve restructuring of GPL composition in biological membranes (Bennet et al., 1997; Ohtsu et al., 1998; Koštál and Šimek, 1998; Hodková et al., 1999; Hodková et al., 2002; Bayley et al., 2001; Holmstrup et al., 2002; Šlachta et al., 2002a; Koštál et al., 2003; Overgaard et al., 2005).

Decreasing ambient temperature is most often considered to be the triggering factor for acclimatory changes in GPL composition. In insects, however, the triggering factors and transduction cascades for GPL compositional changes have not been analyzed in detail. Based on earlier studies, at least three different triggering mechanisms might be involved: ambient temperature, hydration state of the organism

(mediated *via* osmolarity/concentration of specific solutes) and endogenous factors such as developmental hormones. In the field situation, all three factors may operate in parallel, which makes it difficult to distinguish between their effects. Changes in temperature intervene both daily and seasonally. Naturally occurring diurnal variations in temperature were sufficient to induce the nocturnal increase of cold tolerance in adults of *Drosophila melanogaster* through the process of 'rapid cold hardening' (Lee et al., 1987) and this was accompanied by small but significant changes in the GPL composition (Overgaard et al., 2005). Many insects that overwinter in temperate and polar habitats enhance their cold hardiness during the acclimatization process, which starts upon entering to diapause and continues during autumnal decrease of ambient temperatures (Denlinger, 1991; Šlachta et al., 2002b). Changes in GPL composition were observed both in the field and in the laboratory studies during such winter cold hardening (Bennet et al., 1997; Hodková et al., 1999; Hodková et al., 2002; Koštál et al., 2003). Partial dehydration often represents an inseparable part of the complex acclimatory change related to cold acclimation (Ring and Danks, 1994; Block, 1996; Danks, 2000; Williams et al., 2004). It raises the question of whether the GPL changes observed during cold acclimation may be triggered by changes in hydration state of the organism. Indeed, typical membrane alterations resulting in a higher degree of unsaturation were induced by mild desiccation stress applied to the collembolan *Folsomia candida* (Bayley et al., 2001; Holmstrup et al., 2002). Many insects change their hormonal milieu seasonally, in response to specific token stimuli such as photoperiod, and enter diapause (Denlinger, 1985; Tauber et al., 1986; Danks, 1987; Koštál, 2006). Similar remodelling of GPL composition was observed in response to cold exposure and to transition from direct development to diapause in two insects, adult *P. apterus* (Hodková et al., 2002), and larvae of the fly *Chymomyza costata* (Koštál et al., 2003).

The main objective of this study was to describe the nature and triggering factors of seasonal changes in GPL composition in the bug *Pyrrhocoris apterus* L. (Insecta: Heteroptera). The changes observed during autumnal *acclimatization* of wild bugs were compared with the *acclimation* changes induced under controlled laboratory conditions, where three potential triggering/modulating factors were examined separately: low temperatures, desiccation and progression of diapause. Survival at sub-zero temperatures, and several other physiological parameters related to it, were measured in parallel and linked to the changes in GPL composition

Materials and methods

Insects, acclimations

In the present study, wild and laboratory-reared insects were compared. Wild adults were collected in České Budějovice (Czech Republic) on two occasions during 2005, on 23rd October and 27th December. All insects were in reproductive diapause and they passed through the process of winter cold hardening between October and December (Hodek, 1968; Koštál and Šimek, 2000). Fig. 1A shows the air temperatures as recorded by the weather station of the Czech Institute of Hydrometeorology in České Budějovice. The insect samples were transported to the laboratory (10 min) without changing their temperature and were then processed immediately.

Laboratory-reared insects came from a culture that originated from adults collected in České Budějovice during spring 2005. F_2 embryos and nymphs were reared under conditions inducing reproductive diapause in the adults, i.e. constant temperature of 25°C and a short-day photoperiod of 12 h:12 h, L:D (Hodek, 1968). Dry seeds of the linden tree

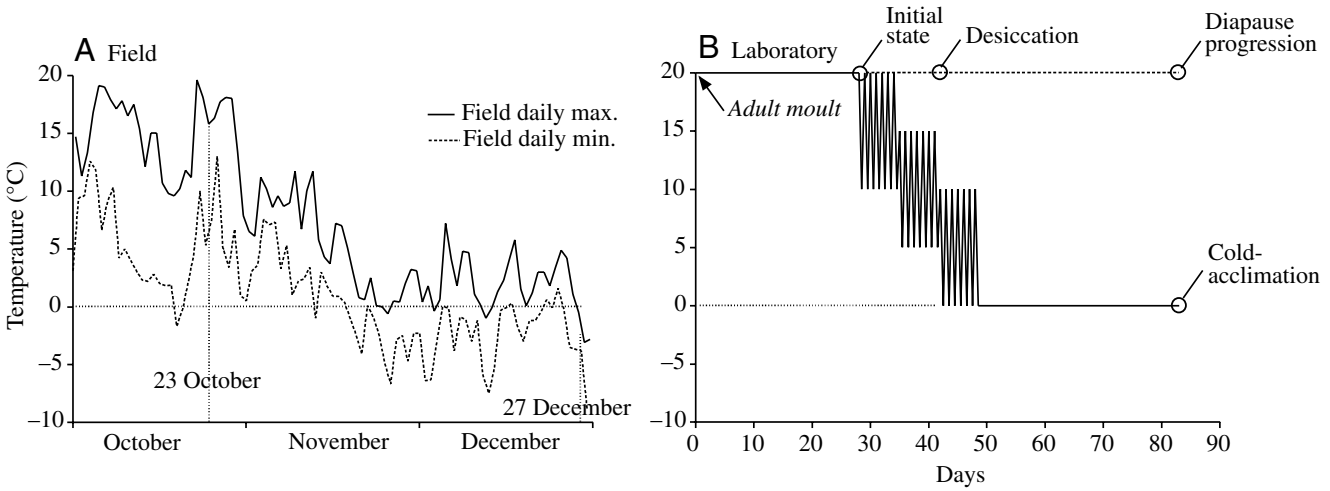

Fig. 1. Course of air temperatures in the field (A) and the temperature protocol used for laboratory acclimation (B) of *Pyrrhocoris apterus*. The adult insects were sampled on 23 October and 27 December in the field. Laboratory adults were maintained under short days conditions (12 h:12 h, L:D). Their physiological state was considered as the initial state when they reached age of 4 weeks. Three different treatments were then applied: progression of diapause, desiccation, and cold acclimation (see text for detailed descriptions).

(*Tilia parviflora* Ehrh.) and water were provided *ad libitum*. Adult F_2 insects were kept at a constant 20°C, with a short-day photoperiod and with access to food and water, for 4 weeks. Such adults were considered to be in deep reproductive diapause and their physiological state was considered as the *initial state*. Three different treatments were then applied (depicted in Fig. 1B). (1) Progression of diapause: the insects were kept under constant conditions (access to food and water) for another 8 weeks. (2) Desiccation: the insects were kept at 20°C, with a short-day photoperiod, and denied access to liquid water and food for 14 days. Relative humidity of the air fluctuated between 40–60%. Such a treatment results in approximately 50% mortality (Tollarová and V.K., unpublished data), and in the present study only survivors were taken for analyses. (3) Cold acclimation: the insects were exposed to gradually decreasing temperatures: 20°C (day)/10°C (night) during the first week, followed by 15°C/5°C and 10°C/0°C during the second and third weeks, respectively (all 3 weeks in short days), followed by 5 weeks at constant 0°C and continuous darkness. Such an acclimation protocol simulates the natural drop of temperatures during autumn (see Fig. 1A) and the bugs achieve higher chill tolerance than if constant temperatures (in contrast to thermoperiodic regime) are used for acclimation (Koštál et al., 2001).

Physiological parameters

The fresh mass (FM) was measured in five males and five females (individually) using a Sartorius balance with sensitivity of 0.1 mg. Dry mass (DM) was measured after drying the specimens at 65°C for 3 days. Hydration (in mg H_2O mg^{-1} DM) was calculated from the gravimetric data. Haemolymph samples for osmolality measurements were collected from 10 insects (five males, five females) by cutting off one of the antennae and allowing it to bleed into a calibrated capillary tube. Osmolalities were measured in 10–15-nl droplets using the Clifton Nanoliter Osmometer (Clifton Technical Physics, USA).

The concentrations of the two most abundant 'winter' polyols (ribitol and sorbitol) that typically accumulate in diapausing cold-acclimated *P. apterus* adults (Koštál and Šimek, 2000; Koštál et al., 2001), were measured in 70% ethanol extracts of whole body samples (five males, five females, taken individually). The extraction, derivatization and analytical procedures (gas chromatography coupled to mass spectrometry) were the same as described by Koštál and Šimek (Koštál and Šimek, 1995). All chemicals used for analysis were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

The temperature of crystallization of body fluids (supercooling point, SCP) was measured in individual insects (16 males, 16 females) at a cooling rate of 0.3° min^{-1} using the method described (Nedvěd et al., 1995). Chill tolerance was assessed by exposing groups of 20 insects (10 males, 10 females) to a temperature of –15°C (maximum, –14.0°C; minimum, –15.3°C) for various periods (1, 4, 7, 10, 13, 20 or 28 days). The capacity for coordinated crawling at 25°C, 2 days after the exposure, was used as a criterion for survival. Survival rate was described using the parameter LT50, which gives the time of exposure that is subsequently survived by 50% of the population sample. The parameter Lt50 was calculated from nonlinear logistic regression with the sigmoid curve as described (Koštál et al., 2001).

