MINI-REVIEW

Eur. J. Entomol. **101**: 445–458, 2004
ISSN 1210-5759

Photoperiod, diapause and cold-hardiness

MAGDALENA HODKOVA and IVO HODEK

Institute of Entomology, Academy of Sciences, Ceske Budejovice, CZ-370 05 Czech Republic; e-mail: hodkova@entu.cas.cz

Key words. Photoperiod, temperature, diapause, cold-hardiness, hormones, survival, supercooling, cryoprotectants, *Pyrrhocoris apterus*

Abstract. Great progress has recently been made in cryobiology. One field, however, has been neglected: the temporal sequence of the effects of photoperiod and temperature, and their relative importance in cold hardening. This is relevant to the question of importance of diapause in cold-hardiness. Denlinger (1991) outlined the categories of such relations and stressed a great need for further detailed research. A survey of studies done over the past decade revealed many gaps in the evidence and the ambiguous nature of the data on the photoperiodic regulation of cold-hardiness. We hope that this review will stimulate further research in this field. Among several directions where research is most needed we have stressed (1) simultaneous recording of changes in survival and dynamics of suspected cryoprotectants (stressed also by Danks, 1996), (2) checking the regulation of different phases of cold hardening, and (3) discrimination between direct and indirect (mediated via neuroendocrine system) effects of environmental cues on cold hardening.

INTRODUCTION

There was a lengthy debate about the relation between diapause and cold-hardiness. Contrasting views were expressed, ranging from "diapause as a prerequisite for cold-hardiness" to "both phenomena as independent events". The central contradiction was resolved in a very helpful review (Denlinger, 1991) in which the different categories were delimited. Larvae of *Ostrinia nubilalis*, induced to diapause by short daylength, were reported not to become more cold hardy than the non-diapause controls. Cold-hardening develops later by a process of cold acclimation. In another category, flesh flies of the genus *Sarcophaga* become cold hardy due to the induction of diapause, even if temperature is not decreased. Their cold-hardiness can be only intensified later, by cold acclimation (Denlinger, 1991, pp. 179–180). The linden bug, *Pyrrhocoris apterus*, shows a similar development of cold-hardiness in two steps. The first step is regulated by hormones, the second requires low temperature (Hodkova et al., 1992; Hodkova & Hodek, 1994). Adaptations, in which cold-hardiness and diapause are not linked are also recorded (Danks, 1987, p. 41, 2000; Bale, 1996, 2002). For example, in larvae of *Dendroides canadensis* cold-hardiness is induced by photoperiod and regulated by juvenile hormone, but does not appear to be associated with diapause (Horwath & Duman, 1983 and subsequent papers). "Simple" effects of low temperature on cold-hardiness, not associated with photoperiodically induced diapause, have recently been reviewed by Bale (2002).

Although Denlinger (1991) raised important questions in his review (e.g. pp. 179, 189, 193–194), and Danks (1996, p. 397) suggested three fruitful approaches for further research, it does not seem that these reviews stimulated studies on the link between diapause and cold-hardiness. Perhaps researchers had the false impression that no important discoveries remained to be made in the field of the environmental regulation of cold-hardiness. Another important fact, leading to this neglect was the great range of methodical innovations. They enabled deeper biochemical and molecular studies, including molecular genetics of the heat and cold shock proteins or enzymes regulating the synthesis of cryoprotectants, that promised more interesting and more rewarding results. However, mechanisms of cold hardening cannot be understood without a knowledge of the whole sequence of events, starting with the transfer of environmental information into a cell (directly or via neuroendocrine system) and terminating with the final output of the cold hardening process, i.e. survival at low temperatures.

In our team, we have partly followed the global trend and studied both the role of cryoprotectants (Kostal & Simek, 2000; Kostal et al., 2001; Slachta et al., 2002a, b) and changes in phospholipids in cell membranes (Hodkova et al., 1999, 2002; Slachta et al., 2002a; Kostal et al., 2003). However, due to our earlier discovery of biphasic cold hardening in *P. apterus* (Hodkova et al., 1992; Hodkova & Hodek, 1994) we have continued our eco-physiological studies on cold-hardiness.

In this minireview we survey the research done since Denlinger's review (1991) on the link between photoperiodically induced diapause and cold-hardiness. We adhere to the opinion, expressed by Danks (1996) that cold-hardiness should be studied in a complex way. We have therefore considered only those species, in which parameters of cold-hardiness were studied in relation to photoperiodically induced diapause. We hope this review will incite a revival of research on the environmental regulation of cold-hardiness.

HORMONAL REGULATION OF COLD-HARDINESS

Hormonal regulation of cold-hardiness is still poorly understood. Early papers have been summarised by Den-

TABLE 1. Hormonal regulation of parameters related to cold hardiness.

Species	Developmental stage	Treatment	Effect	Reference
	LARVA			
Chilo suppressalis	non-diap. larva	JH I	glycerol ↑	Tsumuki & Kanehisa, 1980
	diap. larva	20-HE	SCP ↑	Tsumuki & Hirai, 1999
		JH I (0.01 μg)	SCP ↓	Tsumuki & Kanehisa, 1981
		JH I (1 μg)	glycerol ↑	
		β-E	glycerol ↓	
			glycerol ↓	
Ceruchus piceus	larva (mid winter)	AKH	LPIN ↑	Xu et al., 1990
	larva (spring, autumn)	JH I	SCP ↑	
			INA ↓	
			SCP ↓	
Dendroides canadensis	larva (summer)	JH I	AP ↑	Horwath & Duman, 1983 Xu & Duman, 1991
Tenebrio molitor	larva	JH I	AP ↑	Xu et al., 1992
Eurosta solidaginis	larva	JH I	SCP ↑	Rojas et al., 1987
	ligated larva	head ligation	SCP ↓	Hamilton et al., 1986
		JHA	glycerol ↓ (at low T)	
			glycerol ↑ (at high T)	
			glycerol↑ (at low T)	
Sarcophaga crassipalpis	larva	20-HE	SCP ↑	Lee et al., 1988
	pupa	JHA	glycerol ↑	
		20-HE (0.1 μg)	glycerol ↑	
		20-HE (0.5 μg)	SCP ↑	
	PUPA			
Pieris brassicae	non-diap. pupa	JH	glycerol ↑	Pullin & Bale, 1989a
	diap. pupa	JH	sorbitol ↑ glycerol ↓	Pullin, 1992
		E	sorbitol ↓ glycerol ↑	
		20-HE	sorbitol ↓ trehalose ↓	
	ADULT			
Aulacophora nigripennis	diap. adult	JHA	survival ↓	Watanabe & Tanaka, 1998b, 1999a, 2000
			myo-inositol ↓	
Pyrrhocoris apterus	non-diap adult	extirp. CC-CA	SCP ↓	Hodkova & Hodek, 1994
	diap. adult	extirp. CA	SCP ↓ (at low T)	Hodkova et al., 1992
		extirp PI	PL restructuring	Hodkova et al., 2002
			SCP ↑	Hodkova, unpubl.

JH – juvenile hormone; JHA – juvenile hormone analogue; E – ecdysone; HE – hydroxyecdysone; AKH – adipokinetic hormone; CC – corpora cardiaca; CA – corpora allata; PI – pars intercerebralis of the brain; LPIN – lipoprotein ice nucleators; INA – ice nucleator activity; AP – antifreeze (hysteresis) proteins; PL – phospholipids; SCP – supercooling point. Up-arrow – increase; down-arrow – decrease.

linger (1991) and Zachariassen & Lundheim (1992). Thereafter, only a few new studies have been published. It seems that the effect of hormonal treatment depends on species, developmental stage, dose of hormones, freeze tolerance/intolerance and experimental conditions (Table 1). Most information concerns the role of JH. Since diapause individuals are generally more cold hardy than non-diapause individuals (see below), it is not surprising that hormonal treatments mimicking the diapause state induce changes that are thought to enhance cold-hardiness. For example, *Chilo suppressalis* has a larval diapause characterised by a high level of JH (Chippendale, 1977), and the application of JH I to non-diapause larvae results in an increase in the content of glycerol (Tsumuki & Kanehisa, 1980, 1981). On the other hand, the termination of adult diapause by JHA in *A. nigripennis* is associated with a decrease in both *myo*-inositol level and survival at low temperatures (Watanabe & Tanaka, 1998b, 1999a, 2000). Hormonal effects may not be always related to diapause. In larvae of *D. canadensis* (Horwath & Duman, 1983; Xu & Duman, 1991) and *Tenebrio molitor* (Xu et al., 1992) the application of JH I results in an increase of anti-freeze (thermal hysteresis) proteins, although these species are supposed to have no diapause. The response to hormonal treatment obviously depends on the strategy of cold-hardiness that differs in freeze tolerant and intolerant insects (Zachariassen, 1985). For example, the application of JH I to freeze intolerant larvae of *Ceruchus piceus*

