The Journal of Experimental Biology 207, 1509-1521
Published by The Company of Biologists 2004
doi:10.1242/jeb.00923

On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*

V. Koštál[1,*], J. Vambera[2] and J. Bastl[3]

[1]*Institute of Entomology, Academy of Sciences, České Budějovice, Czech Republic,* [2]*Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic* and [3]*Agricultural Faculty, University of South Bohemia, České Budějovice, Czech Republic*

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

Accepted 4 February 2004

Summary

Three acclimation groups [i.e. non-diapause (LD), diapause (SD) and diapause, cold-acclimated (SDA)] of the adult bugs *Pyrrhocoris apterus* differed markedly in their levels of chill tolerance. Survival time at a sub-zero, but non-freezing, temperature of –5°C (Lt50) extended from 7.6 days, through 35.6 days, to >60 days in the LD, SD and SDA insects, respectively. The time necessary for recovery after chill-coma increased linearly with the increasing time of exposure to –5°C, and the steepness of the slope of linear regression decreased in the order LD>SD>SDA. The capacity to prevent/counteract leakage of Na^+ down the electrochemical gradient (from haemolymph to tissues) during the exposure to –5°C increased in the order LD<SD<SDA. As a result, the rates of counteractive outward movement of K^+, and of the E_K dissipation, decreased in the same order. The least chill-tolerant insects (LD) showed the highest rate of body-water loss. Most of the water was lost from the haemolymph compartment. The ability to regulate a certain fraction of ion pools into the hindgut fluid was the highest in the SDA group, medium in the SD group and missing in the LD group. The adenylate energy charge in the fat body cells was constant in all three groups. The total pools of ATP, ADP and AMP, however, decreased in the SD and SDA groups but remained constant in the LD group. The inability of insects to maintain ion gradients at sub-zero temperature is discussed as an important cause of pre-freeze mortality.

Key words: chill tolerance, diapause, water loss, ion gradient, pre-freeze mortality, Heteroptera, *Pyrrhocoris apterus.*

Introduction

Insects adopt three basic strategies of survival at sub-zero temperatures. They can either (1) supercool their body fluids and avoid freezing (‘freeze avoidance’), (2) concentrate solutes in body fluids by extensive dehydration and, consequently, also avoid freezing (‘cryoprotective dehydration’) or (3) tolerate formation of ice crystals in extracellular spaces (‘freeze tolerance’) (Lee, 1991; Holmstrup and Westh, 1994; Nedved, 2000; Ramløv, 2000; Zachariassen and Kristiansen, 2000; Duman, 2001; Bale, 2002; Holmstrup et al., 2002; Renault et al., 2002; Sinclair et al., 2003). In most freeze-avoiding insects, mortality occurs well above the temperature of spontaneous ice crystallization [supercooling point (SCP)] (Knight et al., 1986; Bale, 1987). The physiological nature of chilling-injury, which results in pre-freeze mortality, is poorly understood in insects. Two processes probably play an important role in survival at sub-zero temperatures of the hydrated, supercooled insect organism: (1) maintaining regulated metabolism and (2) maintaining ion gradients (Hochachka, 1986; Knight et al., 1986). A disruption of metabolic regulation may occur at low temperatures as a result of specific thermal dependence of different metabolic pathways, thus disrupting the fine balance between substrates and products. The regulation of ion concentrations outside and inside the cells, or across epithelia, requires maintenance of the membrane integrity and active pumping (ATP-dependent) of ions across the cell membranes. Failure to maintain specific ion concentrations inside the cell and/or dissipation of membrane potentials may lead to severe metabolic perturbations such as loss of excitability in nerves, inability to keep cell volume, failure of secondary transports, opening of voltage-dependent Ca^{2+} channels, leakage of Ca^{2+} from endoplasmic reticulum and, ultimately, cell disintegration and death (for review, see Hochachka, 1986).

Despite the fact that chilling-injury is generally recognized as a widespread and serious cause of death in insects that overwinter in a supercooled state (Knight et al., 1986; Bale, 1987, 2002; Lee, 1991; Nedved, 2000; Ramløv, 2000), surprisingly few attempts have been made to characterize physiological mechanisms involved in pre-freeze mortality. Several studies focused on electrical activity of the nervous

system during chilling. It has been observed that, at a sufficiently low temperature, the insects enter a state of chill-coma when excitability of muscles and nerves is severely altered or lost (Anderson and Mutchmor, 1968; Bradfisch et al., 1982; Hosler et al., 2000). The loss of nervous membrane excitability was attributed to the preceding decrease of resting potential due to the effect of low temperature on transport mechanisms involved in ion balance (Heitler et al., 1977; Kivivouri et al., 1990; Cossins et al., 1995). Thus, the neuronal damage is one of the likely causes of injury inflicted by chilling (Yocum et al., 1994). Pullin and Bale (1988) and Pullin et al. (1990) studied pre-freeze mortality in the nettle aphid *Microlophium carnosum*. They found that ATP content and energy charge declined relatively slowly at low temperature, which they interpreted as insusceptibility of catabolic respiratory processes to chill-injury. Nevertheless, chilled aphids displayed almost doubled levels of ATP in comparison with the control (non-chilled) group during the first day after the start of chilling (i.e. before any substantial mortality occurred). This might mean that ATP could not be normally processed, which would indicate mismatching among various metabolic pathways (Hochachka, 1986; Knight et al., 1986). The same authors observed no evidence of rapid leakage of electrolytes during chilling of aphids (Pullin and Bale, 1988). However, the method they used (measuring conductivity of water in which the aphids were submerged) was rather imprecise and might have been influenced by the relative impermeability of the cuticle. Membrane failure due to the phase transition in the membrane lipids (Hazel, 1989), oxidative stress (Rojas and Leopold, 1996) and protein denaturation or incorrect folding (Yocum, 2001) were suggested as other potential mechanisms of chilling-injury.

The main purpose of the present study was to follow the changes in water and ion balances and energy status during exposure to a sub-zero, but non-freezing, temperature of –5°C in the adult bugs of *Pyrrhocoris apterus* (Insecta: Heteroptera: Pyrrhocoridae). Such changes were correlated with pre-freeze mortality and chilling-injury (assessed as the time necessary for recovery from chill-coma). *P. apterus* was a convenient model because relatively extensive knowledge on its diapause and physiology of cold hardiness has been gathered (Sláma, 1964; Hodek, 1968, 1983; Hodková and Hodek, 1994, 1997; Šula et al., 1995; Socha et al., 1997; Koštál and Šimek, 2000; Koštál et al., 2001; Šlachta et al., 2002; Hodková et al., 1999, 2002). Here, bugs in three acclimation groups (non-diapause, diapause and diapause, cold-acclimated), which differed markedly in the level of chill-tolerance, were compared. We found that during exposure to –5°C, the least chill-tolerant (non-diapause) insects, in comparison with the other two groups, displayed relatively rapid loss of water from the haemolymph compartment, an inability to maintain ion gradients across the fat body membrane, an inability to regulate ions into the hindgut fluid and no spending of total adenylate pools in the fat body. These observations are discussed with respect to their potential role in chilling-injury and pre-freeze mortality.

Materials and methods

Insects, acclimation groups

The adults of *Pyrrhocoris apterus* L. came from the laboratory culture, which is renewed each spring by collecting adults in the field near Chelcice, South Bohemia, Czech Republic. Nymphs produced by the field-collected insects were reared at a constant temperature of 25°C and photoperiodic conditions that either promote continuous non-diapause development, i.e. long-day photoperiod of 18 h:6 h L:D, or induce reproductive diapause, i.e. short-day photoperiod of 12 h:12 h L:D (Hodek, 1968). Dry seeds of the linden tree (*Tilia parviflora* Ehrh.) and water were provided *ad libitum*. Three acclimation groups (experimental variants) of insects were obtained, as depicted in Fig. 1: (1) non-diapause adults (LD) were kept at 20°C in long days for 2 weeks (most females started to lay eggs); (2) diapause adults (SD) were kept at 20°C in short days for 6 weeks and (3) diapause adults, cold-acclimated (SDA) were kept at 20°C in short days for 2 weeks and were then acclimated at 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 three weeks in short days), followed by the fourth week at constant 0°C and continuous darkness. Such an acclimation protocol simulates the natural drop of temperatures during autumn, and the bugs achieve higher chill tolerance than if constant temperatures (in contrast to thermoperiodic regime) are used for acclimation (Koštál et al., 2001).