Analysis of glycerophospholipids

Thoracic muscle and fat body tissues were rapidly (3 min) dissected from the adults. Tissues from three individuals were pooled to obtain one sample, and each sample was replicated six times (three times with males, three times with females). Total lipids were extracted in ice-cold chloroform:methanol (2:1) solution using the method of Folch et al. (Folch et al., 1957) slightly modified according to Koštál and Šimek (Koštál and Šimek, 1998). After the extraction, the solvents were evaporated under a stream of nitrogen, and lipids were stored at –80°C until analysis.

High performance liquid chromatography (HPLC) combined with electrospray ionisation mass spectrometry (ESI-MS) (Han and Gross, 1995; Hsu and Turk, 2000; Brooks et al., 2002; Wang et al., 2005) was performed on a quadrupole ion LCQ mass spectrometer (Thermo, San Jose, CA, USA) coupled to a Rheos 2000 ternary HPLC system (Flux, Basel, Switzerland), equipped with a FAMOS autosampler and Thermos thermostat (both LC Packings-Dionex, Amsterdam, The Netherlands). The stored dry samples were dissolved in 1 ml of methanol and 5 μl aliquots were injected into a 150×2 mm i.d. 3.5 μm Synergi Polar HPLC column (Phenomenex, Torrance, CA, USA). The mobile phase was composed of (A) 10 mmol l^{-1} ammonium acetate (NH_4Ac) in methanol, (B): 10 mmol l^{-1} NH_4Ac in water and (C) isopropanol. A linear gradient of A:B:C changing from 90:10:0 to 70:0:30 within 14 min was used with a flow rate of 300 μl min^{-1}. The column temperature was maintained at 30°C. The mass spectrometer was operated either in positive or negative ion detection modes at +4 kV or –3.6 kV, respectively. Capillary temperature was 240°C; nitrogen was used as both the sheath and the auxiliary gas. For MS2 and MS3 fragmentations, ion isolation windows were 5 and 2 Da, respectively. Maximum ion injection time was 100 ms, collision energies were 30% (MS2) or 35% (MS3), mass range of 600–800 Da was scanned each 0.5 s. All chemicals used for extraction and analysis were purchased from Sigma-Aldrich Co.

Data processing

As there were no significant differences between males and females, the data sets for both sexes were pooled. Owing to the complexity of instrument response to particular GPL molecular species, which is dependent not only on features of the chemical structure but also on experimental conditions, results of GPL analysis were preferably expressed in relative values, i.e. relative molar proportion of each lipid species from the total of 100%. Our analysis was focused on major GPEtn and GPChol molecular species, which were present at or above 1%. It is known that GPEtns plus GPChols represent more than 80% of all glycerophospholipids in *P. apterus* tissues (Hodková et al.,

1999). The ratio of total GPEtns to total GPChols (GPEtn/GPChol) was calculated for each tissue/treatment and the relative proportions of individual fatty acyls were calculated based on GPL data. Unsaturation ratio (UFA/SFA) was calculated from the proportional composition of fatty acyls.

Unpaired two-tailed *t*-tests (parametric) were used to compare means of two variables with normal Gaussian distribution (*F*-tests were used to verify equality of variances). Mann–Whitney *U*-tests (non-parametric) were used to compare the medians of SCP variables (with skewed distribution of data). To investigate if there were any general trends in the changes of GPL composition caused by different treatments, all data were analysed together using a multivariate statistical analysis, Principal component analysis (PCA; Simca-P software, Umetrics, Umea, Sweden). Using this method, the data are represented in *K*-dimensional space (where *K* is equal to the number of variables) and reduced to a few principal components (two principal components, PC1 and PC2, in this study) that describe the maximum variation within the data. The principal components are then displayed as a set of scores, which highlight clustering. Differences between the mean scores for different treatments along the axes PC1 and PC2 were tested using one-way ANOVA followed by a *post-hoc* Student–Newman–Keuls test (SNK).

Results

Changes in chill tolerance and related parameters

Wild adults of *P. apterus* collected in the field during October and December had similar dry masses (Table 1). Although their hydration decreased from October to December, this change was statistically insignificant (P=0.0601; *t*-test). Osmolality of haemolymph, however, increased almost threefold (from 326 to 904 mosmol kg^{-1}) and high amounts of winter polyols accumulated: 27.0 and 3.8 μg mg^{-1} DM of ribitol and sorbitol, respectively. The temperature of crystallization of body fluids (SCP) decreased from –11.0 in October to –19.2°C in December and the chill tolerance (measured as a Lt50 at –15°C) increased from <1 to 18.9 days.

Table 1. *Physiological parameters in the field-collected adults of Pyrrhocoris apterus*

Parameter	Value in October	Value in December
Dry mass (mg)	18.1±6.3	18.6±3.7
Hydration (mg water mg^{-1} DM)	1.78±0.41	1.45±0.32
Haemolymph osmolality (mosmol kg^{-1})	326±21	904±55***
Conc. of ribitol (μg mg^{-1} DM)	trace	26.96±8.14
Conc. of sorbitol (μg mg^{-1} DM)	trace	3.80±2.88
Supercooling point (°C)	–11.0	–19.2***
	(–4.8, –15.2)	(–16.4, –20.3)
Lt50 at –15°C (days)	<1	18.9±3.4

DM, dry mass.

Each value represents either a mean ± s.d. or, in the case of supercooling points, median (25%, 75% percentile); (see text for *N*). The differences between October and December means or medians were statistically analyzed using unpaired two-tailed *t*-tests or two-tailed Mann–Whitney *U*-tests, respectively (*P<0.05; **P<0.001; ***P<0.0001).

There were no significant differences in the measured physiological parameters between the laboratory-reared insects during their initial state and those collected in the field in October (compare Tables 1 and 2; P>0.05 for all parameters, *t*-tests or *U*-test). When laboratory adults were subjected to various treatments, their dry mass did not change significantly (Table 2). Hydration remained stable during the progression of diapause at 20°C, but it significantly decreased during the desiccation and cold-acclimation treatments. Desiccating conditions caused the loss of 51% of initial water content and an approximate twofold increase in haemolymph osmolality. Cold acclimation was accompanied by the loss of 34% of initial water content and more than a threefold increase in osmolality. No winter polyols accumulated during the progression of diapause. Small amounts of polyols (slightly above the analytical thresholds: 2.5 or 1.0 μg of ribitol or sorbitol, respectively, in the sample) were found in desiccated insects and high amounts of polyols, i.e. 15.9 and 11.4 μg mg^{-1} DM of ribitol and sorbitol, respectively, were found in the cold-acclimated specimens. Supercooling capacity and chill tolerance at –15°C remained relatively low during diapause progression and desiccation while cold acclimation caused the SCP to decrease from –13.9 to –18.9°C and the Lt50 at –15°C to increase from <1 to 14.6 days.

Glycerophospholipid composition

Gradient HPLC efficiently separated GPLs from other lipid classes, particularly from triacylglycerols, which were dominant in the sample extracts. In this way, highly reproducible full-scan positive ESI mass spectra of the GPChol and GPEtn fractions were obtained with minimal outward peak overlapping and ion suppression. As shown in Fig. 2, $[M+H]^+$ ions dominated in the full-scan ESI spectra of GPEtn and GPChol fractions. Twelve to 13 major GPEtn and GPChol molecular species were consistently detected in the tissue samples of *P. apterus* (Figs 2, 3). Fig. 3 shows a typical HPLC chromatogram (upper trace), and extracted chromatograms of principal GPEtn and GPChol species. The identity of each major ion was verified by fragmenting the ion and studying the MS2 and MS3 spectra containing preferential losses of headgroups and fatty acyls from the *sn*-1 or *sn*-2 positions of the glycerol backbone. All GPL species were composed of only five different fatty acyls (FAs): palmitoyl (16:0), heptadecanoyl (17:0), stearoyl (18:0), oleyl (18:1) and linoleyl (18:2). The GPL compositions were qualitatively similar in the wild (October) and laboratory-reared (initial state) insects in both tissues (compare Fig. 4A and Fig. 5A with Fig. 6A and Fig. 7A, respectively). There were, however, quantitative differences at the level of individual GPL species. The single largest difference was found in the proportion of 18:2/18:2-GPChol in the muscle tissue, which was 28.2% in the wild

Table 2. *Physiological parameters in the laboratory-reared adults of Pyrrhocoris apterus, and the effects of diapause progression, desiccation and cold acclimation*

Parameter	Value			
	Initial state (day 0)	After diapause progression (2 months)	After desiccation (14 days)	After cold acclimation (2 months)
Dry mass (mg)	21.2±9.1	24.6±8.0	19.4±7.3	21.7±6.5
Hydration (mg water mg^{-1} DM)	1.72±0.21	1.60±0.33	0.85±0.15***	1.13±0.14***
Haemolymph osmolality (mosmol kg^{-1})	342±27	373±40	750±121***	1128±370***
Conc. of ribitol (μg mg^{-1} DM)	trace	trace	0.34±0.27	15.94±6.54
Conc. of sorbitol (μg mg^{-1} DM)	trace	trace	0.11±0.07	11.40±2.20
Supercooling point (°C)	–13.9 (–12.2, –14.8)	–12.9 (–6.2, –15.0)	–11.7 (–7.8. –14.3)	–18.9*** (–14.9, –19.6)
Lt50 at –15°C (days)	<1	<1	<1	14.6±4.1

DM, dry mass.