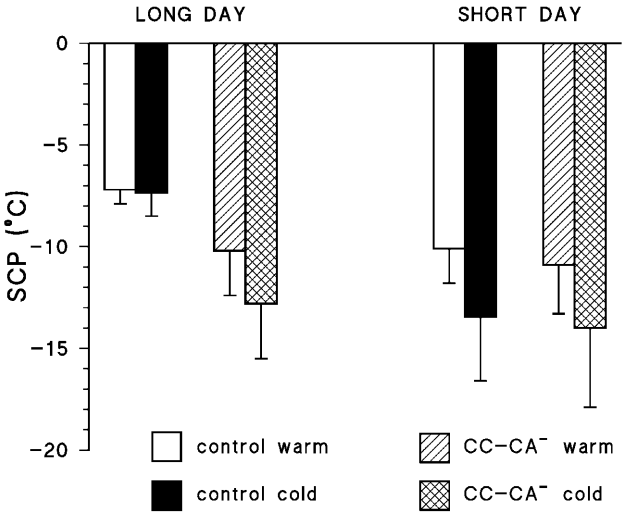


Fig. 1. Effect of photoperiod, cold acclimation, and endocrine glands on supercooling point in adults of *P. apterus*. CC = corpora cardiaca, CA = corpora allata. Long day = 18L : 6D, short day = 12L : 12D, warm conditions = 26°C continuously, cold conditions = thermal cycle of 20 : 5°C under 8L : 16D. Adults were transferred to cold conditions at the age of 1 week. Both control (sham-operated) and operated (CC-CA removed) were measured at the age of 3 weeks. Data from Hodkova & Hodek (1994).

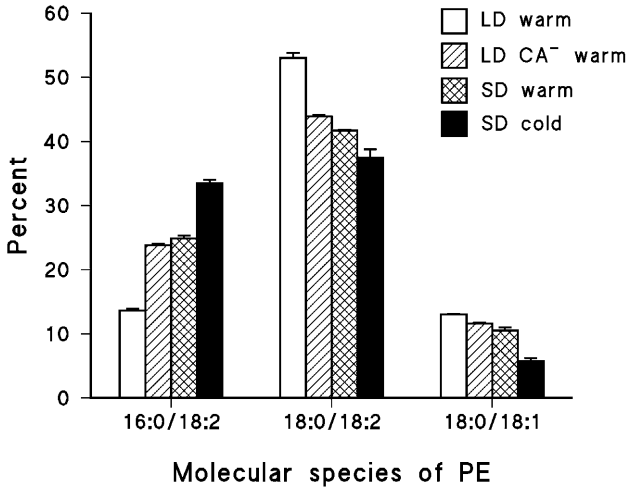


Fig. 2. Effect of photoperiod, cold acclimation, and corpus allatum on molecular species of phosphatidylethanolamine in the fat body of laboratory and field collected adults of *P. apterus*. LD warm = 18L : 6D, 26°C, SD warm = 12L : 12D, 26°C, SD cold = field collected insects in February, 1999 (mean outdoor temperature = –2°C). CA⁻ = corpus allatum removed. Only molecular species showing clear changes are included. Data from Hodkova et al. (2002).

results in a decrease in the supercooling point (SCP) (Xu et al., 1990), while freeze tolerant larvae of *Eurosta solidaginis* respond to a similar treatment by an increase of SCP (Rojas et al., 1987).

In adults of *P. apterus*, the decrease in SCP and the winter remodelling of membrane lipids are triggered by short days and changes due to temperature drop represent the later phase of adaptation (overwintering strategy of this species see the next chapter). The effect of photoperiod is mediated by the neuroendocrine system. At a high temperature, the extirpation of the complex of corpora cardiaca + corpus allatum (CC+CA) from long-day insects induced a depression of the SCP, similar to that seen in short days. This operation had no effect in short-day insects, probably because hormones affecting SCP are absent (Fig. 1, Hodkova & Hodek, 1994). Extirpation of the CA alone had no effect at a high temperature but enabled depression of the SCP during cold acclimation (Hodkova et al., 1992). Thus it seems that the absence of the CC (the source of several hormones – Raabe, 1982) triggers a decrease in SCP at a high temperature and the absence of the CA (the source of juvenile hormone) enables a further decrease in SCP during cold

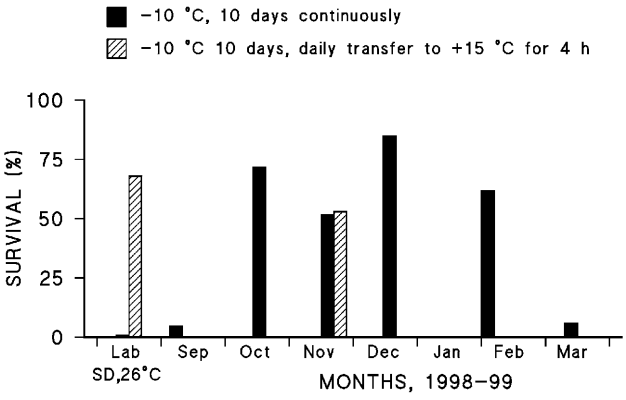


Fig. 3. Survival of laboratory and field collected adults of *P. apterus* at subzero temperature; effect of intermittent transfers to higher temperature. One sample of laboratory adults was reared at a short-day photoperiod (SD) of 12L : 12D and 26°C and then transferred to either constant –10°C or –10°C/+15°C at the age of 4–5 weeks (after 1-week exposure to 5°C). Most field collected adults were transferred from outdoor temperature to constant –10°C. Only the November samples were also exposed to –10°C/+15°C.

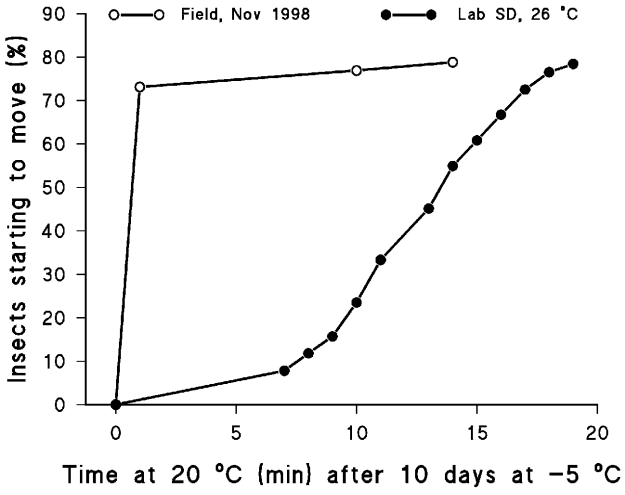


Fig. 4. Rate of recovery of laboratory and field collected adults of *P. apterus* after exposure to subzero temperature. Laboratory adults were reared at a short-day photoperiod (SD) of 12L : 12D and 26°C and then transferred to –5°C at the age of 4–5 weeks (after 1-week exposure to 5°C). Field collected adults were transferred from outdoor temperature to –5 °C.