Insects from the three acclimation groups were then exposed to a constant temperature of –5°C (fluctuations between –4.3°C and –5.7°C) for different periods of time (up to 60 days). The temperature of –5°C was selected as it is well above the SCP level of any acclimation group: LD, –9.4±0.4°C (N=154); SD, –11.6±0.9°C (N=32); SDA, –15.9±0.8°C (N=32) (means ± S.E.M.; taken from Koštál et al., 2001; Šlachta et al., 2002) and, thus, the occurrence of freezing events was minimized. Exposure to a constant temperature of –15°C (fluctuations

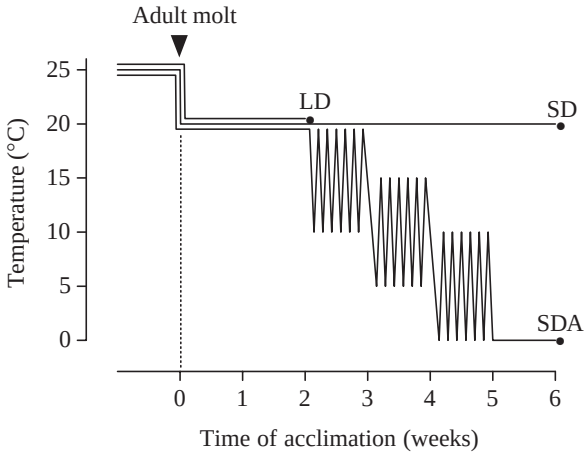

Fig. 1. Schematic depiction of the acclimation protocols used to obtain three different acclimation groups (LD, SD, SDA) of the adults of *Pyrrhocoris apterus*. See Materials and methods for a detailed description.

between –14.5°C and –15.5°C) was used for one additional experiment with the SDA group. Samples (with equal proportions of males and females) were taken at three time points: (1) just prior to the exposure (day 0); (2) after the exposure, which caused pre-freeze mortality in ~20% of the population sample (LD, day 4; SD, day 20); (3) after the exposure, which caused mortality in ~50% of the population sample (LD, day 8; SD, day 35). In the SDA group, mortality rates did not exceed 10% during 60-day exposure; therefore, the two samples were taken arbitrarily at days 40 and 60. The following analyses were performed.

Size, hydration and osmolality of body compartments

Ten specimens of each acclimation group (sampled at day 0) were carefully dissected on a cold stage at 0°C under a binocular microscope without adding any buffer. The fresh mass (FM) of each single tissue/compartment was measured using a Sartorius balance with a sensitivity of 0.1 mg. The following tissues (compartments) were considered: gut (oesophagus and midgut), hindgut + rectum (subsequently called hindgut), abdominal fat body (~90% of the tissue was collected), gonads (only in the LD group), 'remains' after the dissection (skeleton, epidermis, muscles, nervous system, etc.). Dry mass (DM) was measured after drying the specimens at 65°C for 3 days. Hydration (in mg water mg^{-1} DM) and total water content (WC) were calculated from the gravimetric data. Total volume of haemolymph was estimated by subtraction of the sum of the water contents of all dissected tissues/compartments from the value of the whole body-water content (see below). Some additional haemolymph rested in the remains after the dissection; thus, half (our estimation) of the amount of water from the remains was added to the calculated total volume of haemolymph. Volume of the hindgut could be measured only in some specimens of the SD and SDA groups (where the hindgut was relatively big and spherical in shape) by measuring its radius (r) and using it in a sphere volume (V) formula: $V=4/3\pi r^3$. In the insects with a small, tubular hindgut (mostly LD group), its volume was estimated to represent 0.1 µl.

Whole body FMs and DMs were obtained from another 30 insects of each acclimation group. Loss of whole-body FM was measured in yet another 30 adults (per acclimation group) that were individually marked by a pencil on their elytra and weighed two (or three) times: prior to, (during) and at the end of the exposure to –5°C.

Haemolymph samples for osmolality measurements were collected from 10 individual insects by cutting off one of the antennae and allowing it to bleed into a calibrated capillary tube. Similarly, samples of the hindgut fluid were obtained by piercing the hindgut wall and collecting the fluid into a capillary tube. Osmolalities were measured in 10–15 nl droplets using the Clifton Nanoliter Osmometer, according to the manufacturer's instructions (Clifton Technical Physics, Hartford, NY, USA).

Mortality and recovery time

Groups of 10–30 insects were exposed to –5°C for different periods of time. They were then transferred to 20°C, supplied with water and linden seeds, and their survival was scored 5 days later. Only the individuals capable of rapid coordinated crawling were considered to be survivors (our preliminary experiments verified that such insects were later capable of normal reproduction).

Recovery time was defined as the time necessary for resumption of locomotion after the chill-coma caused by the exposure to –5°C. Insects (10 per sample) that were previously exposed to –5°C for different periods of time were placed in glass Petri dishes on their backs and the time taken for them to turn over unaided and resume a normal position at 20°C was measured.

Na^+ and K^+ concentrations, equilibrium potentials

Concentrations of Na^+ and K^+ ions were determined in the extracts of haemolymph samples taken as described above. The whole amount of haemolymph that bled spontaneously (without pressing the animal) was collected from each specimen. The haemolymph collected from 10 insects was pooled, and the volume of each pooled sample was measured (depicted in Fig. 3A) and used to estimate the mean haemolymph volume in one specimen. Each pooled sample was taken in 6 (LD group) or 3 (SD and SDA groups) replications. Haemolymph was then extracted in 100 µl of a 1 mol l^{-1} solution of trichloroacetic acid (Sigma Chemical Co., St Louis, MO, USA) in deionized water (conductivity <0.1 µS cm^{-1}). Samples of hindgut fluid (10 tissues pooled, three replications) were taken as described above. Fat body tissues [10 tissues pooled, 6 (LD) or 3 (SD, SDA) replications] were weighed (FM), dried and weighed again (DM) in order to calculate the amount of water. Samples of hindgut fluid and fat body tissue were extracted in 100 µl of 65% nitric acid. After the extraction, the samples were centrifuged at 20 000 ***g*** for 10 min and the concentrations of ions were measured in supernatants by atomic emission spectrophotometry (Na^+ at 589.0 nm; K^+ at 766.5 nm) using a spectrophotometer SpectrAA 640 (Varian Techtron, Mulgrave, Australia).

The equilibrium potentials (E) across the fat body membrane were calculated for each ion separately using the Nernst equation:

$$E=(RT/zF)\ln(C_o/C_i)\,, \quad (1)$$

where R is the gas constant, T is the absolute temperature, z is the charge of the ion, F is the Faraday constant, and C_o is the outer (haemolymph) and C_i is the inner (fat body) concentration of the ion. For the sake of simplicity, the fat body was considered as a single compartment having direct communication with the haemolymph. Indeed, our preliminary studies on the morphological structure of the fat body in *P. apterus* (V.K. and J.V., unpublished results) revealed that individual fat body cells (mean diameter of 50 µm) are uniform and are very tightly packed inside the fat body lobes so that the intercellular spaces are negligible.

Adenylates and energy charge

The abdominal fat body tissues from 3–5 specimens were

pooled for each sample (taken in four replications). They were collected in liquid nitrogen and, upon nitrogen evaporation, weighed. Ice-cold $HClO_4$ (6%) solution containing 1 mmol l^{-1} EDTA was added and the sample was quickly homogenized using a plastic pestle and a hand-held battery-driven homogenizer. After centrifugation at 22 000 ***g*** at 4°C for 5 min, the supernatant was neutralized to pH 7 by adding a solution of 1.5 mol l^{-1} KOH, 0.4 mol l^{-1} imidazole and 0.3 mol l^{-1} KCl. Precipitated $HClO_4$ was removed by centrifugation at 22 000 ***g*** at 4°C for 5 min and the supernatant was stored at –80°C until analysis.

The concentrations of ATP, ADP and AMP were measured using enzymatic methods, which couple the interconversions between the adenylates with the reduction/oxidation of NAD(P)/NAD(P)H (Passonneau and Lowry, 1993). Absorbance at 340 nm was measured using a Pye Unicam SP8-100 spectrophotometer.

The contents of ATP, ADP and AMP were used to calculate the adenylate energy charge (AEC) according to the formula: AEC=[ATP]+0.5[ADP]/[ATP]+[ADP]+[AMP]. AEC represents a linear measure of the metabolic energy stored in the adenine nucleotide system (Atkinson, 1968).