Each value represents either a mean ± s.d. or, in the case of supercooling points, median (25%, 75% percentile); (see text for *N*). The differences between the initial state and the treatments were statistically analyzed using unpaired two-tailed *t*-tests or two-tailed Mann–Whitney *U*-tests, respectively (**P*<0.05; ***P*<0.001; ****P*<0.0001).

insects and only 19.4% in the laboratory-reared insects (*P*<0.0001, *t*-test). Wild specimens showed similar GPEtn/GPChol ratios in both tissues (muscle, 1.0±0.03; fat body, 0.9±0.2). By contrast, laboratory-reared insects had a ratio of 1.5±0.3 in the muscles and 0.5±0.1 in the fat body.

Seasonal change in glycerophospholipid composition

The complex change in GPL composition during seasonal acclimatization in the field is shown in Figs 4 and 5. Two most noticeable alterations were: (1) the increase of 16:0/18:2-GPEtn by 6.6 or 5.3% (muscle or fat, respectively), and (2) the

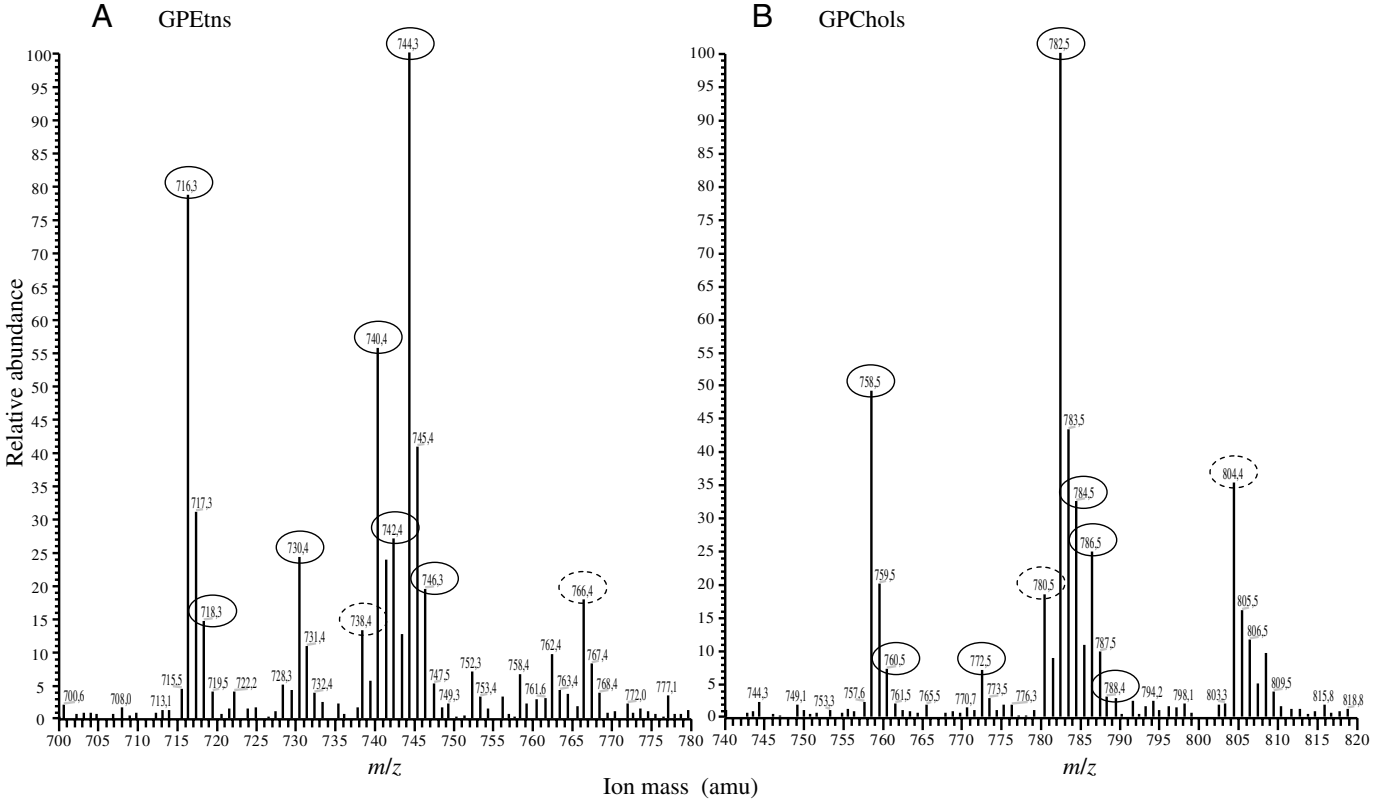

Fig. 2. Electrospray ionisation mass spectra showing molecular species composition of *P. apeterus* thoracic muscle tissue. Positive full mass spectra of (A) glycerophosphoethanolamine (GPEtn) and (B) glycerophosphocholine (GPChol) fractions are depicted separately. Identities of all major $[M+H]^+$ molecular ions (solid ovals) were verified by MS2 and MS3 fragmentations and are shown in Fig. 3. Note that each major ion occurs as a pair of masses differing by 1 amu (^{13}C isotopic contribution). All species formed the adducts with sodium $[M+Na]^+$ in relatively low and constant proportions (broken ovals).

decrease of 18:2/18:2-GPChol by 4.7% (both tissues). The GPEtn/GPChol ratios significantly increased from October to December: to 1.4±0.2 in the muscles (P=0.0011, t-test), and to 1.2±0.2 in the fat body (P=0.0293, t-test). FA composition changed relatively little during autumnal acclimatization (Table 3). Statistically significant increases in the proportion of palmitoyl (16:0) were registered in both tissues, and the proportions of linoleyl (18:2) tended to decrease. Such changes resulted in a slight, but statistically significant, decrease in UFA/SFA ratio in both tissues.

Changes in glycerophospholipid composition during laboratory acclimations

The diapause progression and desiccation treatments were accompanied by relatively small changes in GPL composition of the muscle (Fig. 6B,C) and fat body (Fig. 7B,C). The relative proportions of GPEtn/GPChol remained unchanged (P>0.05, t-test): muscle after diapause progression, 1.4±0.3; muscle after desiccation, 1.6±0.4; fat body after diapause progression, 0.6±0.1; and fat body after desiccation 0.6±0.1. Relatively small changes were observed in FA composition, and the UFA/SFA ratios did not change significantly (Table 4).

The cold-acclimation treatment resulted in considerable restructuring of GPL composition. The general patterns of changes were similar in both tissues (compare Fig. 6D with Fig. 7D). The proportion of 16:0/18:2-GPEtn increased by 8.1% and 3.3% in the muscle and fat body tissue, respectively; 18:0/18:2-GPEtn was the second most increased species (by 5.9% and 3.5%). Also, 18:2/18:2-GPChol decreased by 8.8% and 6.5%, and 16:0/18:2-GPChol decreased by 3.5% and 2.5%. The GPEtn/GPChol ratios significantly increased in both tissues, to 3.6±1.1 in the muscle (P=0.0010, t-test), and to 0.7±0.1 in the fat body (P=0.0037, t-test). Changes were observed also at the level of