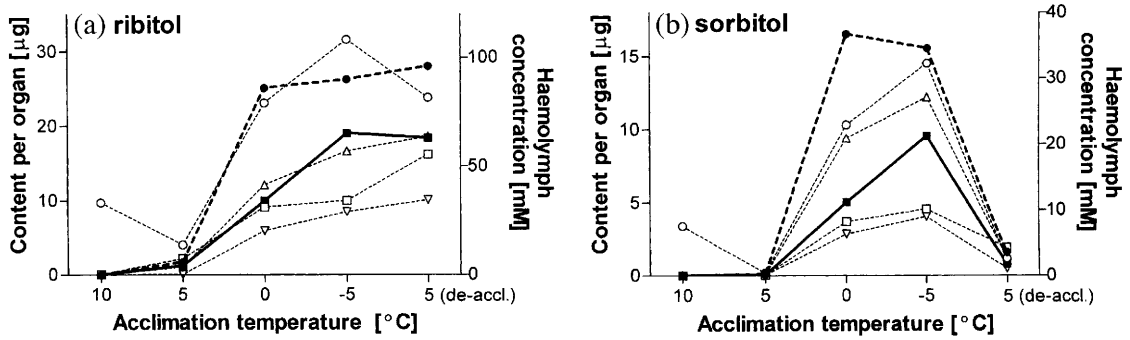


Fig. 5. Accumulation of (a) ribitol and (b) sorbitol in different organs of diapausing adults of *P. apterus* in response to acclimation temperature. Larvae were reared at 12L : 12D (SD) and 25°C until adult ecdysis. Adults were subjected to the following acclimation protocol: SD, 20°C (3 weeks), SD, 10°C (1 week), SD, 5°C (1 week), continuous darkness (DD), 0°C (1 week), DD, –5°C (1 week), DD, 5°C (de-acclimation, 1 week). Haemolymph (closed circles), fat body (closed squares), gut (open circles), muscle (open squares), Malpighian tubes ×10 (open triangles △), ovaries (reversed open triangles ▽). From Kostal et al. (2001).

acclimation. On the other hand, the CC seem to have no effect on the composition of membrane lipids at a high temperature. The extirpation of the CA alone from long-day insects had almost the same effect as short days (Fig. 2, Hodkova et al., 2002). Effects of hormonal treatments seem to be affected by diapause completion. After the transfer of post-diapause *P. apterus* to a high temperature, the CA is still necessary for reproductive activity, but the SCP remains high even after extirpation of the CC+CA (Hodkova & Hodek, 1994).

PYRRHOCORIS APTERUS

The linden bug, *P. apterus* has been for several decades the main experimental model insect for the authors of this review and their colleagues. The research, concentrated at first on the environmental regulation of diapause and later on endocrinological and chronobiological aspects, enabled a better insight particularly into the relation between the phases of overwintering and changes in cold-hardiness during the development of diapause and the subsequent post-diapause quiescence. *P. apterus* is a multivoltine species with facultative diapause, having, however, usually only one complete generation per year in our region (48–50°N, 15°E). At 25 or 26°C, 50% of individuals enter diapause under a photophase of approximately 16.5 h. Temperature plays a secondary role in diapause induction. The responsiveness to photoperiod gradually disappears during the development of diapause; diapause is terminated around the winter solstice. We used 12L : 12D as diapause inducing and 18L : 6D as diapause preventing photoperiods.

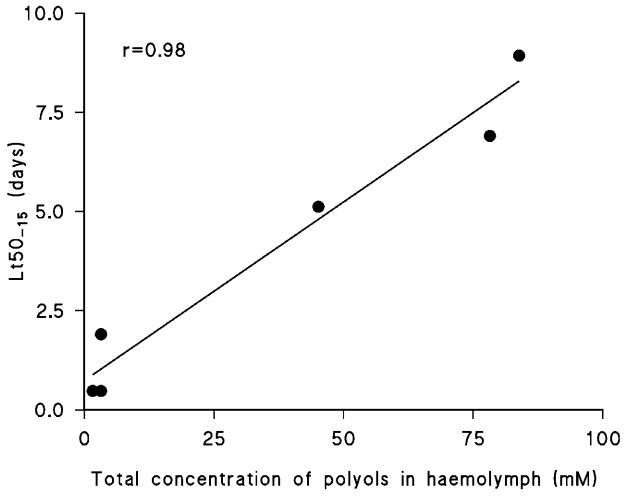


Fig. 6. Linear regression analysis of the correlation between survival at –15°C ($Lt50_{-15}$) and total concentration of polyols (ribitol, sorbitol, arabinitol, mannitol) in haemolymph of adult *P. apterus*. Data from Kostal et al. (2001).

Supercooling capacity

P. apterus does not survive freezing of its body fluids, i.e. it is freeze avoiding (intolerant) according to the classes by Bale (1996). There is a good correlation between short-term (24 h) survival at subzero temperatures and the supercooling point (SCP) (Nedved et al., 1995; Hodkova & Hodek, 1997). The SCP is about –7°C during prediapause. An increase in supercooling capacity is associated with the induction of diapause, in spite of a high temperature of 26°C. Diapause is also a prerequisite for a further increase in supercooling capacity by cold acclimation, further decreasing the SCP by about 5–6°C (Fig. 1, Hodkova & Hodek, 1994, 1997). Post-diapause adults show low values of the SCP still in February, although their developmental potential is fully restored after the termination of diapause and the loss of photoperiodic responsiveness. Evidently the ability to supercool associated with the diapause syndrome is maintained by low ambient temperature in spite of diapause termination. The SCP increases rapidly and irreversibly when these adults are transferred to a high temperature of 26°C, and cold re-acclimation is then no longer possible (Hodkova & Hodek, 1994). An intermediate temperature of 15°C, or fluctuating outdoor temperatures (at short days) are more effective in causing a decrease of the SCP than 5°C in continuous darkness. The SCPs of hemolymph, gut, fat body and gonads were compared to whole-body SCP. The gut was thus identified as the primary site of ice nucleation because its value was very similar to the value for the whole body in both short-day and long-day insects. The SCPs of other organs, including hemolymph,

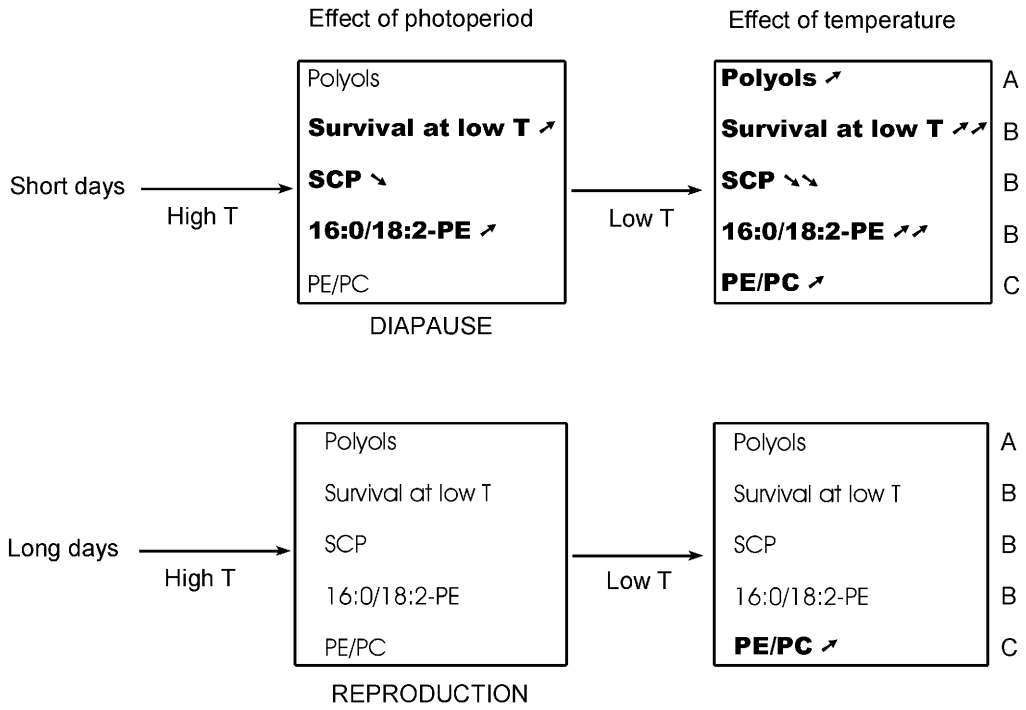


Fig. 7. Interaction of photoperiod and temperature in the regulation of diapause and parameters of cold hardiness in *P. apterus*. Parameters affected by photoperiod and/or temperature are in bold. Up arrows = increase, down arrows = decrease. A – photoperiod has no direct effect on this parameter, but the induction of diapause by short days is a pre-requisite for the response to low temperature. B – changes in these parameters are induced by short days and diapause is a pre-requisite for the more marked changes that occur at low temperatures. C – photoperiod has no effect on this parameter, which is only regulated by temperature. SCP = supercooling point, 16 : 0 = palmitic acid, 18 : 2 = linoleic acid, PE = phosphatidylethanolamine, PC = phosphatidylcholine. For details see the text.

were always lower than the whole-body SCP. Food was not the source of ice nucleating agents: SCP of freshly ecdysed adults remained high after 2 weeks of starvation. In contrast, feeding was a prerequisite for the decrease of SCP during diapause induction. In post-diapause insects, the SCP increased at high temperature in spite of the absence of food (Hodkova & Hodek, 1997).