Results

Water balance and osmolality prior to exposure to –5°C

Data on size, hydration and osmolality in different body compartments are summarized in Table 1. The differences between the insects in the LD and SD groups reflect the basic differences between two developmental modes: reproduction in LD and diapause in SD. The LD adults had relatively big (functional) gonads and the SD adults had a bigger fat body (stores of lipids and glycogen) and a smaller (‘empty’) gut. Whole-body FM was higher in the SD group than in the LD

Table 1. *Size, hydration and osmolality in body compartments of the adults of* Pyrrhocoris apterus *of three acclimation groups prior to their exposure to –5°C*

		Acclimation-group*		
Compartment* (sex)	Parameter†	LD	SD	SDA
Whole body	FM	52.4±10.7^{b}	71.8±11.3^{a}	63.2±10.7^{a,b}
	Hydration	1.90±0.88^{a}	1.60±0.33^{b}	1.29±0.14^{c}
	WC	34.3±7.0^{b}	44.2±6.9^{a}	35.6±6.0^{b}
Gut	FM	7.5±3.8^{a}	4.3±0.8^{b}	4.7±1.5^{b}
	Hydration	2.11±0.37	2.33±0.58	1.93±0.76
	WC	5.1±2.6^{a}	3.0±0.6^{b}	3.1±1.0^{b}
Gonads (female)‡	FM	6.7±2.3	–	–
	Hydration	2.66±0.84	–	–
	WC	4.9±1.7		
Gonads (male)	FM	1.4±0.5	–	–
	Hydration	2.90±1.60	–	–
	WC	1.0±0.4		
Abdominal fat	FM	4.5±1.8^{b}	12.3±4.8^{a}	12.4±4.4^{a}
	Hydration	0.93±0.19^{b}	1.20±0.32^{a}	0.83±0.19^{b}
	WC	2.3±0.9^{b}	6.5±2.5^{a}	5.6±2.0^{a}
Haemolymph	*V*	16.3±6.0^{b}	24.2±7.0^{a}	11.6±3.8^{b}
	Osmolality	370±11^{b}	373±40^{b}	626±64^{a}
Hindgut	*V*	–	–	3.9±3.4
	Osmolality	–	–	195±83
	Na^{+}	17.3±10.9	18.4±10.8	5.7±4.5
	K^{+}	46.7±23.6	12.8±6.5	17.5±8.1
Remains	FM	26.4±2.2^{b}	37.4±4.4^{a}	38.7±5.8^{a}
	Hydration	1.49±0.14^{a}	1.60±0.14^{a}	1.21±0.09^{b}
	WC (1/2)	7.9±0.7^{b}	11.5±1.4^{a}	10.6±1.6^{a}

*See text for description of body compartments and acclimation groups: LD (non-diapause), SD (diapause) and SDA (diapause, cold-acclimated).

†Parameters: FM, fresh mass (mg); hydration (mg water mg^{-1} dry mass); WC, water content (µl); *V*, volume (µl); osmolality (mmol kg^{-1}), Na^{+}, K^{+}, concentrations of ions (mmol l^{-1}). Each value represents mean ± S.D. (see text for *N*). The means in rows were statistically analysed using ANOVA and, where the differences were significant, by Tukey’s multiple comparison test. The means flanked by different letters are statistically different at $P<0.05$. Where no value is shown, the compartment was too small to allow reliable measurement.

‡Only those females that have laid batch of eggs one day before the sampling were dissected.

group. The SD adults also had a higher volume of haemolymph and higher FM of remains after dissection compared with the LD adults. Such differences suggest that the bugs achieve a slightly bigger body size when reared under the short-day conditions. The whole-body hydration rate was higher in the LD group, but the SD adults contained more water in whole body and in haemolymph due to their larger size. Osmolalities of haemolymph were almost equal (~370 mmol kg^{-1}) in both groups. Relatively small (tubular) hindguts were present in both groups. The osmolality of hindgut fluid could not be reliably measured and its volume was estimated to be approximately 0.1 µl.

The differences found between the SD and SDA groups were considered to represent the physiological changes that accompany the process of cold acclimation. Partial dehydration was the most obvious change (Table 1) in SDA adults: (1) whole-body FM decreased, although not significantly; (2) the difference in WC indicated that an average of 8.6 µl (19.5%) of the whole-body water disappeared during acclimation (this amount practically equals the loss of whole body FM) and (3) total volume of haemolymph significantly decreased by 12.6 µl (52%). Insignificant decreases of water content were seen in the other compartments (gut, fat, remains). Haemolymph osmolality almost doubled during the cold acclimation. The volume of hindgut increased from 0.1 µl to 3.9 µl on average, its shape became spherical and the hindgut fluid was hypo-osmotic (195 mmol kg^{-1}) to the haemolymph (626 mmol kg^{-1}). Thus, the decrease in haemolymph volume (12.6 µl) could be fractioned almost perfectly between the evaporative/excretory loss of water (8.6 µl) and the transfer of water to the hindgut (3.8 µl).

Pre-freeze mortality and chilling injury

Fig. 2A documents the considerable differences in the level of chill tolerance between three acclimation groups of insects. While a sufficient time to kill 50% of LD adults was only 7.6 days at –5°C, 35.6 days were required in the SD group, and mortality did not exceed 10% after a 60-day exposure in the SDA group. We believe that freezing events were very exceptional in our experiments. First, the temperature of –5°C was well above the mean SCP of any group; second, it was always possible to sample liquid haemolymph from all specimens (sampling took place within 3 s after withdrawing the specimen from –5°C); third, if stochastic freezing events occurred with high frequency, the relationship between mortality and exposure time would be expected to take a logarithmic rather than a sigmoid shape.

Recovery time necessary for resumption of locomotion after chill-coma linearly increased with increasing time of exposure to –5°C. After exposure for a given duration, recovery time was always shortest in the most chill-tolerant variant (SDA) and longest in the least chill-tolerant variant (LD) of the three variants tested (Fig. 2B).

Changes in hydration and osmolality at –5°C

Insects of all acclimation groups tended to decrease their whole-body FM during exposure to –5°C (Fig. 3A, inset). As we could observe no defecation during the exposure and the loss of DM was probably negligible because of extremely low metabolism, we suppose that the changes in whole-body FM primarily reflected losses of water. The rate of water loss was clearly highest in the LD group. The loss of 12.7% of water during the 8-day exposure would correspond to 4.4 µl when 34.3 µl (Table 1) is taken as the initial total amount of water. It seems that the haemolymph compartment was particularly prone to lose water in the LD group. Fig. 3A shows that the mean volume of haemolymph rapidly decreased in the LD group and, after 8 days, dropped to the volume that was typically obtained from the SDA group (SDA group was characterised by its low hydration and low haemolymph volume; see Table 1). By contrast, no significant change in

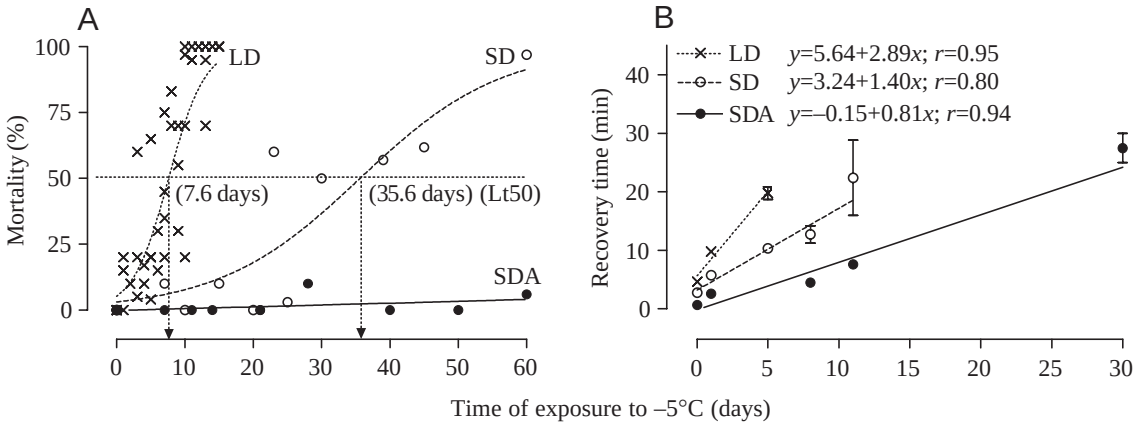

Fig. 2. Relationships between the duration of the exposure to –5°C and (A) the pre-freeze mortality or (B) the recovery time after chill-coma in the adults of *Pyrrhocoris apterus* belonging to three different acclimation groups (LD, SD, SDA; see Fig. 1). In A, each data point represents a mortality rate in the group of 10–30 individuals [Boltzmann sigmoidal curves were used to fit the data: LD, R^2=0.7103 (goodness of fit); SD, R^2=0.7900]. The arrows show times necessary to kill 50% of the population sample (Lt50). In B, each data point represents a mean ± S.E.M. of 10 individuals. The probabilities that slopes of linear regressions deviate from zero were P<0.001 for all groups.