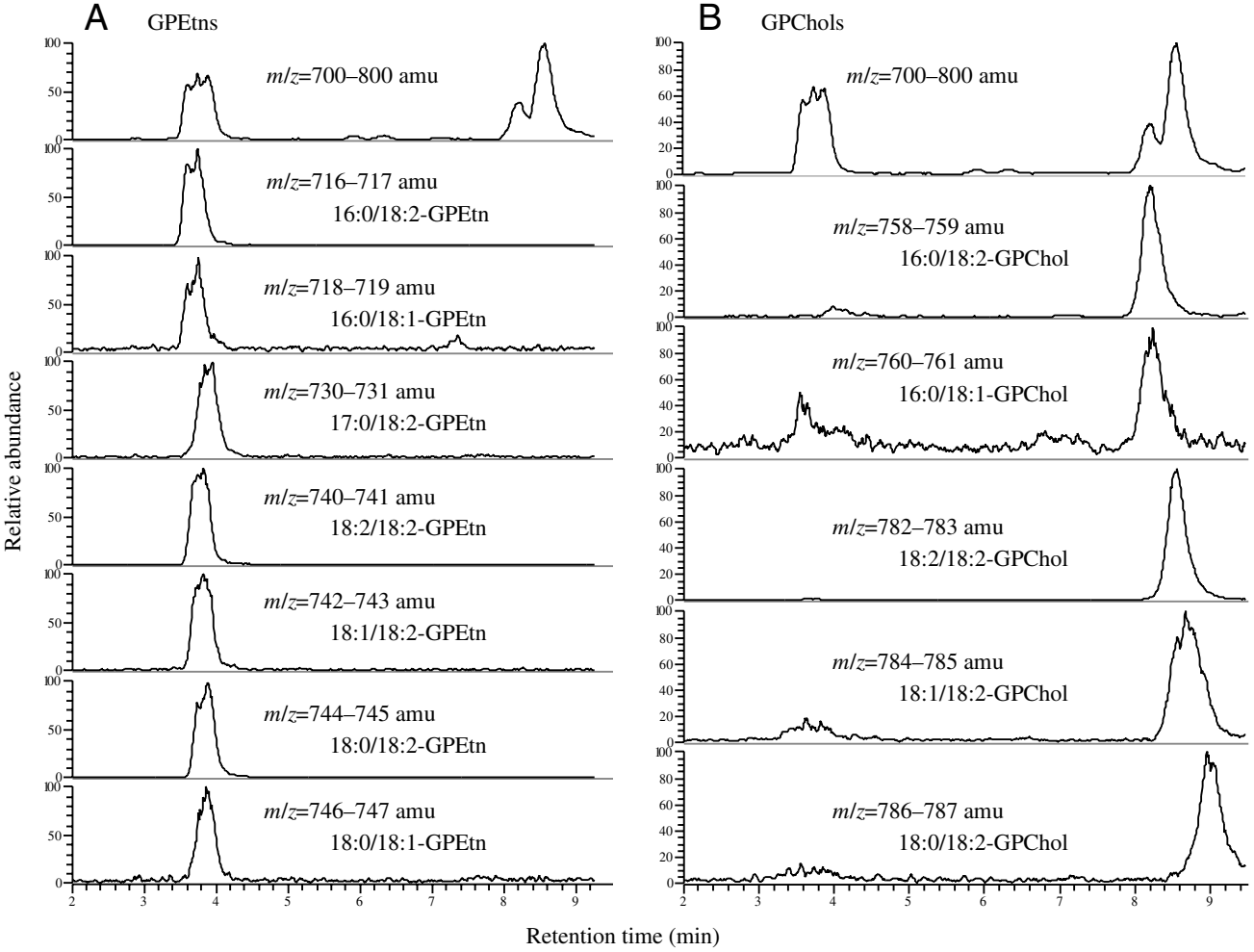

Fig. 3. HPLC separation of major molecular species of (A) glycerophosphoethanolamines (GPEtns) and (B) glycerophosphocholines (GPChols) of *P. apeterus* thoracic muscle tissue. The upper most trace (in A and B) shows total GPEtn and GPChol fractions and the lower traces show individual ion masses (molecules), as they were extracted using the MS ion trap. Note that a relative y axis are used in all chromatograms, where the highest peak is taken as 100% response. That is why the signal/noise is relatively low in the rare species, e.g. 16:0/18:1-GPChol. For quantification purposes, the areas under chromatographic peaks of individual molecular species derived from a total chromatogram (upper traces) were compared.

FA composition in both tissues; the UFA/SFA ratios significantly decreased (Table 4).

PCA analysis

PCA identified two principal components (PC1 and PC2), which together accounted for 82.8% of the total variation seen among different treatments (field and laboratory data were analyzed in a single run). Fig. 8A presents the scores of the two principal components for individual treatments. In the two-dimensional space described by PC1 and PC2 scores, vectors may by drawn, which connect an initial state with a state after the application of a treatment. Similar patterns in changes of GPL composition appear as similar orientations of the vectors. The length of each vector is proportionally related to the significance of the change. The vectors displaying the autumnal acclimatization in the field and the cold acclimation in the laboratory were relatively long and of similar orientation in both tissues. The differences between the relevant scores of principal components were significant at α=0.05 (ANOVA followed by SNK test) (Fig. 8B,C). In contrast, the vectors related to diapause progression and desiccation (these were not drawn in Fig. 8A) were relatively short and the differences between the scores were mostly insignificant.

Discussion

Complex nature of cold acclimation

Insect cold acclimation is a complex physiological change consisting of many components (Lee, 1991; Danks, 1996; Renault et al., 2002; Sinclair et al., 2003) of which the membrane restructuring is just one. Hence, it is difficult to estimate the effect of any single component in isolation. In *P.*

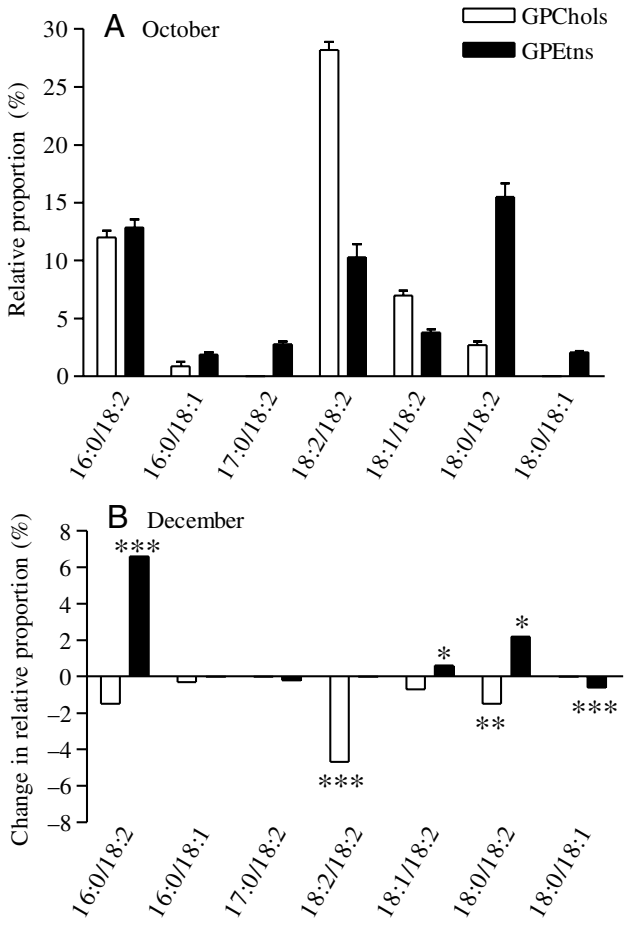

Fig. 4. Restructuring of glycerophospholipid composition in thoracic muscle membranes of *Pyrrhocoris apterus* during field acclimatization between 23 October and 27 December. (A) Mean (± s.d.; *N*=6 independent samples) relative proportion of phosphatidylethanolamine (GPEtn) and phosphatidylcholine (GPChol) molecule (all species make 100% in total) in October. (B) The difference from the initial state at the end of the acclimatization period. The differences were statistically analyzed using unpaired two-tailed *t*-tests (**P*<0.05; ***P*<0.001; ****P*<0.0001).

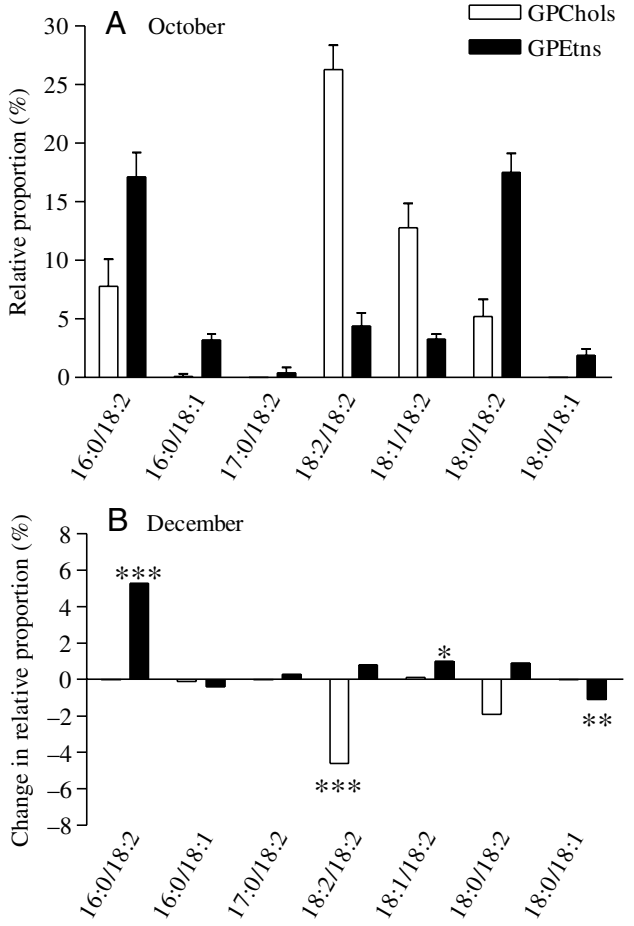

Fig. 5. Restructuring of glycerophospholipid composition in fat body tissue membranes of *Pyrrhocoris apterus* during the field acclimatization between 23 October and 27 December. (A) Mean (± s.d.; *N*=6 independent samples) relative proportion of phosphatidylethanolamine (GPEtn) and phosphatidylcholine (GPChol) molecule (all species make 100% in total) in October. (B) The difference from the initial state at the end of the acclimatization period. The differences were statistically analyzed using unpaired two-tailed *t*-tests (**P*<0.05; ***P*<0.001; ****P*<0.0001).