Survival

Diapause adults of *P. apterus* show considerably higher survival at low temperatures compared to non-diapause adults. Although the short-term survival of adults of *P. apterus* at subzero temperatures is well correlated with the SCP (see above), the difference between diapause and non-diapause adults was also recorded at low temperatures above SCP and, therefore, features other than the SCP may influence survival, particularly if a long-term (10 days) survival is considered. Two-week exposure of non-diapause adults of *P. apterus* to a low temperature of +4°C resulted in 73% mortality, while all diapause individuals survived a four-week exposure to the same temperature and only 13% mortality was recorded after six weeks (Hodkova, unpubl.). The time of survival at a subzero temperature of –5°C in adults of *P. apterus* acclimated at warm temperature of 26°C was much longer in diapause (Lt_{50} = 28.6 days, Kostal et al., 2001) than in non-diapause (Lt_{50} = 7.8 days, Slachta et al., 2002a) adults, although the SCP was lower than –5°C in both groups of insects. While cold acclimation resulted in a substantial increase in the survival at subzero temperatures in diapause adults (Lt_{50} at –5°C increased to > 50 days, Kostal et al., 2001), non-diapause adults showed only a slight increase in survival in approximately 50% of the population (Slachta et al., 2002a). Thus, similar to the supercooling capacity, the survival at low temperatures is enhanced in two steps: (1) during the induction of diapause, (2) during cold acclimation. It seems that diapause is a prerequisite for the second step.

The above examples demonstrate that non-freeze injury may be the cause of mortality at low temperatures. Non-freeze mortality at low temperatures can be avoided by short intermittent transfers to a higher temperature (Fig. 3). For example, when diapause adults of *P. apterus* acclimated at warm temperature of 26°C were exposed to –10°C for 10 days, their survival was much enhanced by daily 4-h tranfers to +15°C. A similar beneficial effect of alternating temperatures was observed in larvae (i.e. a non-diapause stage) of *P. apterus* (Hanc & Nedved, 1999). On the other hand, most diapause adults of *P. apterus* acclimated at low outdoor temperatures survived continuous exposure to –10°C and daily transfers to +15°C had no effect on their survival (Fig. 3). Transfers from subzero temperature probably enabled a reparation of chill injury in warm acclimated insects. The time needed for a reparation of chill injury seems to delay the recovery of warm acclimated insects from chill coma. Most diapause adults of *P. apterus* survived continuous

10-day exposure to –5°C. However, their recovery after the transfer to 20°C was much slower in warm acclimated adults, compared to cold acclimated field insects collected in November (Fig. 4).

Membrane lipids

Non-freeze mortality due to low temperature exposure is frequently associated with damage of cellular membranes (Drobnis et al., 1993). Therefore, the thermal adaptation of membranes is considered to play a central role in the adaptation of poikilothermic organisms to seasonal fluctuations in temperature (Hazel, 1997). Similar to changes in the SCP and survival, winter remodelling of membranes in *P. apterus* proceeds in two steps. Short days trigger an increase in the proportion of the phospholipid molecular species with paired saturated palmitic acid ($C_{16:0}$) and di-unsaturated linoleic acid ($C_{18:2}$) at the expense of phospholipids with 18-carbon fatty acids (C_{18}), both saturated and unsaturated. Further increase in the proportion of the same molecular species ($C_{16:0}/C_{18:2}$) and changes in the composition of the phospholipid head group (increase in the level of phosphatidylethanolamine at the expense of phosphatidylcholine) are induced by low temperatures and represent the later phase of adaptation (Fig. 2, Hodkova et al., 2002). In contrast to most organisms studied so far, the winter remodelling of membrane lipids in *P. apterus* is not associated with an increase in the proportion of unsaturated fatty acids (Hodkova et al., 1999). While diapause is a prerequisite for a further increase in the proportion of palmitic acid at low temperatures, changes in the head group composition are induced by low temperatures in both diapause and non-diapause adults of *P. apterus* (Hodkova et al., 2002, Slachta et al., 2002 a). The higher the proportion of molecular species with paired $C_{16:0}$ and $C_{18:2}$ fatty acids, the lower the non-freeze mortality. Based on known physical properties of phospholipids (Lewis et al., 1989), it is hypothesized that the replacement of C_{18} fatty acids with the 16-carbon palmitic acid may extend the temperature range at which membranes are fluid and thus counteract a potential dehydration of membranes at low temperatures (Hodkova et al., 1999, 2002).

Polyols

Although polyols are synthesized in spite of high temperatures in several species, e.g. in pupae of *Pieris brassicae* at 20°C or in adults of *Aulacophora nigripennis* at 20 or 25°C (see Table 2), temperatures below 5°C are necessary for synthesis of polyols (mainly ribitol and sorbitol) in adults of *P. apterus* (Fig. 5, Kostal & Simek, 2000; Kostal et al., 2001). However, diapause is a prerequisite for the accumulation of polyols at low temperatures. No accumulation of polyols was observed during cold acclimation in non-diapause *P. apterus* (Slachta et al., 2002 a). There was a tight correlation (r = 0.98) between the concentration of polyols in haemolymph and the time of survival at a subzero temperature of –15°C (Fig. 6, Kostal et al., 2001). Furthermore, the time of survival increased when a mixture of ribitol and sorbitol was injected into the haemolymph (Kostal et al., 2001).

Conclusions

It may be concluded for *P. apterus* that the relative importance of photoperiod and temperature depends on the parameter we measure (Fig. 7). Regulation of SCP and phospholipid acyl chain remodelling is regulated in two steps, first by photoperiod and later by temperature. Survival at low temperatures also belongs to this category (category *Sarcophaga*, see Introduction). Photoperiod has no direct effect on polyol synthesis, but the photoperiodic induction of diapause is a prerequisite for the later effect of low temperature (category *Ostrinia nubilalis*, see Introduction). Changes in phospholipid head group composition seem to depend exclusively on temperature.

AULACOPHORA NIGRIPENNIS

The freeze intolerant (avoiding) chrysomelid *A. nigripennis* is one of the rare models where the relation between diapause and cold-hardiness was studied in quite a detail (Watanabe & Tanaka, 1998a, b, 1999a, b, 2000). Although this species is univoltine and is distributed in sub-tropical and warm-temperate regions, its "cold tolerance appears to be linked to diapause program similar to adults of *P. apterus*" (Watanabe & Tanaka, 1999a, p. 179). The life cycle and the role of photoperiod are similar to *P. apterus*, comprising overwintering between October and April, photoperiodic induction and maintenance of diapause in autumn, and absence of photoperi-

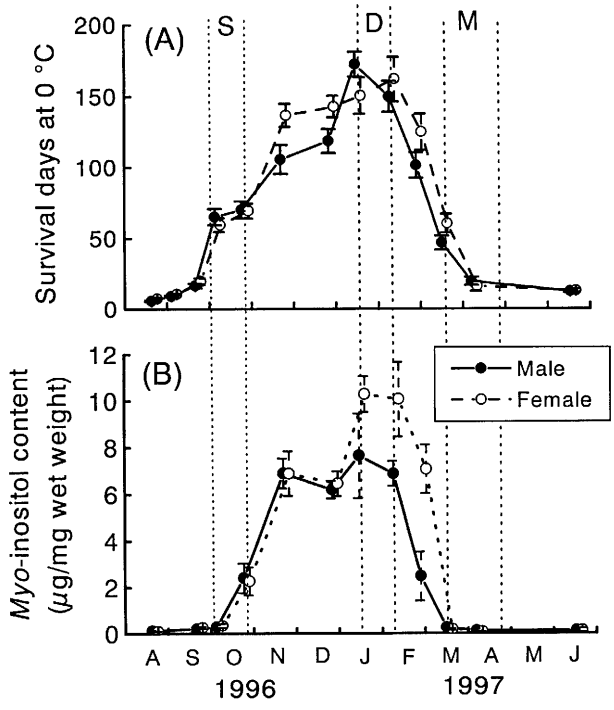


Fig. 8. Seasonal changes of chill tolerance at 0°C (A) and *myo*-inositol content (B) in adults of *A. nigripennis* collected in the field. Periods of swarming (S), diapause termination (D) and mating (M) are indicated by vertical lines. From Watanabe & Tanaka (1999a).