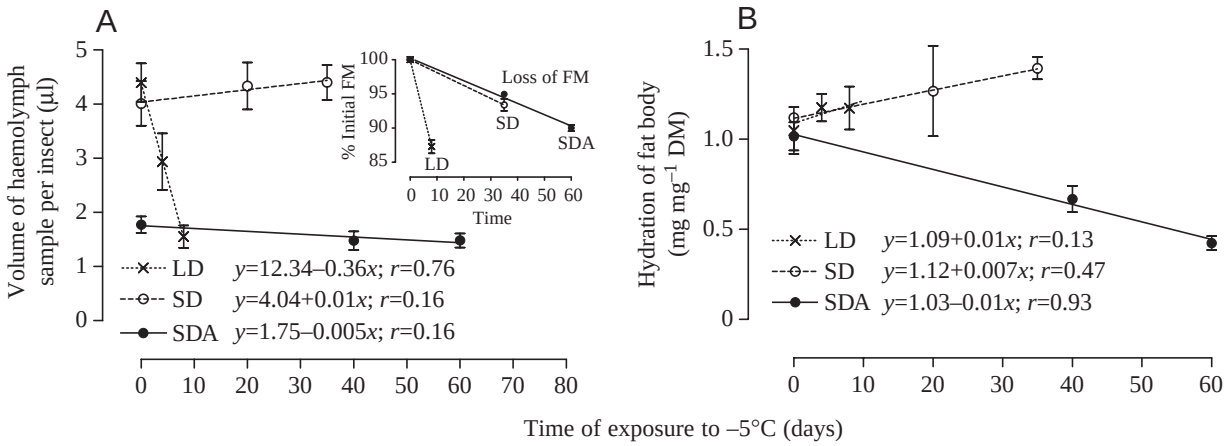

Fig. 3. Relationships between the duration of the exposure to –5°C and the volume of haemolymph sample (A), the loss of fresh mass (FM) (inset) and the hydration of fat body cells (DM, dry mass) (B) in the adults of *Pyrrhocoris apterus* belonging to three different acclimation groups (LD, SD, SDA; see Fig. 1). In A, each data point represents a mean ± S.E.M. of 6 samples (LD) or 3 samples (SD, SDA), each consisting of pooled haemolymph taken from 10 insects. The probabilities that slopes of linear regressions deviate from zero were: LD, $P<0.001$; SD, $P=0.469$; SDA, $P=0.136$. In the inset, each data point represents a mean ± S.E.M. of 30 individuals weighed prior to and after the exposure to –5°C. In B, each data point represents a mean ± S.E.M. of 6 samples (LD) or 3 samples (SD, SDA), each consisting of pooled fat body tissues taken from 10 insects. The probabilities that slopes of linear regressions deviate from zero were: LD, $P=0.567$; SD, $P=0.204$; SDA, $P<0.001$.

hydration or water content was observed in the fat body (in fact, an insignificant tendency to increase water content was registered in the fat body; Fig. 2B). Considering that all 4.4 µl of water were lost from the haemolymph compartment, this would shrink from an initial 16.3 µl (Table 1) to 11.9 µl, which is 1.37-fold. Indeed, a 1.34-fold increase in haemolymph osmolality (from 370 mmol kg^{-1} to 497 mmol kg^{-1}) was observed concomitantly (Table 2). Thus, we suppose that most of the water was really lost from the haemolymph compartment in the LD group insects. The hindgut remained small and tubular in all specimens of the LD group during the exposure to –5°C and its volume was estimated to be 0.1 µl.

Compared with the LD group, less rapid loss of whole-body FM was observed in the SD group: 6.66% (i.e. 2.9 µl) during 35 days of exposure (Fig. 3A, inset). The mean volume of haemolymph remained practically constant (Fig. 3A). No sign of dehydration was observed in the fat body, its total WC remained constant and hydration rate showed an insignificant increase (Fig. 2B). We presume that most of the water was again lost from the haemolymph compartment. But, because of its large initial volume of 24.2 µl (Table 1), the loss of 2.9 µl would correspond to a 1.14-fold decrease only. Haemolymph osmolality increased from 373 mmol kg^{-1} to 737 mmol kg^{-1}, which is 1.98-fold (Table 2). The average volume of hindgut increased from 0.1 µl to 1.1 µl in the SD group (Table 2). In fact, the hindgut remained tubular in six out of 12 specimens, while it became spherical, with an average volume of 2.15±1.52 µl , in the other six specimens.

In the SDA group, the slowest rate of FM loss of the three groups was observed: 5.0% (1.8 µl) or 8.9% (3.2 µl) during 35 or 60 days, respectively (Fig. 3A, inset). The volume of average haemolymph sample remained constant (Fig. 3A). In contrast to the other two groups, a significant trend of fat body dehydration was observed in the SDA insects (Fig. 3B). The FM of fat body decreased from an initial 12.4 mg to 10.7 mg or 9.3 mg during 35 or 60 days, respectively. The hydration rate decreased from

Table 2. *Changes in some physiological parameters during the exposure of adult* Pyrrhocoris apterus *of three acclimation groups to –5°C*

		Acclimation group/time of exposure to –5°C (days)			
Compartment	Parameter	LD/8	SD/35	SDA/35	SDA/60
Haemolymph	Osmolality	497±36 (6)***	737±94 (9)***	1065±324 (7)***	1247±386 (5)^{ns}
Hindgut	*V*	–	1.1±1.6 (12)	3.6±2.2 (7)^{ns}	3.6±2.3 (5)^{ns}
	Osmolality	–	–	344±149 (5)*	523±149 (4)^{ns}
	Na^+	19.3±10.4 (3)^{ns}	38.7±15.3 (3)^{ns}	19.2±1.4 (3)**	31.0±4.3 (3)*
	K^+	57.8±10.7 (3)^{ns}	49.6±14.9 (3)*	66.1±18.1 (3)*	50.7±5.9 (3)^{ns}

Each value represents mean ± S.D. (*N*). The means were statistically compared with corresponding initial (see Table 1) or preceding (in the case of SDA/60) values using unpaired two-tailed *t*-tests (ns, not significant; *$P<0.05$; **$P<0.001$; ***$P<0.0001$). Other descriptions as in Table 1.

1.12 mg water mg^{-1} DM to 0.66 or 0.42 mg water mg^{-1} DM. The total WC decreased from 5.6 µl to 4.3 or 2.8 µl. Thus, the amount of water lost from fat body tissue alone could almost completely explain the total loss of water from the whole body. This indicates that the haemolymph volume did not change significantly; nevertheless, its osmolality reached very high levels of 1065 mmol kg^{-1} or 1247 mmol kg^{-1} during 35 or 60 days, respectively (Table 2). No significant change in the hindgut volume was found in the SDA group. The osmolality of hindgut fluid increased significantly from 195 mmol kg^{-1} to 344 mmol kg^{-1} during the first 35 days of the exposure, and a further increase (statistically insignificant) to 523 mmol kg^{-1} was observed during the additional 25 days (Table 2).

Na$^+$ and K$^+$ concentrations

The following changes in ion concentrations were observed in the haemolymph and fat body tissue during the exposure to –5°C (Fig. 4).

Sodium in haemolymph (Fig. 4A). A significant decrease of the Na$^+$ concentration was found in the LD group. In the other two groups, the concentrations were relatively constant. Considering the changes in haemolymph volume (see above) and the concentrations measured, we could estimate the changes in total pools of Na$^+$: it decreased from 0.43 µmoles to 0.24 µmoles in the LD group; a relatively small decrease from 0.62 µmoles to 0.51 µmoles was observed in the SD group; and the pool was stable in the SDA group [from 0.43 µmoles to 0.37 µmoles (day 35) or 0.40 µmoles (day 60)].

Sodium in fat body (Fig. 4B). No significant changes in the concentration were detected with increasing time of exposure by linear regression analysis in any acclimation group. Also the pools were rather stable: no change in the LD group (0.03 µmoles); a small change in the SD group (from 0.07 µmoles to 0.08 µmoles); and a decrease in the SDA group [from 0.10 µmoles to 0.08 µmoles (day 35) or 0.05 µmoles (day 60)].

Potassium in haemolymph (Fig. 4C). The concentrations significantly increased with increasing time of exposure in all three acclimation groups. The total pools also increased in all groups: from 0.26 µmoles to 0.46 µmoles (LD group); from 0.37 µmoles to 0.46 µmoles (SD group); and from 0.13 µmoles to 0.21 µmoles (day 35) or 0.31 µmoles (day 60) (SDA group).

Potassium in fat body (Fig. 4D). A non-significant decrease in concentration was observed in the LD group. A significant