Table 3. *Composition of fatty acyls in glycerophospholipids extracted from two tissues of the field-collected adults of Pyrrhocoris apterus*

Fatty acyl	Relative proportion (%) of the fatty acyl	
	In October	In December
Muscle		
FA 16:0	13.8±0.6	16.2±0.6***
FA 17:0	1.4±0.1	1.3±0.1
FA 18:0	10.2±0.8	10.2±0.9
FA 18:1	7.8±0.2	7.3±0.5
FA 18:2	66.8±0.9	65.0±0.8**
UFA/SFA	2.9±0.1	2.6±0.1***
Fat body		
FA 16:0	14.1±1.5	16.5±0.6**
FA 17:0	0.2±0.2	0.3±0.4
FA 18:0	12.3±0.7	11.3±0.8*
FA 18:1	10.7±0.6	10.4±0.7
FA 18:2	62.8±1.3	61.6±0.6
UFA/SFA	2.8±0.2	2.6±0.1*

FA, fatty acyl; UFA, unsaturated FA; SFA, saturated FA; 16:0, palmitoyl; 17:0, heptadecanoyl; 18:0, stearoyl; 18:1, oleyl; 18:2, linoleyl.

Each value represents the mean ± s.d. of the relative proportion of the FA (or UFA/SFA ratio) calculated from phospholipid species composition. The differences between October and December means were statistically analyzed using unpaired two-tailed *t*-tests (*P<0.05; **P<0.001; ***P<0.0001).

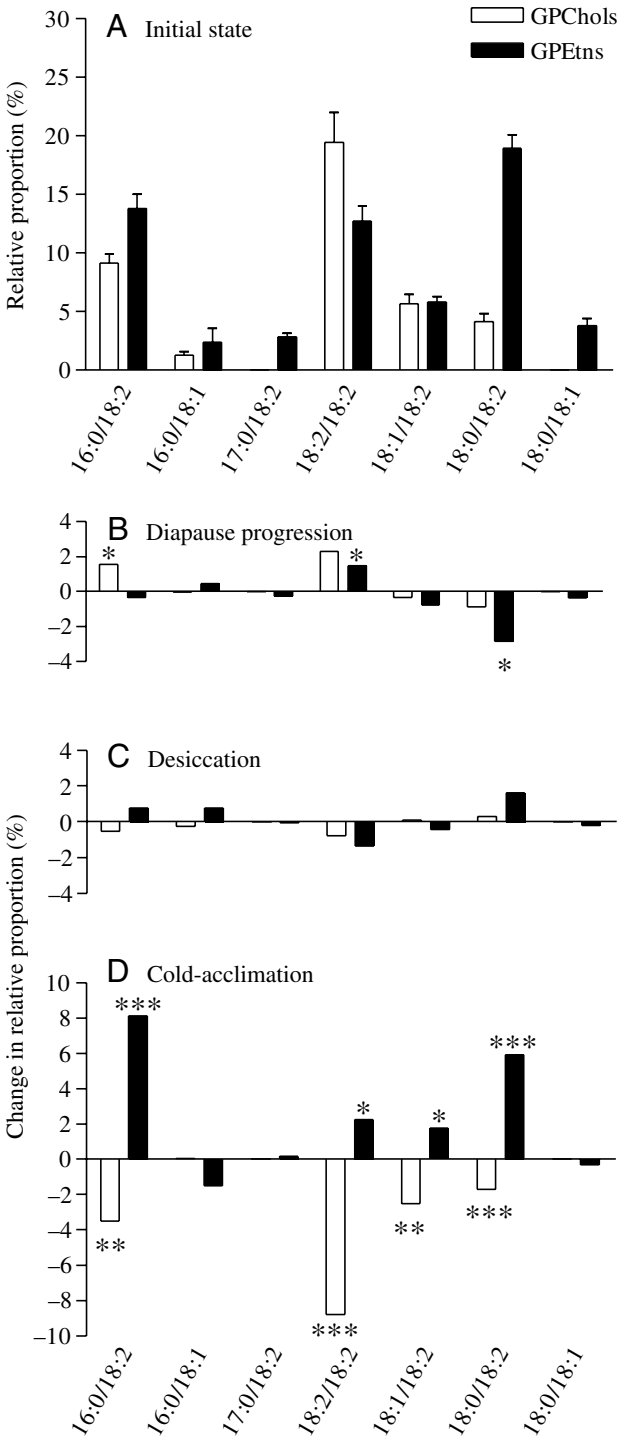

Fig. 6. Restructuring of glycerophospholipid composition in thoracic muscle membranes of *Pyrrhocoris apterus* after various laboratory acclimations. (A) Initial state; (B) dipause progression; (C) desiccation; (D) cold acclimation. The differences were statistically analyzed using unpaired two-tailed *t*-tests (*P<0.05; **P<0.001; ***P<0.0001).

apterus, depression of SCP temperature down to between –15 and –20°C is necessary to extend the range of survival temperatures. This phenomenon was observed repeatedly in the field (Koštál and Šimek, 2000) (Table 1) and in the laboratory cold-acclimation experiments (Hodková and Hodek, 1997; Koštál et al., 2001) (Table 2). The low SCP alone, however, does not guarantee high levels of chill tolerance (Bale, 1987). About 25% of diapausing *P. apterus* adults showed SCP lower than –15°C already prior to cold acclimation (both in the field and in the laboratory, see the values of 75 percentiles in Tables 1 and 2). The minimum SCP values in such adults was –20°C. Despite that, none of those adults survived longer than 1 day at –15°C (Tables 1, 2). This shows clearly that additional adjustments are required to reach the maximum chill tolerance. Among them, accumulation of ribitol and sorbitol play an important role (Koštál and Šimek, 2000; Koštál et al., 2001; Koštál et al., 2004a). Both compounds were also found in cold-acclimated *P. apterus* in relatively high concentrations in this study (Tables 1, 2). Enhancing the capacity to maintain ion gradients across cell membranes and epithelia at sub-zero temperatures is another adjustment that probably contributes to increasing chill tolerance of cold-acclimated *P. apterus* (Koštál et al., 2004b). The potential contribution of membrane restructuring to the increasing chill tolerance of this insect was hypothesized first by Hodková et al. (Hodková et al., 2002). They described the differences in the GPL composition of membranes between laboratory, warm-acclimated insects and those collected in the field during February [fig. 4 in Hodková et al. (Hodková et al.,

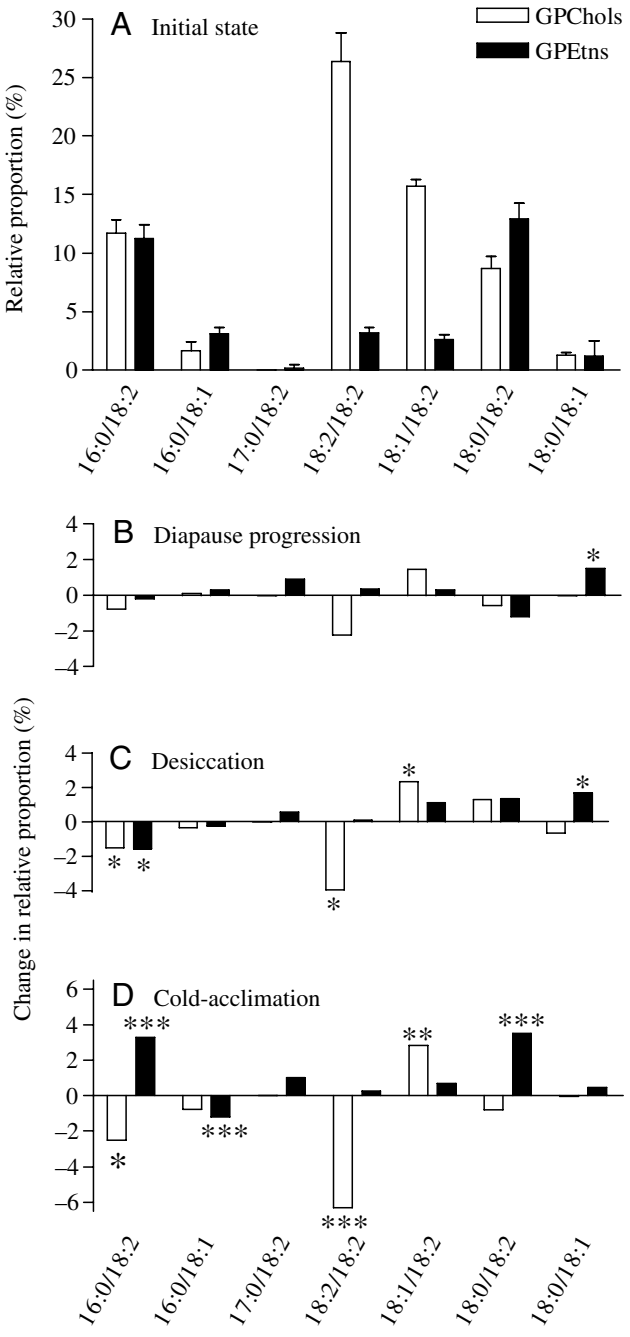

Fig. 7. Restructuring of glycerophospholipid composition in fat body tissue membranes of *Pyrrhocoris apterus* after various laboratory acclimations. (A) Initial state; (B) dipause progression; (C) desiccation; (D) cold acclimation. The differences were statistically analyzed using unpaired two-tailed *t*-tests (*P<0.05; **P<0.001; ***P<0.0001).