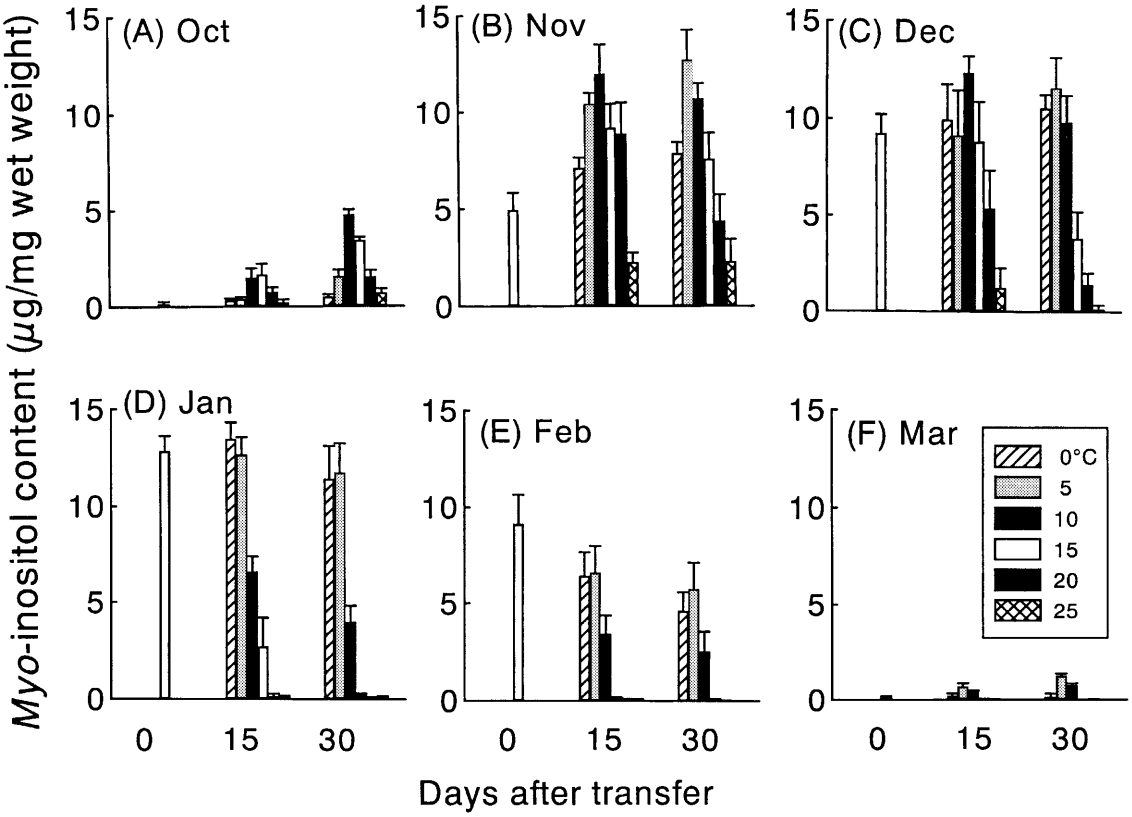


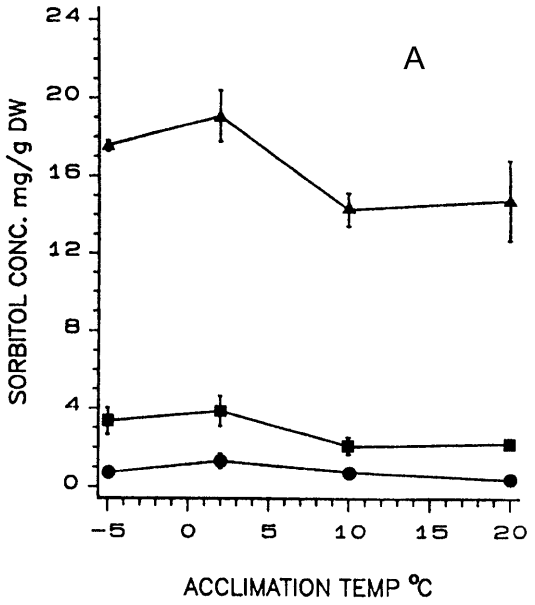
Fig. 9. Seasonal changes of thermal responses in relation to *myo*-inositol metabolism in adult females of *A. nigripennis*. Adults collected on 9 October (A), taken from outdoors on 27 November (B), 27 December (C), 27 January (D), 27 February (E) or 27 March (F) were incubated for 15 or 30 days at temperatures indicated under continuous darkness (0°C) or 12L : 12D (5–25°C). From Watanabe & Tanaka (1999a).

odic responsiveness after February. After diapause termination the SCP increases and survival at 0°C steeply decreases. The content of glucose is rather low also during winter and decreases to almost zero in spring, but adults accumulate a relatively large amount of *myo*-inositol (Watanabe & Tanaka, 1998a). *Myo*-inositol decreased from the winter level of about 10 μg/mg ww to zero in March (i.e. it returned to the state of August – September). However, even during mid-winter when adults accumulate high amounts of *myo*-inositol, the SCP still remains relatively high (about –7°C). Therefore, the SCP seems not to be affected by a colligative effect of *myo*-inositol. In a natural population of *A. nigripennis*, the chill tolerance is closely correlated with the titre of *myo*-inositol (Fig. 8, Watanabe & Tanaka, 1999a). Although it is clear that cold-hardiness and *myo*-inositol accumulation are linked with the photoperiodically induced diapause (even at 15–25°C), both parameters are further enhanced by the fall in temperature later in the season, in November/December (Fig. 9, Watanabe & Tanaka, 1999 a).

PIERIS BRASSICAE

The cabbage white butterfly, *Pieris brassicae*, overwinters as a pupa and diapause is induced by short photoperiods experienced during the 4th and 5th larval instars. Although diapause and cold-hardiness in *P. brassicae* were studied earlier (for references see Pullin & Bale, 1989b), it became a subject of a complex study at the beginning of the period covered by this review (Pullin & Bale, 1989a, b; Pullin et al., 1991; Pullin, 1992). Cryoprotectant synthesis and cold-hardiness were examined as a response to diapause and exposure to low temperature. Diapause pupae accumulate sorbitol (15–20 mg/g dry weight) even when kept at 20°C (Fig. 10A, Pullin & Bale, 1989b). One week exposure to –15°C is successfully endured by diapause pupae, whilst this treatment causes 100% mortality of non-diapause pupae (Pullin & Bale, 1989 b). Glycerol content is consistently low and unlikely to function as a cryoprotectant: it is highest in non-diapause group and decreases after exposure to low temperature (Pullin & Bale, 1989b).

In this 1989 study the diapause pupae were kept at 20°C and exposed to several temperatures in a laboratory, whereas in the next study (Pullin et al., 1991) the diapause pupae were exposed to overwintering conditions in the field, in two winters. They were sampled over the whole dormancy period from October to May and the changes in the level of solutes were recorded. Sorbitol showed the same pattern in both winters, increasing after the onset of diapause in October and peaking at around 40nMol near the winter solstice. From February it decreased steeply to the same negligible level in May as in prediapause (Fig. 10B, Pullin et al., 1991). The



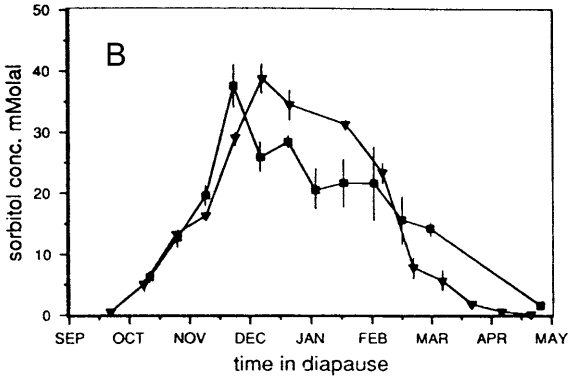


Fig. 10A–B. Concentration of sorbitol in pupae of *Pieris brassicae*. A – 91-day-old diapause pupae (triangles), 8-day-old diapause pupae (squares), 8-day-old non-diapause pupae (circles) after exposure to –5, 2, 10 and 20°C (n = 5–6). The level of sorbitol is high even in the 20°C treatment (From Pullin & Bale, 1989b). B – Pupae overwintering outdoors (England, two subsequent years) (n = 6) (From Pullin et al., 1991).

increase in pre-freeze cold tolerance in mid-winter coincided with the increased concentration of sorbitol.