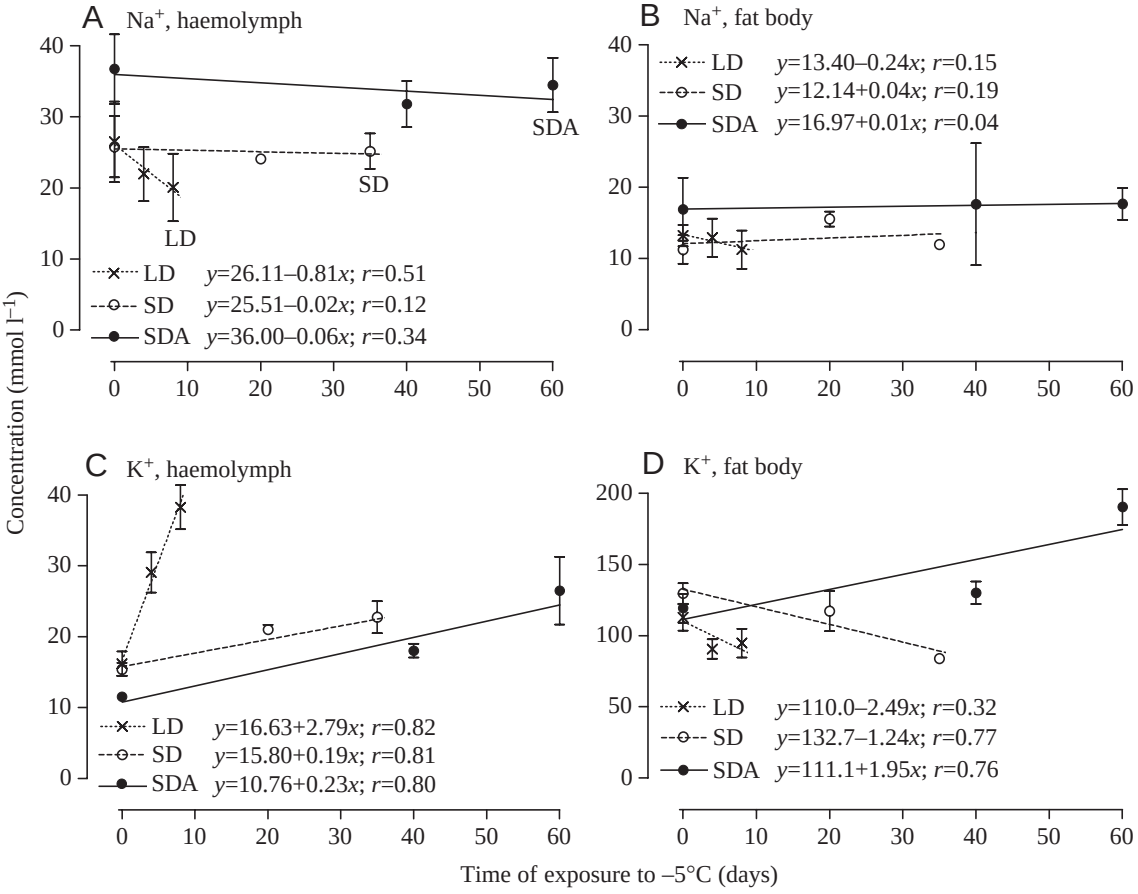

Fig. 4. Relationships between the duration of the exposure to –5°C and the concentrations of sodium (A,B) or potassium ions (C,D) in either haemolymph (A,C) or fat body cells (B,D) in the adults of *Pyrrhocoris apterus* belonging to three different acclimation groups (LD, SD, SDA; see Fig. 1). Each data point represents a mean ± S.E.M. of 6 samples (LD) or 3 samples (SD, SDA), each consisting of pooled tissues taken from 10 insects. The probabilities that slopes of linear regressions deviate from zero were: (A) LD, P=0.016; SD, P=0.724; SDA, P=0.274; (B) LD, P=0.527; SD, P=0.602; SDA, P=0.917; (C) LD, P<0.001; SD, P=0.003; SDA, P<0.002; (D) LD, P=0.142; SD, P=0.009; SDA, P=0.017.

decrease was observed in the SD group and a significant increase in the SDA group. The total pool was rather stable in the LD group (from 0.26 μmoles to 0.24 μmoles); it decreased in the SD group (from 0.84 μmoles to 0.58 μmoles) and also in the SDA group [from 0.67 μmoles to 0.55 μmoles (day 35) or 0.52 μmoles (day 60)].

In an additional experiment, the changes of haemolymph ion concentrations were measured in the SDA insects when exposed to –15°C (instead of –5°C), where the duration of exposure that causes mortality in 50% of the population sample (Lt50) shortens dramatically to 9.0 days (Koštál et al., 2001). Under such conditions, the K^+ concentration increased significantly and rapidly while the Na^+ concentration significantly decreased. The rates of change in both concentrations were very similar to the rates observed in the LD group exposed to –5°C (Fig. 5).

In the hindgut fluid (Table 2), no significant changes in the ion concentrations or total pools were registered during the exposure to –5°C in the LD group, and the pools were relatively small (~0.002 μmoles of Na^+ and ~0.005–0.006 μmoles of K^+). In the SD group, the initial pools of both ions were small (~0.002 μmoles of Na^+ and ~0.001 μmoles of K^+). During the exposure, the concentrations increased (significantly in the case of K^+; see Table 2) and the pools increased too (to 0.04 μmoles of Na^+ and 0.05 μmoles of K^+). In the SDA group, the highest increases (of the three groups) of concentration and, especially, of the pools of both ions were observed. After 35 days, 0.07 μmoles of Na^+ and 0.24 μmoles of K^+ were accumulated in the hindgut fluid, and after 60 days it was 0.10 μmoles of Na^+ and 0.17 μmoles of K^+.

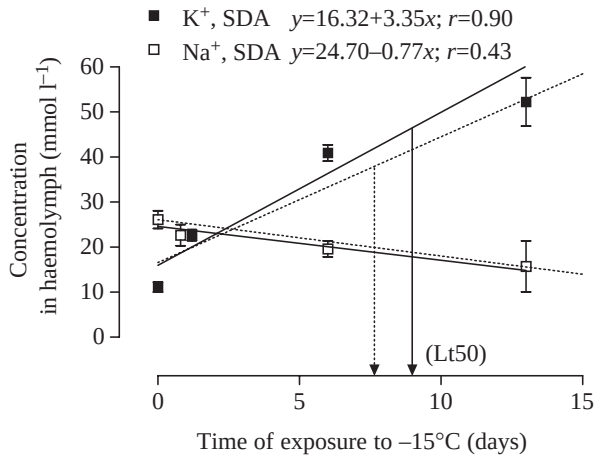

Fig. 5. Relationship between the duration of the exposure to –15°C and the concentrations of sodium and potassium ions in haemolymph in the adults of *Pyrrhocoris apterus* belonging to the acclimation group SDA (see Fig. 1). Each data point represents mean ± S.E.M. of 10 samples of haemolymph taken from individual insects. The probabilities that slopes of linear regressions deviate from zero were: K^+, $P<0.001$; Na^+, $P=0.013$. The solid arrow shows time necessary to kill 50% individuals of the population sample (Lt50). For comparison, the Lt50 (broken arrow) and linear regressions (broken lines) of the haemolymph concentrations of the two ions in the acclimation group LD exposed to –5°C are shown (redrawn from Fig. 4A,C).

Na^+ and K^+ equilibrium potentials

Equilibrium potentials of sodium (E_{Na}) across the fat body membrane remained relatively constant at approximately +20 mV during the exposure to –5°C in all the acclimation groups (Fig. 6).

Equilibrium potentials of potassium (E_K) decreased from –49.5 mV on day 0 to –24.7 mV on day 8 in the LD group. A slower rate of decrease in E_K was observed in the SD group (from –57.5 to –34.9 mV on day 35). The slowest rate of E_K decrease, from –60.6 to –52.4 mV on day 60, was registered in the SDA group (Fig. 6). The values of E_K taken at days 0 and 60 did not differ statistically (unpaired two-tailed *t*-test, $P=0.2726$).

Adenylates and energy charge

The concentrations of adenylates in the fat body cells showed no statistically significant changes (in most cases) during the exposure to –5°C in all three acclimation groups (Table 3). The trends of decrease, nevertheless, were apparently indicated in the groups SD and SDA, and these were significant in the case of AMP. Considering the decreasing FM

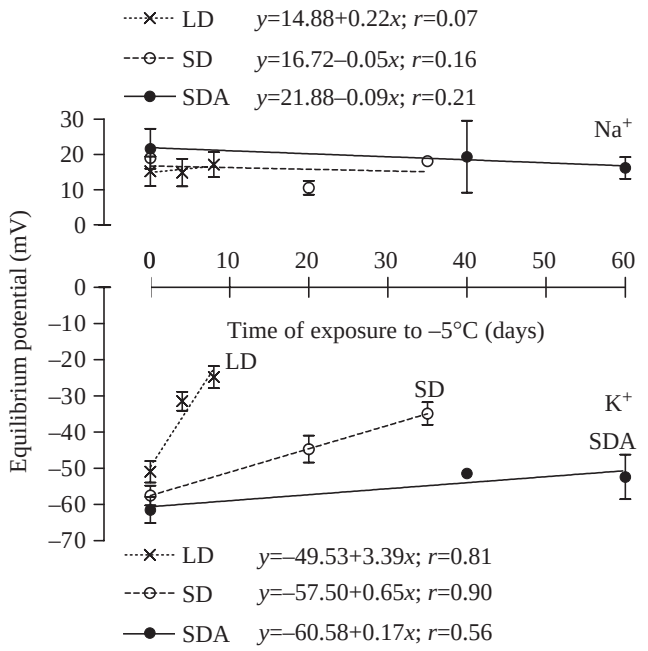

Fig. 6. Relationships between the duration of the exposure to –5°C and the equilibrium potentials of sodium (upper part) and potassium ions (lower part) across the fat body membrane in the adults of *Pyrrhocoris apterus* belonging to three different acclimation groups (LD, SD, SDA; see Fig. 1). Each data point represents a mean ± S.E.M. of 6 (LD) or 3 (SD, SDA) equilibrium potentials calculated using the data shown in Fig. 4. The probabilities that slopes of linear regressions deviate from zero were: Na^+: LD, $P=0.756$; SD, $P=0.684$; SDA, $P=0.584$; K^+: LD, $P<0.001$; SD, $P=0.001$; SDA, $P=0.117$.