2002)]. We verified and further extended their results using more advanced analytical techniques and comparing the responses in the field and variously treated laboratory animals.

Autumnal acclimatization and changes in GPL composition

The increase in the relative proportion of 1-palmitoyl-2-linoleyl-*sn*-GPEtn at the expense of 1,2-dilinoleyl-*sn*-GPChol was the most prominent specific change observed in the wild adults of *P. apterus* during their autumnal acclimatization in the field. It was one component of a more general trend, i.e. the increase in the total proportion of GPEtn species counteracted by the decrease in the total proportion of GPChol species (Fig. 3B, Fig. 4B), which also can be expressed as an increase in the GPEtn/GPChol ratio. Similar changes were registered in two tissues, thoracic muscle and fat body. It should be noted here that whereas the locomotory function of muscle does not change seasonally, the physiology of fat body changes profoundly. The fat body is the principal tissue for intermediary metabolism in insects (Keeley, 1985). Its major function in reproductively active females is the synthesis and release of vitellogenic proteins for yolk formation during oocyte maturation (Telfer, 2003). At early phases of diapause, the fat body synthesizes large amounts of energy reserves in the form of diapause proteins, triacylglycerols and glycogen. During the later phases of diapause, the synthetic activity of the fat body declines and the lipidic and glycogen reserves are slowly utilized (Tauber et al., 1986). Upon stimulation by low temperature, the fat body glycogen becomes the main source for the rapid biosynthesis of polyol cryoprotectants (Storey and Storey, 1991).

The extensive seasonal restructuring of GPL composition in *P. apterus* occurred without major changes in the proportions of the FAs. A moderate increase (by 2.4%) in the proportion of palmitoyl (16:0) was found in both tissues (Table 3). The most common response of ectotherm membranes to cold is to increase the proportion of unsaturated FAs and/or increase the number of double bonds per FA. This response was not seen in *P. apterus*. In fact, autumnal acclimatization led to a slight *decrease* in the UFA/SFA ratio in both tissues (Table 3). Our FA data, however, were not obtained by direct analysis of FAs but were derived from original analytical data of GPL species. Nevertheless, direct GC/MS analysis of FAs in *P. apterus* [performed by Hodková et al. (Hodková et al., 1999)] confirmed a positive correlation between the unsaturation ratio and ambient temperature, and a negative correlation between the proportion of palmitoyl and temperature.

In the field, various factors triggering/stimulating GPL restructuring may not be easily distinguished. Our laboratory experiments allowed us to separate three potential factors: low temperatures, desiccation, and endogenous factors related to diapause progression. Whereas the low temperatures stimulated significant changes (Fig. 5D, Fig. 6D), the later two factors appeared to have relatively small effects on GPL composition in *P. apterus* membranes.

Triggering by low temperatures

The changes in GPL composition triggered by low temperatures in the laboratory were similar to the acclimatization changes observed under the field situation. The GPEtn/GPChol ratio increased, as a result of the increases in 16:0/18:2-GPEtn and 18:0/18:2-GPEtn countered by the decrease in 18:2/18:2-GPChol. Results of multivariate statistical analysis (PCA) further supported the overall

similarity between the restructurings seen in the field and laboratory and in both tissues (Fig. 8). This was so despite the fact that the initial states widely differed between the two situations and between the two tissues as well. Such uniformity in the response suggests a common adaptive value of the underlying GPL changes in relation to cold.

The ratio of GPEtn/GPChol generally tends to be higher in cold- than in warm-acclimated ectotherms (Hazel, 1989; Hazel, 1995; Hazel, 1997). Molecules of the two lipid classes differ in their geometries: GPChols assume a cylindrical shape and pack efficiently in a lamellar gel phase; GPEtns adopt a more conical form, which is readily accommodated in the reverse hexagonal phase. Consequently, less efficient packing of GPEtns in the lamellar phase disrupts the organization of the membrane and thus counters the effects of low temperatures, which tend to organize the membrane in a gel phase (Hazel, 1989). The increasing GPEtn/GPChol ratio in a membrane may thus help to prevent the formation of a gel phase in membranes at low ambient temperatures. In our earlier work (Šlachta et al., 2002a), we detected a slight increase in the GPEtn/GPChol ratio in response to low temperatures in the non-diapausing adults of *P. apterus*. It increased from 1.0 to 1.1 (NS, *t*-test) in the muscles, and from 0.7 to 1.0 (P=0.0032, *t*-test) in the fat body. Non-diapausing adults were not able, however, to accomplish the other cold-acclimation adjustments seen in diapausing adults (SCP depression, polyol accumulation and ion gradients regulation) and no, or only a weak, increase in their chill tolerance was observed (Šlachta et al., 2002a).

It is more difficult to search for an adaptive explanation for the observed changes in relative proportions of specific molecular species, such as the 16:0/18:2-GPEtn. The range of temperatures at which membranes composed of GPEtns remain

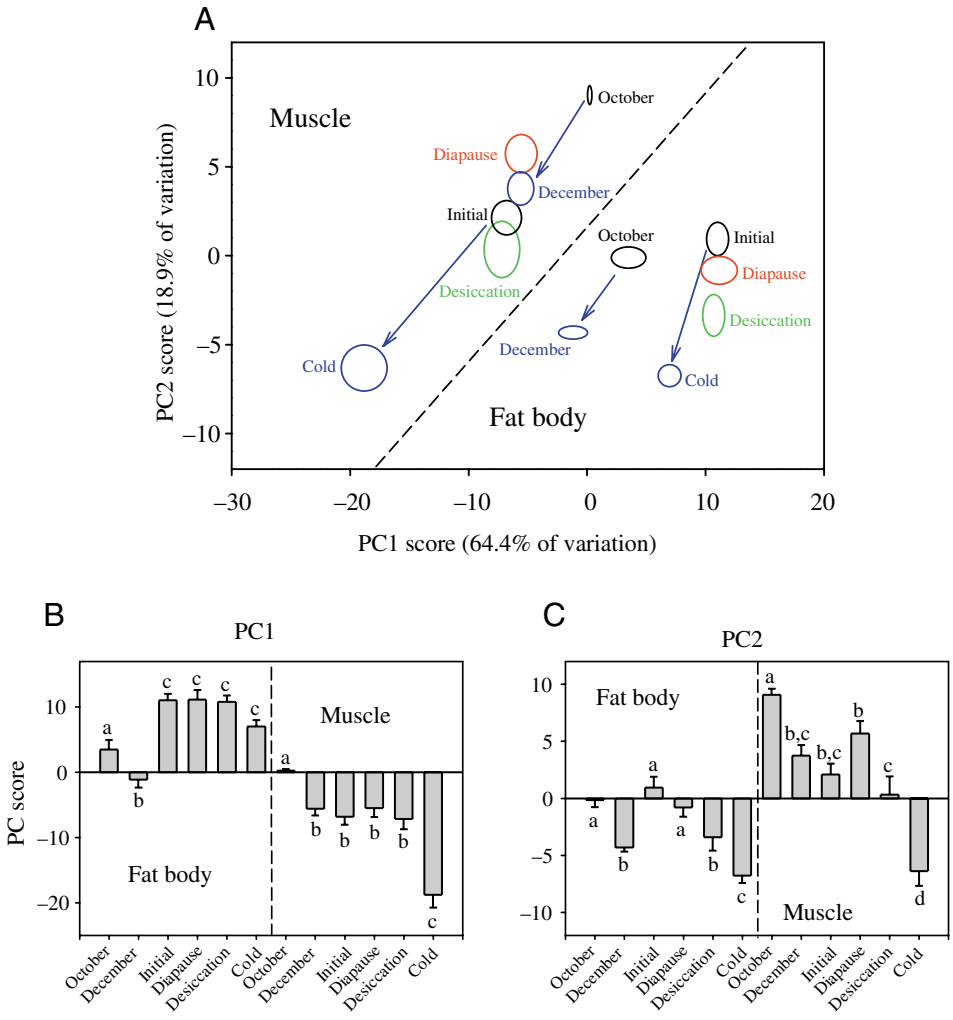

Fig. 8. Results of multivariate principal component analysis of the differences between membrane glycerophospholipid compositions in thoracic muscles and fat body tissues of *Pyrrhocoris apterus* sampled in the field (23 October and 27 December) or variously acclimated in the laboratory (initial state, diapause progression, desiccation, cold acclimation). (A) The centres of elipses represent the means, and the borders the standard errors, of the scores from the first and second principal component. The dashed line separates the data for muscles and fat-body tissues. (B,C) The differences among the scores of PC1 and PC2, respectively, were tested using one-way ANOVA followed by a Student–Newman–Keuls test (different letters indicate statistically different values).