In neither the laboratory nor field experiments, was the supercooling ability much affected by the sorbitol content. Laboratory reared diapause pupae had a mean SCP only 2°C lower than non-diapause pupae (–23 vs –21°C) (Pullin & Bale, 1989b) and in the field the SCP remained between –23 and –25°C throughout the winter (Pullin et al., 1991). Pullin et al. (1991) partly agree with Zachariassen (1985) that the low SCP may be achieved by voiding ice nucleators from the gut and masking of proteins that may act as intracellular ice nucleators. These authors suggest that this process may be related to pupation, but that the diapause state may be responsible for a slight depression of the SCP in diapause vs. non-diapause pupae of *P. brassicae*. Correlation between cold tolerance and high concentrations of sorbitol lead the authors to speculate about the possible cryoprotective role of this polyol, other than by the suppression of the SCP, e.g. by preventing denaturation of proteins. It is also argued that sorbitol accumulates as a result of diapause-associated metabolic suppression and this itself provides the cryoprotection in diapause pupae of *P. brassice* (Pullin et al., 1991).

DROSOPHILIDAE

Most recent studies are focused on heat shock proteins that are implicated in the regulation of diapause and stress resistance in several insect species (Denlinger, 2002). However, these proteins appear not to be associated with either photoperiodically induced reproductive diapause or stress resistance in *D. triauraria* (Goto et al., 1998; Goto & Kimura, 1998, 2004). Nor is there a correlation between trehalose content and survival at subzero temperatures. Levels of trehalose are similar in diapause and non-diapause individuals of *D. triauraria* and, although the synthesis of trehalose at low temperatures is not affected by diapause state, cold tolerance is higher in diapause individuals (Kimura et al., 1992; Goto et al., 1998). On the other hand, energy stores, such as glycogen (Kimura et al., 1992) or triacylglycerols (Ohtsu et al., 1995), are important for winter survival of *Drosophila* spp. Diapause individuals of the cool-temperate species, *D. triauraria* and *D. subauraria*, maintain a high glycogen content, even in mid winter, and survive until spring. In contrast, the warm-temperate species entering less intensive diapause, *D. rufa* and *D. lutescens*, and a non-diapause strain of *D. triauraria*, lost more than half of their stored glycogen by mid-winter and died before spring when exposed to cool temperatures in the field (Kimura et al., 1992).

Another drosophilid fly, *Chymomyza costata*, enters larval diapause under short-day photoperiod. Both diapause and non-diapause larvae become more tolerant to freezing after one-month acclimation to 4°C. However, non-diapause larvae are more sensitive to a fast cooling rate than diapause larvae. This cold-induced freezing tolerance is accompanied by the accumulation of trehalose and proline (Fig. 11, Shimada & Riihimaya, 1990). As in freeze intolerant *P. apterus* (see ch. *P. apterus*), the level of cold-hardiness of freeze tolerant *C. costata* is positively correlated with the proportion of the phospholipid molecular species containing the palmitic/linoleic ($C_{16:0}/C_{18:2}$) fatty acid pairs (Kostal et al., 2003). It is noteworthy that the more cold tolerant *Drosophila* species from northern regions have higher proportion of C_{16} fatty acids and lower proportion of C_{18} fatty acids in total phospholipids than species from southern regions (Ohtsu et al., 1998). For more details on cold acclimation in *Drosophila* spp. see the recent review by Hoffmann et al. (2003).

TWO TYPES OF LINKAGE BETWEEN DIAPAUSE AND COLD-HARDINESS

It was unambiguously proved in many insects that the induction of diapause is a pre-requisite for cold hardening. In several species the diapause state is a necessary condition for later processes producing cold-

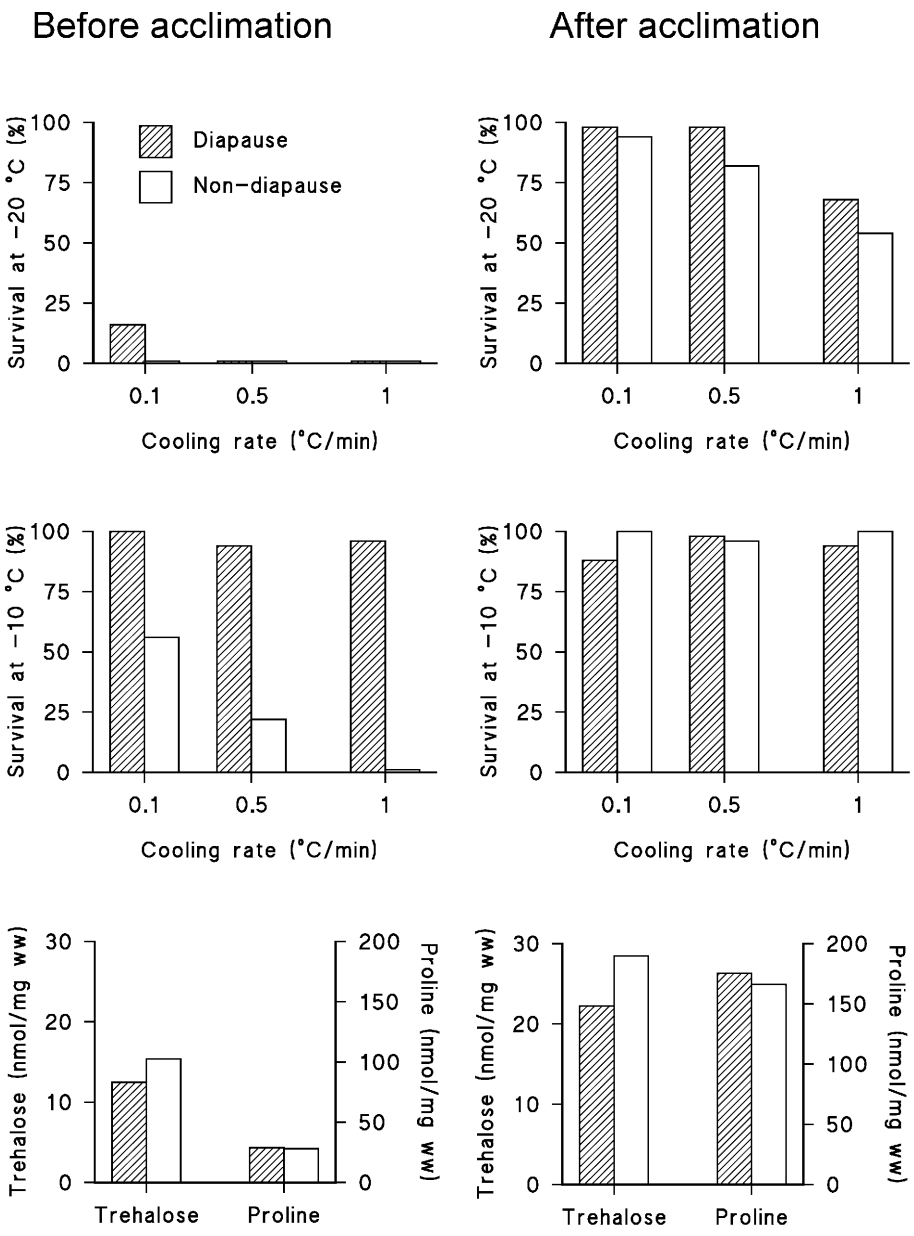


Fig. 11. Effects of diapause and cold acclimation on the freeze tolerance and trehalose and proline contents in *Chymomyza costata* larvae. Before acclimation, larvae were reared at 18°C under 10 h (diapause) or 16 h (non-diapause) light/day for 1 month. Larvae were then acclimated at 4°C under constant darkness for 1 months. After freezing and thawing they were cultured on a fresh medium and puparia were counted to estimate the percentage of survival (after the data from Shimada & Riihimaya, 1990).

hardiness by cold acclimation, but diapause does not provoke any immediate changes of cold-hardiness. For example, the bug *Graphosoma lineatum* appears to have this type of link (Slachta et al., 2002b). Crucial evidence for a direct link between diapause and cold-hardiness is an increase in cold-hardiness without any decrease in temperature, i.e. without cold acclimation. Such a link has been proved in *P. apterus*, where the diapause itself is associated with a certain level of cold-hardiness that is later, in the second phase, increased by low temperatures. The first phase of cold hardening, however, can be induced by photoperiod without a temperature decrease (see ch. *P. apterus*). In addition to *P. apterus*, evidence for a direct link between diapause and cold-hardiness was reported in the aphid parasitoid *Aphidius ervi*, reared at 15°C, when survival was recorded at –10°C (Langer & Hance, 2000) and in *Euseius finlandicus*, where the survival at –4°C was higher in diapause mites, in spite of high rearing temperature of 20°C (Broufas & Koveos, 2001) (Table 2).