Table 3. *Changes in energy status of the fat body during the exposure of adult* Pyrrhocoris apterus *of three acclimation groups to –5°C*

		Acclimation group/time of exposure to –5°C (days)				
	Parameter	LD/0	SD/0	SDA/0	ANOVA	*P*
Initial state	ATP	1.30±0.17[b]	1.98±0.35[a]	1.44±0.35[a,b]	**	0.0241
	ADP	0.40±0.23	0.78±0.99	0.54±0.49	ns	0.7151
	AMP	0.08±0.05	0.22±0.14	0.18±0.22	ns	0.4457
	AEC	0.85±0.06	0.83±0.10	0.83±0.09	ns	0.9295
	Parameter	LD/8	SD/35	SDA/35	SDA/60	
After exposure at –5°C	ATP	1.26±0.12[ns]	1.47±0.86[ns]	0.96±0.26[ns]	1.22±0.14[ns]	
	ADP	0.50±0.50[ns]	0.17±0.11[ns]	0.50±0.05[ns]	0.49±0.13[ns]	
	AMP	0.13±0.13[ns]	0.02±0.01*	0.15±0.02[ns]	0.08±0.03**	
	AEC	0.84±0.12[ns]	0.93±0.06[ns]	0.75±0.04[ns]	0.82±0.08[ns]	

Each value represents mean ± S.D. (N=4) expressed in nmol mg^{-1} fresh mass of fat body tissue (ATP, ADP, AMP) or as a ratio (AEC, adenylate energy charge).

The means in each row in the upper part of the table were statistically treated with ANOVA and, where the differences were significant, by Tukey's multiple comparison test. The means flanked by different letters are statistically different at P<0.05. The means in the lower part of the Table were statistically compared with the corresponding initial or preceding (in the case of SDA/60) values using unpaired two-tailed *t*-tests (ns, not significant; *P<0.05; **P<0.001). Other descriptions as in Table 1.

of fat body during the exposure of SDA insects to –5°C, there must have been a significant decrease in the total content (pools) of all adenylates in the fat body. By contrast, because the FM of the fat body was stable in the LD and SD groups, the total pools probably did not decrease at all (LD) or only slightly (SD).

The adenylate energy charge (AEC) was similar in all three groups (ranging between 0.83 and 0.85) prior to their exposure to –5°C and it was also fairly stable during the exposure (Table 3).

Discussion

In this paper, the chilling-injury and pre-freeze mortality in adult bugs of *Pyrrhocoris apterus* were correlated with the changes of water, osmotic and ion balance and energetic status during exposure to sub-zero, but non-freezing, temperatures. Insects of three different physiological states (acclimation groups) were compared throughout the study: non-diapause (LD), diapause (SD) and diapause, cold-acclimated (SDA). When exposed to –5°C, the three acclimation groups differed substantially in the levels of chilling-injury and pre-freeze mortality. The level of chilling-injury was estimated based on the time necessary for recovery from chill-coma. Entering a state of torpor, called chill-coma, is a general response of insects to a species-specific low temperature (Goller and Esch, 1990; Hosler et al., 2000). We found a linear relationship between the duration of the exposure to –5°C (in days) and the time needed for resumption of locomotion (in min) after the chill-coma in *P. apterus*. Moreover, for each specific exposure time, the recovery times differed depending on acclimation group in the order: LD>SD>SDA. We interpret these results as showing that the level, or 'sum', of chilling-injury increased with the increasing duration of the exposure and that the resistance to chilling-injury was dependent on physiological status. Such observations are in accordance with general theory of insect cold hardiness (Lee, 1991; David et al., 1998). The duration of exposure to –5°C that causes mortality in 50% of the population sample (Lt50) increased in the order LD<SD<SDA. Thus, the main question that we attempted to answer was: are there any differences between the three acclimation groups in their ability to regulate water, ions and energy that could be correlated with (and causally linked to) the differences in chill tolerance? First, the differences among the three acclimation groups in their initial physiological state (prior to exposure to –5°C) will be discussed. Second, we turn to the differences in their response to exposure to –5°C.

Initial physiological state prior to exposure to –5°C

The non-diapause insects (LD) were actively moving, feeding and reproducing while the diapause insects (SD) had arrested reproduction, and their locomotion and feeding activities were minimal. Such 'overt' differences were reflected in the sizes of gonads, gut and fat body and were undoubtedly based on fundamental differences in gene expression and hormonal milieu that are typical for each of the two alternative states in insects (Tauber et al., 1986; Danks, 1987; Flannagan et al., 1998; Denlinger, 1985, 2002). What concerns the parameters assessed in this study are that no significant differences were found between the LD and SD groups in their hydration, osmolality, ion concentrations, adenylate concentrations and adenylate energy charge prior to the exposure to –5°C. However, the ways in which individual parameters changed during the exposure to –5°C differed substantially between the two groups and will be discussed later.

Cold acclimation of the SD group of *P. apterus* is known to result in various transformations, which lead to an increased level of chill-tolerance (Hodková and Hodek, 1997; Hodková et al., 1999, 2002; Koštál et al., 2001; Šlachta et al., 2002). Here, we showed that the process of cold-acclimation (SD → SDA) is also accompanied by the following changes: (1) partial dehydration affecting especially the haemolymph compartment, which was reduced to one half of its pre-acclimation level; preferential loss of water from the haemolymph was reported previously in insects subjected to drying of their habitats (Zachariassen and Einarson, 1993; Hadley, 1994; Zachariassen and Pedersen, 2002); (2) redistribution of water; a 'reserve' (~4 μl) of hypo-osmotic fluid accumulated in the hindgut; the principal role of the hindgut in insect water balance is widely recognised (Hadley, 1994; Danks, 2000; Coast, 2001); (3) regulation of ion concentrations; despite the loss of 52% of water from the haemolymph, the concentrations of Na^+ and K^+ were maintained almost constant because the ion pools decreased by 0.19 μmoles or 0.24 μmoles of Na^+ or K^+, respectively. Ion pools increased during cold acclimation in the hindgut fluid (by 0.02 μmoles or 0.07 μmoles of Na^+ or K^+, respectively) and they remained rather constant in the fat body. Thus, we suppose that some fraction of ions from dehydrating haemolymph could be excreted. No significant changes in the concentrations of adenylates or in the AEC in the fat body were observed during the cold acclimation of *P. apterus*.

Changes of physiological parameters during the exposure to –5°C

First, the changes observed in the LD group will be discussed. Although the insects of all acclimation groups tended to lose body water during the exposure to –5°C, the rate of water loss was clearly the highest in the LD group. Such a difference between acclimation groups might be caused by a different quality and/or quantity of the cuticular hydrocarbons, which form the crucial barrier against water loss (Lockey, 1985; Yoder et al., 1992, 1995; Hadley, 1994). Respiratory water loss was probably minor thanks to a deep depression of metabolism at –5°C. Nevertheless, we assume that the loss of body water *per se* was not the likely cause of pre-freeze mortality in the LD group. Most of the body water was lost from the haemolymph compartment while the intracellular compartments remained normally hydrated. The final amount of haemolymph after the 8-day exposure to –5°C in the LD group was at least as high as in the SDA group prior to the exposure. And furthermore, the SDA insects were substantially more dehydrated during their exposure to –5°C for 60 days but still did not die. During the exposure to –5°C, the LD insects displayed a dramatic decrease (to 56%) of the Na^+ pool in the haemolymph, which was counteracted by a similarly massive influx of K^+. As the volume of haemolymph decreased, the efflux of Na^+ did not result in a big change in its concentration (but this was still significant) while the influx of K^+ led to a >2-fold increase in its concentration. At the same time, neither the concentrations nor the pools of ions changed significantly in the fat body. This was probably because the fat body represented a relatively small compartment in the LD insects (~12.5% of total intracellular water). We suggest that the other tissues (muscles, gut, gonads) served as more important sinks for Na^+ and as sources of K^+. No significant regulation of ions into the hindgut fluid (and no excretion by defecation) was observed in the LD insects. These results might indicate that, at –5°C, the activity of Na^+/K^+-ATPases in the membranes of tissues was not sufficient to counteract the inward movement of Na^+ down the electrochemical gradient, which caused a (partial) depolarization of cell membranes followed immediately by an outward movement of K^+ (documented by a sharp decrease of E_K across the fat body membrane).

In comparison with the LD insects, the rates of water loss at –5°C were low in the SD and SDA insects. The initial water content in haemolymph was considerably lower (by half) in the SDA than in the SD insects. Perhaps, in order to protect a minimal haemolymph volume serving the basic transporting function, the water gradually disappeared from the intracellular compartments (fat body) of the SDA insects during their exposure to –5°C. With respect to ion regulation, the SD insects showed ion fluxes of a similar direction as detected in the LD insects but with much lower rates. Thus, the Na^+ pool decreased while the K^+ pool increased, both 1.2-fold, in haemolymph. The opposite changes were registered in the fat body (the fat body water represented ~33% of the total intracellular water pool in the SD group). In contrast to LD insects, at least some (~50%) of the SD insects were able to build a reserve of hindgut fluid and to regulate a certain fraction of ions into it. Such a capacity was even better expressed in the SDA insects. They showed no decrease of the Na^+ pool and a slight increase of the K^+ pool in haemolymph during exposure to –5°C. Despite reduction of the water content in the fat body to half, the Na^+ concentration there did not increase (its pool halved too) and the concentration of K^+ increased (despite its pool tending to decrease). Thus, it seems that the capacity to prevent/counteract a leakage of Na^+ down the electrochemical gradient (from haemolymph to the tissue cells) increased in the order LD<SD<SDA. As a result, the rates of counteractive outward movement of K^+, and of the E_K dissipation, decreased in the same order. Such a capacity might also be supported by regulation of a certain fraction of ions into the hindgut fluid (to counteract dehydration).