Table 4. *Composition of fatty acyls in glycerophospholipids extracted from two tissues of the laboratory-reared adults of Pyrrhocoris apterus, and the effects of diapause progression, desiccation and cold acclimation*

	Relative proportion (%) of the fatty acyl			
Fatty acyl	Initial state (day 0)	Diapause progression (2 months)	Desiccation (14 days)	Cold acclimation (2 months)
Muscle				
FA 16:0	13.3±0.9	14.2±0.7	13.7±0.7	14.9±0.5**
FA 17:0	1.4±0.2	1.3±0.3	1.4±0.2	1.5±0.1
FA 18:0	13.4±0.7	11.4±1.0**	14.3±1.5	15.4±1.8*
FA 18:1	9.5±0.8	9.0±0.5	9.5±0.6	8.2±1.3
FA 18:2	62.3±1.8	64.2±1.3	61.1±2.2	60.0±1.9*
UFA/SFA	2.6±0.1	2.7±0.2	2.4±0.2	2.2±0.1***
Fat body				
FA 16:0	13.9±0.3	13.6±0.8	12.0±0.7	13.3±0.6
FA 17:0	0.1±0.1	0.6±0.5	0.4±0.7	0.6±0.4
FA 18:0	12.1±0.8	11.9±0.6	13.6±0.7**	13.6±0.6**
FA 18:1	12.9±1.0	14.7±1.1*	14.6±1.0*	13.7±0.9
FA 18:2	61.1±2.0	59.3±1.2	59.4±1.1	58.8±1.2*
UFA/SFA	2.8±0.1	2.8±0.1	2.8±0.1	2.6±0.1*

Each value represents the mean ± s.d. of the relative proportion of the FA (or UFA/SFA ratio) calculated from phospholipid species composition. The differences between the initial state and the treatments were statistically analyzed using unpaired two-tailed *t*-tests (*P<0.05; **P<0.001; ***P<0.0001).

fluid, i.e. T_m–T_h (where T_m is a temperature of transition from lamellar gel to liquid-crystalline phase and T_h is a temperature of transition from liquid-crystalline to reverse hexagonal phase) markedly increases with a decrease in acyl chain length (Lewis et al., 1989). Hodková et al. (Hodková et al., 2002) suggested that a relative increase of 16:0 FA and its specific pairing with 18:2 FA in the GPEtn molecule may help to maintain the ambient temperature within a suitable interval above T_m yet below T_h, and protect membranes from unregulated phase transitions caused by either a drop of ambient temperature or partial dehydration, or both occurring at the same time, as is normal in overwintering insects. Generally, the phase behaviour, fluidity and other properties of membranes are finely tuned to serve various purposes in different environmental situations. Changing the lipidic composition is the main tool of such tuning. Some changes may represent just a side effect without any explicit adaptive value. However, increasing the relative proportion of 16:0/18:2-GPEtn, as a specific response to cold, might be of some adaptive value as it was found in two tissues with different physiological functions, and it was observed also in the fly, *Chymomyza costata* (Koštál et al., 2003), and water strider (M. Hodková, unpublished results), which are ecologically and phylogenetically distant to *P. apterus*.

Triggering by desiccation

It has been proposed that some of the physiological adaptations to cold might be derived from more ancestral adaptations to desiccation (Ring and Danks, 1994; Block, 1996; Danks, 2000; Williams et al., 2004). Populations of *P. apterus* probably colonized central Europe from Asia Minor and the Near East relatively recently, during the climate warming after the end of the last glacial period. The ancestral habitats were prone to drying, which could drive the evolution of physiological adaptations to desiccation in this species. Moreover, *P. apterus* adults typically lose up to one third of body water during cold acclimation and overwintering (Koštál and Šimek, 2000; Koštál et al., 2004b) (Tables 1, 2). In this study, we attempted to test whether desiccation triggers some changes in GPL composition. In the collembolan, *Folsomia candida*, a mild desiccation stress was sufficient to elicit the restructuring of membrane GPLs (Bayley et al., 2001; Holmstrup et al., 2002). In addition to alteration of membrane lipids, *Folsomia candida* responded to desiccating conditions by accumulating glucose and myo-inositol, which led to the increase in osmolality, and subsequently influenced the rates of water uptake/loss (Bayley and Holmstrup, 1999). Our experiments showed that the loss of one half of body water, associated with doubling the haemolymph osmolality, induced relatively small changes in GPL composition in *P. apterus* and that the overall pattern of these changes differed from the changes induced by low temperatures (Fig. 7). For example, a decrease in the proportion of 16:0/18:2-GPEtn was noted in response to desiccation whereas an increase was detected as a result of cold acclimation (see above). No, or negligible, accumulation of polyhydric solutes was observed in response to desiccation in *P. apterus* (Table 2).

Triggering by endogenous factors related to diapause

The preparation for winter survival starts long before the autumnal decline of environmental temperatures in *P. apterus* (Koštál and Šimek, 2000), and in many other insects as well

(Danks, 1987; Koštál, 2006). Hodková et al. (Hodková et al., 2002) found that the relative proportion of 16:0/18:2-GPEtn was higher in short-day reared (diapausing) than in long-day reared adults of *P. apterus* (non-diapausing, reproducing) that were maintained in the laboratory at 26°C. Moreover, when non-diapausing females of *P. apterus* had the corpus allatum surgically removed (which prevents juvenile hormone production and causes entry into a diapause-like state), the GPL profiles in their muscle and fat body tissues changed in a similar manner to those of females in which diapause was induced photoperiodically (Hodková et al., 2002). Similar results were obtained with larvae of the fly *Chymomyza costata*, where relative proportions of 16:0/18:2-GPEtn in total GPLs extracted from the muscle and fat body tissues significantly increased in response to both low temperature stimulus and the transition from direct development to diapause, i.e. without any change in temperature (Koštál et al., 2003). Such results suggest that the seasonal restructuring of GPL composition is photoperiodically activated already *during the entrance into diapause*, which typically happens (long) before the seasonal decline in temperatures, and that the changes are hormonally mediated. By contrast, the changes in GPL composition *during the diapause progression*, as described in this paper, were relatively small. Moreover, the orientations of the vectors of diapause-related changes (Fig. 8A) were opposite in the muscle and fat body tissues. Such a clear difference in the vector orientations might reflect specific (changes of) physiological roles in two different tissues during diapause, which may require synthesizing/maintaining specific GPL species. In this light, relatively deep and uniform restructuring of GPL composition of both tissues in response to cold implies an adaptive mechanism assisting in survival at low temperatures.

The study was supported by the Czech Science Foundation grant no. 206/03/0099.

References

Allakhverdiev, S. I., Nishiyama, Y., Suzuki, I., Tasaka, Y. and Murata, N. (1999). Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of *Synechocystis* to salt stress. *Proc. Natl. Acad. Sci. USA* **96**, 5862-5867.

Bale, J. S. (1987). Insect cold hardiness: freezing and supercooling – an ecophysiological perspective. *J. Insect Physiol.* **12**, 899-908.

Bayley, M. and Holmstrup, M. (1999). Water vapor ansorption in arthropods by accumulation of myoinositol and glucose. *Science* **285**, 1909-1911.

Bayley, M., Petersen, S. O., Knigge, T., Köhler, H.-R. and Holmstrup, M. (2001). Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. *J. Insect Physiol.* **47**, 1197-1204.

Bennett, V. A., Pruitt, N. L. and Lee, R. E., Jr (1997). Seasonal changes in fatty acid composition associated with cold-hardening in third instar larvae of *Eurosta solidaginis*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **167**, 249-255.

Block, W. (1996). Cold or drought – the lesser of two evils for terrestrial arthropods? *Eur. J. Entomol.* **93**, 325-339.

Brooks, S., Clark, G. T., Wright, S. M., Trueman, R. J., Postle, A. D., Cossins, A. R. and Maclean, N. M. (2002). Electrospray ionisation mass spectrometric analysis of lipid restructuring in the carp (*Cyprinus carpio* L.) during cold acclimation. *J. Exp. Biol.* **205**, 3989-3997.