If an increase in solutes with an assumed cryoprotective function is also considered, similar reports on the direct effect of photoperiod were published on *Pieris rapae* (sorbitol – Pullin et al., 1991), *Cylindrocopturus adspersus* (trehalose, at 20°C – Rojas et al., 1994), *Achaearanea tepidariorum* (inositol, at 17 and 20°C – Tanaka, 1995, 1996), *A. nigripennis* (*myo*-inositol, at 20 and 25°C – Watanabe & Tanaka, 1997, 1999a), *Delia antiqua* (trehalose, at 25°C – Nomura & Ishikawa, 2001).

TABLE 2. Examples of cases where cold-hardiness is linked to diapause state (often it increases without temperature decrease; photoperiodic induction of diapause proved or assumed).

Species (family, order)	Parameter measured (higher than in non-diapausing) (diapausing vs non-diapausing)	Reference
Sarcophaga crassipalpis (Sarcophag., Dipt.)	glycerol from <10 to >70 mM	Lee et al., 1987
Pieris brassicae (Pier., Lep.)	sorbitol 47× increase c-h at 20°	Pullin & Bale, 1989b; Pullin et al., 1991
Leptinotarsa decemlineata (Chrysomel., Col.)	proline	Lefevere et al., 1989
Cylindrocopturus adspersus (Curculion., Col.)	trehalose from 32 to 42 µg/mg ww	Rojas et al., 1994
Pyrrhocoris apterus (Pyrrhocor., Het.)	SCC, survival	Hodkova & Hodek, 1994, 1997; Kostal et al., 2001; Slachta et al., 2002a
	membrane PL remodelling	Hodkova et al., 1999, 2002
	ribitol, sorbitol	Kostal & Simek, 2000; Kostal & Slachta, 2001; Kostal et al., 2001
Achaearanea tepidariorum (Therid., Araneae)	inositol, glycogen (but no increase in glycerol)	Tanaka, 1995, 1996
Aulacophora nigripennis (Chrysomel., Col.)	*myo*-inositol at 15, 20, 25°	Watanabe & Tanaka, 1998a, b; 1999a, b
Drosophila triauraria (Drosophil., Dipt.)	survival 24 h at –8° 100 vs 20%	Goto et al., 1998
Syrphus ribesii (Syrph., Dipt.)	SCC, survival	Hart & Bale, 1998
Orius sauteri, *O. minutus* (Anthocor., Het.)	survival at 0° LT_{50}: 140d vs 20d	Ito & Nakata, 1998
Calliphora vicina (Calliphor., Dipt.)	survival at 0, –4, –8°	Saunders & Hayward, 1998
Adoxophyes orana (Tortric., Lep.)	c-h increase at 20° survival at –5°: 37% 30d vs 0% 6d	Milonas & Savopoulou-Soultani, 1999
Aphidius ervi (Bracon., Hym.)	SCC, survival at –10°: 50% 10d vs 5d	Langer & Hance, 2000
Delia antiqua (Anthom., Dipt.)	survival at –15°: 80 vs 5% trehalose: 16 vs 4 mg/mg ww	Nomura & Ishikava, 2001
Euseius finlandicus (Phytos., Acari)	c-h increase at 20° survival at –4°	Broufas & Koveos, 2001
Cornu (*Helix*) *aspersum* (Helic., Gastropoda)	SCP: 16L: –3°, 8L: –5.2° mOsm/kg: 16L: 287, 8L: 342	Ansart et al., 2001; Ansart & Vernon, 2003
Graphosoma lineatum (Pentatom., Het.)	c-h increase from IX to X; survival at –10° 9d: 70 vs 0%; trehalose 0.75 vs 0.1 mg/♀	Slachta et al., 2002b
Chymomyza costata (Drosophil., Dipt.)	1 h survival at –25°: 65 vs 0% membrane PL remodelling	Kostal et al., 2003

c-h – cold hardiness; SCC – supercooling capacity; SCP – supercooling point; PL – phospholipids.

COMPARATIVE VALUE OF COLD-HARDINESS PARAMETERS

There is a substantial difference in the comparative value of parameters (Table 2) used as indicators of cold-hardiness.

Survival

The increase in survival due to diapause is rather straightforward, although the comparison is complicated by the wide range of temperatures used for measuring survival : 0, –4, –10, –15, –23°C. In two anthocorid *Orius* spp., the survival of diapause adults at 0°C was seven times longer than in non-diapause adults at 0°C; LT_{50} was 140 days vs. 20 days in non-diapause adults (Ito &

Nakata, 1998), in the braconid *A. ervi*, the survival of diapause mummies at –10°C was only twice as great (LT_{50} : 10 days vs. 5 days) (Langer & Hance, 2000). When diapause was induced in the third instar larvae of the tortricid *Adoxophyes orana* by short-day photoperiod at a relatively high temperature of 20°C, survival at –5°C was much higher than in non-diapause larvae reared at a long-day photoperiod at 20°C (Table 2). However, the results in non-diapause larvae may be partly affected by the higher rearing temperature of 25°C (Milonas & Savopoulou-Soultani, 1999). In the pupae of the anthomyiid fly *D. antiqua*, survival of 15 days at –15°C was highly increased by diapause: >80% vs. <5% (Nomura & Ishikawa, 2001). Also the increase in survival at a low temperature of –4°C in *E. finlandicus* was substantial: 14 days were survived by 25% of diapause females, while 25% of non-diapause survived only 1 day (Broufas & Koveos, 2001). Although the survival data can be criticized as mere empirical evidence, not providing causal insight into processes leading to cold-hardiness, they are still the most real indicator of low temperature tolerance.

Supercooling point

The values of SCP might seem more useful for comparison. However, the ecological value of this parameter has often been questioned. While in *P. apterus* the value of SCP is well correlated with a short-term survival (see ch. *P. apterus*), in some insects, e.g. *Arctia caja*, i.e. a "non-dynamic" species according to the classification by Merivee (1978), the SCP does not change in different ecological periods and/or ontogenetic stages, while the values of survival at cold do. The generally low use of SCP may be the reason why it has been measured in less studies during the last decade than in the earlier period.

Cryoprotectants

In contrast, the progress of analytical methods has led to a great abundance of data on increase in chemical substances coinciding with the diapause state: polyols, amino-acids and sugars. Earlier findings on seasonal changes in these solutes were reviewed by Danks (1978, 2000).

Some **amino-acids** have cryoprotecting properties (Anchordoguy et al., 1988), apparently by protecting enzymes (Carpenter & Crowe, 1988) and the membrane structure under the stress of freezing and thawing (Leopold, 1991). Increase in proline level was recorded in diapause adults of the chrysomelid *Leptinotarsa decemlineata* (Lefevere et al., 1989) and high levels of alanine were produced in diapause larvae of *Enosima leucotaeniella* when they were exposed to 5 and further 0°C (Goto et al., 1993a, b).

Trehalose and other sugars probably have similar function (mentioned above) as amino-acids. In an older paper (Moreau et al., 1981), a peak in trehalose was reported in diapause *Pieris brassicae* 14 days after pupation at 23°C. Its level was further enhanced at lower temperature. A similar situation was recorded in diapause adults of the curculionid *C. adspersus*: from 32 µg/mg ww in the field (October samplings) the level of trehalose increased in the laboratory at 20°C to 42 and at 0°C to 55 µg/mg ww (Rojas et al., 1994). In larvae of *E. leucotaeniella*, which produce alanine in diapause, the trehalose content was reported to increase as late as in post-diapause (Goto et al., 1993a, b). Pupae of the anthomyiid *D. antiqua* in winter diapause had, in spite of 15°C, 16 µg/mg ww trehalose compared to 4 µg in non-diapause pupae (Nomura & Ishikawa, 2001). Trehalose may protect insects also against desiccation stress, as was reported in pupae of *Operophtera brumata* exposed to dry conditions in summer (Ring & Danks, 1998). See also Ring & Danks (1994) and Danks (2000) for a wider view of the potential roles of trehalose.