When the insects of the most chill-tolerant acclimation group (SDA) were exposed to a more severe low temperature of –15°C instead of –5°C, the rates of change of ion concentrations in their haemolymph became much faster and almost matched those observed in the LD group (the least chill-tolerant group) at –5°C. Interestingly, the Lt50 of the SDA group at –15°C shifted to 9.0 days, which also closely matched that of the LD group at –5°C (7.6 days).

The nature of chilling-injury

Most of the observations presented in this paper share one common denominator and that is maintaining the ion concentrations and ion gradients across membranes at sub-

zero temperature. Entering into a state of chill-coma in insects was previously attributed to the inability of Na^+/K^+-ATPases to function at low temperatures and to maintain/restore the nerve membrane electrochemical potential (Heitler et al., 1977; Kivivouri et al., 1990; Cossins et al., 1995; Hosler et al., 2000). The Na^+/K^+-ATPase is also responsible for maintaining Na^+ gradients across cell membranes in the other insect tissues (Emery et al., 1998). And, finally, the overall ion balance in insects is maintained by the gastrointestinal system including the hindgut and Malpighian tubules (Maddrell and O'Donnell, 1992; Zeiske, 1992). Here again, ion-pumping ATPases coupled to various secondary ion transporters represent the most important structural components (Schweikl et al., 1989; Wieczorek et al., 1989, 2000; Zeiske, 1992). We found that *P. apterus* adults of three different acclimation groups differed significantly in all three aspects (recovery from chill-coma, ion gradients across cell membranes, function of hindgut). The most chill-tolerant insects (SDA group) showed the most rapid recovery from chill-coma, the best capacity to maintain ion gradients across cell membranes and the highest level of ion regulation into the hindgut. Thus, we suggest that the impaired function of ion pumping systems together with the inability to prevent/restrict ion leakage down the electrochemical gradient might be an important cause of chilling-injury and pre-freeze mortality in non-diapause or non-acclimated *P. apterus* adults.

To our knowledge, the ability of cold-hardy overwintering insects to maintain ion gradients in a supercooled state has so far been reported only briefly by Dissanayke and Zachariassen (1980) and Hanzal et al. (1992) but without studying the changes in non-acclimated specimens. It was also shown that freezing of water in haemolymph of the wood fly *Xylophagus cinctus* at –10°C caused loss of function of the ionic pumps and rapid movements (within a few hours) of Na^+, K^+ and Mg^{2+} to electrochemical equilibrium across the cell membranes (Kristiansen and Zachariassen, 2001). There are examples of the effect of low temperature on ion balance in other organisms. Prolonged hypothermic exposure in non-adapted mammals may lead to dissipation of ion gradients across cell membranes, partial membrane depolarization, the opening of voltage-dependent Ca^{2+} channels and the influx of Ca^{2+}, which activates membrane phospholipid hydrolysis in a process that ultimately leads to cell damage (for a review, see Hochachka, 1986). Regulation of ion pumping and maintaining ion gradients is considered to be one of the pre-requisites for successful cold acclimation in overwintering ectothermic vertebrates (Cossins and Kilbey, 1989; Boutilier et al., 1997; Guppy and Withers, 1999; Stinner and Hartzler, 2000). In plants, ion leakage is commonly recognised as a serious consequence and/or cause of chilling-injury (Lyons, 1973; Jennings and Tatar, 1979). Maintenance of ion gradients requires active ion-pumping, which requires energy in the form of ATP. Hence, the whole system is dependent on the ability of an organism to maintain regulated metabolism, where ATP use is balanced with ATP synthesis. We found that *P. apterus* adults of all acclimation groups were able to keep stable adenylate energy charge (AEC) in their fat body cells during exposure to –5°C. The trends to decrease total adenylate pools, however, were registered in the SD and, especially, SDA groups. This observation suggested that the energy could be exploited for maintenance of ion gradients in SD and SDA groups. Thus, the lack of energy does not seem to be the cause of the failure of Na^+/K^+-ATPases to maintain the Na^+ gradient in the LD group. Rather, the ability of ionic pumps to exploit the energy might be impaired. It is a question of whether the ionic pumps were able to function at –5°C at all. Previously, we found that the oxygen consumption rate at 0°C ranged between 8.4 and 17.1 μl O_2 h^{-1} g^{-1} FM in cold-acclimated diapause *P. apterus* adults (Šlachta et al., 2002). Also, the biosynthesis of polyols rapidly proceeded at 0°C (Koštál et al., 2001). These two examples showed that metabolic functions might operate even at temperatures very close to that used in the present study. The ability of SD and SDA insects to increase the concentrations and enlarge the pools of ions in the hindgut fluid when kept at –5°C also indicated that active transport might be still functioning at –5°C. It was suggested (Hochachka, 1986) that maintaining low-permeability membranes (by means of downregulation of the ion-specific channels) could represent an alternative or additional compensating mechanism for the impaired function of ion pumps at a low temperature. The function of ion-transporting systems is also significantly influenced by the lipidic environment, in which the transporter molecules are embedded (Cornelius, 2001; Cornelius et al., 2001; Haines, 2001). Although the cold-acclimation-related changes in lipidic composition of membranes have been described in several insects, including *P. apterus* (Hodková et al., 1999, 2002; Šlachta et al., 2002), they have not been studied directly in relation to the function of ion pumps or transporters.

Collectively, some pieces of correlative evidence have been presented in this paper, which suggest that further study on the (in)ability of insects to maintain ion gradients across cell membranes could bring better understanding of the causes of their pre-freeze mortality and, consequently, their success during overwintering or cold exposure.

We thank Irena Vacková and Jana Mikesová for maintaining the laboratory insect culture. The data on recovery after chill-coma represent a part of Jan Vambera's M.Sc. Thesis, which was supervised by Oldrich Nedved (Faculty of Biological Sciences, University of South Bohemia, Czech Republic), to whom we also wish to express our thanks for his advice and opinion during the early stages of this study. The study was supported by the Grant Agency of the Czech Republic (grant no. 206/03/0099) and by the Academy of Sciences of the Czech Republic (project no. K 600 5114).

References

Anderson, R. L. and Mutchmor, J. A. (1968). Temperature acclimation and its influence on the electrical activity of the nervous system in 3 species of cockroaches. *J. Insect Physiol.* **14**, 243-251.

Atkinson, D. E. (1968). The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* **7**, 4030-4034.

Bale, J. S. (1987). Insect cold hardiness: freezing and supercooling – an ecophysiological perspective. *J. Insect Physiol.* **33**, 899-908.

Bale, J. S. (2002). Insects at low temperatures: from molecular biology to distributions and abundance. *Phil. Trans. R. Soc. Lond. B* **357**, 849-862.

Boutilier, R. G., Donohoe, P. H., Tattersall, G. J. and West, T. G. (1997). Hypometabolic homeostasis in overwintering aquatic amphibians. *J. Exp. Biol.* **200**, 387-400.

Bradfisch, G. A., Drewes, C. D. and Mutchmor, J. A. (1982). The effects of cooling on an identified reflex pathway in the cockroach (*Periplaneta americana*), in relation to chill-coma. *J. Exp. Biol.* **96**, 131-141.

Coast, G. M. (2001). The neuroendocrine regulation of salt and water balance in insects. *Zool. Anal. Complex Syst.* **103**, 179-188.

Cornelius, F. (2001). Modulation of Na^+,K^+-ATPase and Na^+-ATPase activity by phospholipids and cholesterol. I. Steady-state kinetics. *Biochemistry* **40**, 8842-8851.

Cornelius, F., Mahmmoud, Y. A. and Christensen, H. R. Z. (2001). Modulation of Na^+,K^+-ATPase by associated small transmembrane regulatory proteins and by lipids. *J. Bioenerg. Biomemb.* **33**, 415-423.

Cossins, A. R. and Kilbey, R. V. (1989). The seasonal modulation of Na^+/H^+ exchanger activity in trout erythrocytes. *J. Exp. Biol.* **144**, 463-478.

Cossins, A. R., Schwarzbaum, P. J. and Wiesler, W. (1995). Effects of temperature on cellular ion regulation and membrane transport systems. In *Biochemistry and Molecular Biology of Fishes*, vol. 5 (ed. P. W. Hochachka and T. P. Mommsen). Amsterdam: Elsevier Science.

Danks, H. V. (1987). *Insect Dormancy: An Ecological Perspective. Monograph series No. 1. National Museum of Natural Sciences.* Ottawa: Biological Survey of Canada.

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

David, R. J., Gibert, P., Pla, E., Potavy, G., Karan, D. and Moreteau, B. (1998). Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. *J. Therm. Biol.* **23**, 291-299.

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 Press.

Denlinger, D. L. (2002). Regulation of diapause. *Annu. Rev. Entomol.* **47**, 93-122.