Browse, J., Miquel, M., McConn, M. and Wu, J. (1994). *Arabidopsis* mutants and genetic approaches to the control of lipid composition. In *Temperature Adaptations of Biological Membranes* (ed. A. R. Cossins), pp. 141-154. London, Chapel Hill: Portland Press.

Cossins, A. R. (ed.) (1994). *Temperature Adaptations of Biological Membranes.* London, Chapel Hill: Portland Press.

Danks, H. V. (1987). *Insect Dormancy: An Ecological Perspective* (Monograph Series No. 1), 439pp. Ottawa: Biological Survey of Canada (Terrestrial Arthropods).

Danks, H. V. (1996). The wider integration of studies on insect cold-hardiness. *Eur. J. Entomol.* **93**, 383-403.

Danks, H. V. (2000). Dehydration in dormant insects. *J. Insect Physiol.* **46**, 837-852.

Denlinger, D. L. (1985). Hormonal control of diapause. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* Vol. 8 (ed. G. A. Kerkut and L. I. Gilbert), pp. 353-412. Oxford: Pergamon.

Denlinger, D. L. (1991). Relationship between cold hardiness and diapause. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp.174-198. New York, London: Chapman & Hall.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Han, X. and Gross, R. W. (1995). Structural determination of picomole amounts of phospholipids via elctrospray ionization tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **6**, 1202-1210.

Hazel, J. R. (1989). Cold adaptation in ectotherms: regulation of membrane function and cellular metabolism. *Adv. Comp. Environ. Physiol.* **4**, 1-50.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.

Hazel, J. R. (1997). Thermal adaptation in biological membranes: beyond homeoviscous adaptation. *Adv. Mol. Cell Biol.* **19**, 57-101.

Hodek, I. (1968). Diapause in females of *Pyrrhocoris apterus* L. (Heteroptera). *Acta Entomol. Bohemoslov.* **65**, 422-435.

Hodková, M., Šimek, P., Zahradníčková, H. and Nováková, O. (1999). Seasonal changes in the phospholipid composition in thoracic muscles of a heteropteran, *Pyrrhocoris apterus*. *Insect Biochem. Mol. Biol.* **29**, 367-376.

Hodková, M., Berková, P. and Zahradníčková, H. (2002). Photoperiodic regulation of the phospholipid molecular species composition in thoracic muscles and fat body of *Pyrrhocoris apterus* (Heteroptera) via an endocrine gland, corpus allatum. *J. Insect Physiol.* **48**, 1009-1019.

Holmstrup, M., Hedlund, K. and Boriss, H. (2002). Drought acclimation and lipid composition in *Folsomia candida*: implications for cold shock, heat shock and acute desiccation stress. *J. Insect Physiol.* **48**, 961-970.

Hsu, F.-F. and Turk, J. (2000). Characterization of phosphatiylinositol, phosphatiylinositol-4-phosphate and phosphatiylinositol-4,5-biphosphate by electrospray ionization tandem mass spectrometry: a mechanistic study. *J. Am. Soc. Mass Spectrom*. **11**, 9896-9899.

Inaba, M., Suzuki, I., Szalontai, B., Kanesaki, Y., Los, D. A., Hayashi, H. and Murata, N. (2003). Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. *J. Biol. Chem.* **278**, 12191-12198.

Keeley, L. L. (1985). Physiology and Biochemistry of the fat body. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* Vol. 3 (ed. G. A. Kerkut and L. I. Gilbert), pp. 211-248. Oxford: Pergamon.

Koštál, V. (2006). Eco-physiological phases of insect diapause. *J. Insect Physiol.* **52**, 113-127.

Koštál, V. and Šimek, P. (1995). Dynamics of cold hardiness, supercooling and cryoprotectants in diapausing and non-diapausing pupae of the cabbage root fly, *Delia radicum* L. *J. Insect Physiol.* **41**, 627-634.

Koštál, V. and Šimek, P. (1998). Changes in fatty acid composition of phospholipids and triacylglycerols after cold-acclimation of an aestivating insect prepupa. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **168**, 453-460.

Koštál V. and Šimek, P. (2000). Overwintering strategy in *Pyrrhocoris apterus* (Heteroptera): the relations betwen life-cycle, chill tolerance and physiological adjustments. *J. Insect Physiol.* **46**, 1321-1329.

Koštál, V., Šlachta, M. and Šimek, P. (2001). Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of *Pyrrhocoris apterus* (Heteroptera: Insecta). *Comp. Biochem. Physiol.* **130B**, 365-374.

Koštál, V., Berková, P. and Šimek, P. (2003). Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). *Comp. Biochem. Physiol.* **135B**, 407-419.

Koštál, V., Tollarová, M. and Šula, J. (2004a). Adjustments of the enzymatic

complement for polyol biosynthesis and accumulation in diapausing cold-acclimated adults of *Pyrrhocoris apterus*. *J. Insect Physiol.* **50**, 303-313.

Koštál, V., Vambera, J. and Bastl, J. (2004b). On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus. J. Exp. Biol.* **207**, 1509-1521.

Lee, R. E. (1991). Principles of insect low temperature tolerance. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 17-46. New York, London: Chapman & Hall.

Lee, R. E., Chen, C. P. and Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science* **238**, 1415-1417.

Lewis, R. N. A. H., Mannock, D. A., McElhaney, R. N., Turner, D. C. and Gruner, S. M. (1989). Effect of fatty acyl chain length and structure on the lamellar gel to liquid-crystalline and lamellar to reversed hexagonal phase transitions of aqueous phosphatidylethanolamine dispersions. *Biochemistry* **28**, 541-548.

Murata, N. (1994). Genetic and temperature-induced modulation of cyanobacterial membrane lipids. In *Temperature Adaptations of Biological Membranes* (ed. A. R. Cossins), pp. 155-162. London, Chapel Hill: Portland Press.

Nedvěd, O., Hodková, M., Brunnhofer, V. and Hodek, I. (1995). Simultaneous measurement of low temperature survival and supercooling in a sample of insects. *Cryo Letters* **16**, 108-113.

Ohtsu, T., Kimura, M. T. and Katagiri, C. (1998). How *Drosophila* species acquire cold tolerance. Qualitative changes of phospholipids. *Eur. J. Biochem.* **252**, 608-611.

Overgaard, J., Sorensen, J. G., Petersen, S. O., Loeschke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster. J. Insect Physiol.* **51**, 1173-1182.

Renault, D., Salin, C., Vannier, G. and Vernon, P. (2002). Survival at low temperatures in insects: what is the ecological significance of the supercooling point? *Cryo Letters* **23**, 217-228.

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

Sinclair, B. J., Vernon, P., Klok, C. J. and Chown, S. L. (2003). Insects at low temperatures: an ecological perspective. *Trends Ecol. Evol.* **18**, 257-262.

Sinensky, M. (1974). Homeoviscous adaptation – a homeostatic process that regulates viscosity of membrane lipids in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **71**, 522-525.

Šlachta, M., Berková, P., Vambera, J. and Koštál, V. (2002a). Physiology of cold-acclimation in non-diapausing adults of *Pyrrhocoris apterus* (Heteroptera). *Eur. J. Entomol*. **99**, 181-187.

Šlachta, M., Vambera, J., Zahradníčková, H. and Koštál, V. (2002b). Entering diapause is a prerequisite for successful cold-acclimation in adult *Graphosoma lineatum* (Heteroptera: Pentatomidae). *J. Insect Physiol.* **48**, 1031-1039.

Steponkus, P. L., Lynch, D. V. and Uemura, M. (1990). The influence of cold acclimation on the lipid composition and cryobehaviour of the plasma membrane of isolated rye protoplasts. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **326**, 571-583.

Storey, K. B. and Storey, J. M. (1991). Biochemistry of cryoprotectants. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 64-93. New York, London: Chapman & Hall.

Tauber, M. J., Tauber, C. A. and Masaki, S. (1986). *Seasonal Adaptations of Insects.* Oxford: Oxford University Press.

Telfer, W. H. (2003). Vitellogenesis. In *Encyclopedia of Insects* (ed. V. H. Resh and R. T. Cardé), pp. 1171-1173. San Diego: Academic Press.

Wallis, J. G. and Browse, J. (2002). Mutants of *Arabidopsis* reveal many roles for membrane lipids. *Prog. Lipid Res.* **41**, 254-278.

Wang, C., Kong, H., Guan, Y., Yang, J., Gu, J., Yang, S. and Xu, G. (2005). Plasma phospholipid metabolic profiling and biomarkers of type 2 diabetes mellitus based on high-performance liquid chromatography/electrospray mass spectrometry and multivariate statistical analysis. *Anal. Chem.* **77**, 4108-4116.

Williams, J. B., Ruehl, N. C. and Lee, R. E. (2004). Partial link betwen the seasonal acquisition of cold-tolerance and desiccation resistance in the goldenrod gall fly *Eurosta solidaginis* (Diptera: Tephritidae). *J. Exp. Biol.* **207**, 4407-4414.