Dissanayake, P. and Zachariassen, K. E. (1980). Effect of warm acclimation on the cationic concentrations in the extracellular and intracellular body fluid of hibernating *Rhagium inquisitor* beetles. *Comp. Biochem. Physiol. A* **65**, 347-350.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu. Rev. Physiol.* **63**, 327-357.

Emery, A. M., Billingsley, P. F., Ready, P. D. and Djamgoz, M. B. A. (1998). Insect Na^+/K^+-ATPase. *J. Insect Physiol.* **44**, 197-209.

Flannagan, R. D., Tammariello, S. P., Joplin, K. H., Cikra-Ireland, R. A., Yocum, G. D. and Denlinger, D. L. (1998). Diapause-specific gene expression in pupae of the flesh fly *Sarcophaga crassipalpis*. *Proc. Natl. Acad. Sci. USA* **95**, 5616-5620.

Goller, F. and Esch, H. (1990). Comparative study of chill-coma temperatures and muscle potentials in insect flight muscles. *J. Exp. Biol.* **150**, 221-231.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev. Cambridge Phil. Soc.* **74**, 1-40.

Hadley, N. F. (1994). *Water Relations of Terrestrial Arthropods*. San Diego: Academic Press.

Haines, T. H. (2001). Do sterols reduce proton and sodium leaks through lipid bilayers? *Prog. Lipid Res.* **40**, 299-324.

Hanzal, R., Bjerke, R., Lundheim, R. and Zachariassen, K. E. (1992). Concentration of sodium and free amino acids in the haemolymph and other fluid compartments in an adephagous and polyphagous species of beetle. *Comp. Biochem. Physiol. A* **102**, 741-744.

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

Heitler, W. J., Goodman, C. S. and Fraser Rowell, C. H. (1977). The effects of temperature on the threshold of identified neurons in the locust. *J. Comp. Physiol.* **117**, 163-182.

Hochachka, P. W. (1986). Defense strategies against hypoxia and hypothermia. *Science* **231**, 234-241.

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

Hodek, I. (1983). Role of environmental factors and endogenous mechanisms in the seasonality of reproduction in insects diapausing as adults. In *Diapause and Life Cycle Strategies in Insects* (ed. B. K. Brown and I. Hodek), pp. 9-33. The Hague, Boston, London: Dr W. Junk Publishers.

Hodková, M. and Hodek, I. (1994). Control of diapause and supercooling by the retrocerebral complex in *Pyrrhocoris apterus*. *Entomol. Exp. Appl.* **7**, 237-245.

Hodková, M. and Hodek, I. (1997). Temperature regulation of supercooling and gut nucleation in relation to diapause of *Pyrrhocoris apterus* (L.) (Heteroptera). *Cryobiology* **34**, 70-79.

Hodková, M., Berková, P. and Zahradnícková, 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.

Hodková, M., Šimek, P., Zahradnícková, 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.

Holmstrup, M., Bayley M. and Ramlov, H. (2002). Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable arctic invertebrates. *Proc. Natl. Acad. Sci. USA* **99**, 5716-5720.

Holmstrup, M. and Westh, P. (1994). Dehydration of earthworm cocoons exposed to cold: a novel cold hardiness mechanism. *J. Comp. Physiol. B* **164**, 312-315.

Hosler, J. S., Burns, J. E. and Esch, H. E. (2000). Flight muscle resting potential and species-specific differences in chill-coma. *J. Insect Physiol.* **46**, 621-627.

Jennings, P. H. and Tatar, T. A. (1979). Effect of chilling on membrane potentials of maize and oat leaf cells. In *Low Temperature Stress in Crop Plants: the Role of the Membrane* (ed. J. M. Lyons, D. Graham and J. K. Raison), pp. 153-161. New York: Academic Press.

Kivivouri, L., Lehti, S. and Lagerspetz, K. Y. H. (1990). Effect of temperature acclimation on thermal dependence and hysteresis of resting membrane potential of the stretch receptor neuron in crayfish [*Astacus astacus* (L.)]. *J. Therm. Biol.* **15**, 9-14.

Knight, J. D., Bale, J. S., Franks, F., Mathias, S. F. and Baust, J. G. (1986). Insect cold hardiness – supercooling points and prefreeze mortality. *Cryo-Lett.* **7**, 194-203.

Koštál, V. and Šimek, P. (2000). Overwintering strategy in *Pyrrhocoris apterus* (Heteroptera): the relations between 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. B*, **130**, 365-374.

Kristiansen, E. and Zachariassen, K. E. (2001). Effect of freezing on the transmembrane distribution of ions in freeze-tolerant larvae of the wood fly *Xylophagus cinctus* (Diptera, Xylophagidae). *J. Insect Physiol.* **47**, 585-592.

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

Lockey, K. H. (1985). Insect cuticular lipids. *Comp. Biochem. Physiol. B* **81**, 263-273.

Lyons, J. M. (1973). Chilling injury in plants. *Annu. Rev. Plant Physiol.* **24**, 445-466.

Maddrell, S. H. P. and O'Donnell, M. J. (1992). Insect Malpighian tubule: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* **172**, 417-429.

Nedved, O. (2000). Snow White and seven dwarfs: a multivariate approach to classification of cold tolerance. *Cryo-Lett.* **21**, 339-348.

Passonneau, J. V. and Lowry, O. H. (1993). *Enzymatic Analysis. A Practical Guide.* Totowa: The Humana Press Inc.

Pullin, A. S. and Bale, J. S. (1988). Cause and effects of pre-freeze mortality in aphids. *Cryo-Lett.* **7**, 101-113.

Pullin, A. S., Fontaine, X. L. R. and Bale, J. S. (1990). Application of ^{31}P-NMR to the study of pre-freeze mortality in aphids. *Cryo-Lett.* **11**, 127-136.

Ramløv, H. (2000). Aspects of natural cold tolerance in ectothermic animals. *Hum. Repro.* **15**, 26-46.

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-Lett.* **23**, 217-228.

Rojas, R. R. and Leopold, R. A. (1996). Chilling injury in the housefly:

evidence for the role of oxidative stress between pupariation and emergence. *Cryobiology* **33**, 447-458.

Schweikl, H., Klein, U., Schindlbeck, M. and Wieczorek, H. (1989). A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. *J. Biol. Chem.* **19**, 11136-11142.

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.

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

Sláma, K. (1964). Hormonal control of respiratory metabolism during growth, reproduction and diapause in female adults of *Pyrrhocoris apterus* L. (Hemiptera). *J. Insect Physiol.* **10**, 283-303.

Socha, R., Šula, J. and Zemek, R. (1997). Feeding, drinking and digestive enzyme activities in long- and short-day females of *Pyrrhocoris apterus* (Heteroptera). *Physiol. Entomol.* **22**, 161-169.

Stinner, J. N. and Hartzler, L. K. (2000). Effect of temperature on pH and electrolyte concentration in air-breathing ectotherms. *J. Exp. Biol.* **203**, 2065-2074.

Šula, J., Kodrík, D. and Socha, R. (1995). Hexameric haemolymph protein related to adult diapause in the red firebug, *Pyrrhocoris apterus* (L.) (Heteroptera). *J. Insect Physiol.* **41**, 793-800.

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

Wieczorek, H., Gruber, G., Harvey, W. R., Huss, M., Merzendorfer, H. and Zeiske, W. (2000). Structure and regulation of insect plasma membrane H^+ V-ATPase. *J. Exp. Biol.* **203**, 127-135.

Wieczorek, H., Weerth, S., Scindelbeck, M. and Klein, U. (1989). A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **19**, 11143-11148.

Yocum, G. D. (2001). Differential expression of two *HSP70* transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. *J. Insect Physiol.* **47**, 1139-1145.

Yocum, G. D., Zdarek, J., Joplin, K. H., Lee, R. E., Jr, Smith, D. C., Manter, K. D. and Denlinger, D. L. (1994). Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by a low and high temperature stress. *J. Insect Physiol.* **40**, 13-21.

Yoder, J. A., Blomquist, G. J. and Denlinger, D. L. (1995). Hydrocarbon profiles from puparia of diapausing and nondiapausing flesh flies (*Sarcophaga crassipalpis*) reflect quantitative rather than qualitative differences. *Arch. Insect Biochem. Physiol.* **28**, 377-385.

Yoder, J. A., Denlinger, D. L., Dennis, M. W. and Kolattukudy, P. E. (1992). Enhancement of diapausing flesh fly puparia with additional hydrocarbons and evidence for alkane biosynthesis by decarboxylation mechanism. *Insect Biochem. Mol. Biol.* **22**, 237-243.

Zachariassen, K.-E. and Einarson, S. (1993). Regulation of body fluid compartments during dehydration of the tenebrionid beetle *Rhytinota praelonga*. *J. Exp. Biol.* **182**, 283-289.

Zachariassen, K.-E. and Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology* **41**, 257-279.

Zachariassen, K.-E. and Pedersen, S. A. (2002). Volume regulation during dehydration of desert beetles. *Comp. Biochem. Physiol. A* **133**, 805-811.

Zeiske, W. (1992). Insect ion homeostasis. *J. Exp. Biol.* **172**, 323-334.