Polyols became the most often studied cryoprotectants since the findings by Salt on glycerol (see Ring & Riegert, 1991), and it has remained so because of rather easy analytical methods. They were monitored also in some species in the years covered by this survey. Sorbitol increased 47 fold in diapause pupae of *P. brassicae*, in spite of a high temperature of 20°C, and further to 60 fold level after a temperature decrease to 2°C (Pullin et al., 1991). During the first 40 days of diapause, glycerol content in pupae of *Sarcophaga crassipalpis* increased from <10mM to >70mM in spite of 20°C (Lee et al., 1987). Also in adults of the chrysomelid *A. nigripennis* (Watanabe & Tanaka, 1998a, b, 1999a, b), the increase in *myo*-inositol was linked to the diapause programme. Its content increased not only at 15°C, but also at 20°C in the period October – December and at 25°C in October. Only after diapause termination, temperature had a direct effect on the level of polyols: it was maintained at 5°C, while it was catabolized at 15°C. Similar linkage to the diapause program exists in the spider *Achaearanea tepidariorum*: inositol increased even at 20°C, but its level was higher at 17°C (Tanaka, 1995, 1996). It might be assumed that the high levels of polyols, as those in *P. brassicae* (Pullin et al., 1991), exert colligative effects. However, in the case of low order milimolar titres, as found e.g. in *P. apterus*, the effect is apparently non-colligative (Kostal et al., 2001).

The functional relation between the presence of polyols and cold hardening could be questioned by the evidence from insects diapausing in tropics or summer. Long lasting diapause of an endomychid beetle *Stenotarsus ovalis* (= *rotundus*) that was studied in detail in dormancy sites in Panama, is a good example (Tanaka, 2000). During diapause these beetles have a much higher titre of glycerol and glucose (4.5 mg/g, respectively 3.5 mg/g dry weight) than after diapause termination (1.5, respectively 1.0 mg/g dry weight) (Pullin & Wolda, 1993). SCP increased to about –7°C from the much lower level recorded during diapause (about –13°C) (Nedved & Windsor, 1994). In this low altitude tropical region cold-hardiness evidently does not come into consideration. The recorded changes are apparently components of diapause syndrome and represent adaptations to stress in general (Danks, 1978, Ring & Danks, 1994). Further detailed studies of insects diapausing at high/moderate temperatures should reveal which of the reported

"cryoprotective" agents are produced really as adaptations for cold. Alternatively it is possible that they are "by-products" of anaerobic pathways as was suggested by Denlinger (1991).

CONCLUSIONS

The last 15 years brought relatively little research on our topic, i.e. the importance of photoperiodic cue for cold-hardiness, acting through the induction of diapause. Only about half of the studies surveyed here bring clear evidence of the role of photoperiod, while most of them ascertained a linkage between diapause and cold-hardiness, or at least some parameters that may reflect cold hardening. However, direct evidence for a causal link between survival at low temperatures and other parameters of cold-hardiness has not yet been provided. It is not clear to what extent the changes in the titre of "cryoprotectants" are designed as adaptations for the survival at cold temperatures or are merely produced incidentally as a sequel of decrease in metabolic rate due to diapause. The latter possibility might be indicated by findings of increase in "cryoprotectants" in insects diapausing at high temperature. Stimulation of polyol synthesis by anoxia was discussed by Denlinger (1991, p. 189). The above discussion, of course, does not intend to deny direct effects of low temperatures.

Mechanisms responsible for the translation of environmental signals into cold-hardiness are not well understood. Several papers indicate that the effect of photoperiod on cold-hardiness is mediated by endocrine pathways. The role of endocrine system in thermal regulation of cold-hardiness, if any, is not known.

Regulatory mechanisms may be different for different parameters of cold-hardiness in the same species. For example, in *P. apterus*, most parameters of cold-hardiness, including survival, show two-step regulation, while the phospholipid head group composition are exclusively regulated by temperature, independently of photoperiod (Hodkova et al., 2002). Polyols are absent in diapause *P. apterus* at a high temperature, but diapause is a prerequisite for the accumulation of polyols during cold acclimation (Kostal et al., 2001). Although substantial information on several specific aspects of cold-hardiness has accumulated, complex studies on single species are still rare (Danks, 1996).

In several early papers (see Denlinger, 1991) there are indications of two (or more) phases of cold hardening. Such phases were recorded e.g. in *P. apterus* (Hodkova et al., 1992; Hodkova & Hodek, 1994, 1997; Hodkova et al., 1999, 2002) and *A. nigripennis* (Watanabe & Tanaka 1998a, b, 1999a, b). We assume that (1) photoperiodic signals trigger physiological and biochemical changes that (a) directly increase cold-hardiness and/or (b) are a prerequisite for (2) more robust cold hardening induced by temperature decrease below a species/population specific limit. Effects of photoperiod and temperature on cold-hardiness may differ in different phases of diapause. While the changes regulated by photoperiod are associated with the induction of diapause, the later changes influenced by temperature are associated with the decrease in intensity of diapause due to diapause development. After the completion of diapause, the continuing exposure to low temperaure may maintain strong cold-hardiness, but after the increase in temperature the cold-hardiness irreversibly decreases (Hodkova & Hodek, 1994). Although it is likely that such phases in the regulation of cold-hardiness are common in insects with winter diapause, the detailed analysis of the relative importance of photoperiod and temperature and the temporal sequence of their effects have been neglected in most studies.

ACKNOWLEDGEMENTS. The study was supported by the Grant Agency of the Czech Republic (Grants 206/02/0900) and 206/03/0099) and by the Academy of Sciences of the Czech Republic (Projects Z5007907 and 61/5113). We thank for useful comments to H. Danks, D. Denlinger, L. Sømme and M. Watanabe.

REFERENCES

ANCHORDOGUY T.J., RUDOLPH A.S., CARPENTER J.F. & CROWE J.H. 1988: Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* **24**: 324–331.

ANSART A., VERNON P. & DAGUZAN J. 2001: Photoperiod is the main cue that triggers supercooling ability in the land snail, Helix aspersa (Gastropoda: Helicidae). *Cryobiology* **42**: 266–273.

ANSART A. & VERNON P. 2003: Cold hardiness in molluscs. *Acta Oecol.* **24**: 95–102.

BALE J.S. 1996: Insect cold hardiness: A matter of life and death. *Eur. J. Entomol.* **93**: 369–382.

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

BROUFAS G.D. & KOVEOS D.S. 2001: Cold hardiness characteristics in a strain of the predatory mite Euseius (Amblyseius) finlandicus (Acari: Phytoseidae) from northern Greece. *Ann. Entomol. Soc. Am.* **94**: 82–90.

CARPENTER J.F. & CROWE J.H. 1988: The mechanism of cryoprotection of proteins by solutes. *Cryobiology* **25**: 244–255.

CHIPPENDALE G.M. 1977: Hormonal regulation of larval diapause. *Annu. Rev. Entomol*. **22**: 121–138.

DANKS H.V. 1978: Modes of seasonal adaptation in the insects I. Winter survival. *Can. Entomol.* **110**: 1167–1205.

DANKS H.V. 1987: *Insect Dormancy: an Ecological Perspective.* Biol. Survey Canada, Ottawa, 439 pp.

DANKS H.V. 1996: The wider integration of studies on insect cold-hardiness. *Eur. J. Entomol*. **93**: 383–403.

DANKS H.V. 2000: Insect cold hardiness: a Canadian perspective. *CryoLetters* **21**: 297–308.

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

DENLINGER D.L. 2002: Regulation of diapause. *Annu. Rev. Entomol.* **47**: 93–122.

DROBNIS E.Z., CROWE L.M., BERGER T., ANCHORDOGUY T.J., OVERSTREET J.W. & CROWE J.H. 1993: Cold shock damage is due to lipid phase transition in cell membranes: A demonstration using sperm as model. *J. Exper. Zool*. **265**: 432–437.

GOTO S.G. & KIMURA M.T. 1998: Heat- and cold-shock responses and temperature adaptation in subtropical and tem-

perate species of Drosophila. *J. Insect Physiol.* **44**: 1233–1239.

GOTO S.G. & KIMURA M.T. 2004: Heat-shock-responsive genes are not involved in the adult diapause of Drosophila triauraria. *Gene* **326**: 117–122.

GOTO M., TAKAHASHI K. & SUZUKI C. 1993a: Ecological study on the barnyard grass stem borer, Enosima leucotaeniella (Lepidoptera: Pyralidae) VIII. Seasonal changes of carbohydrate contents in overwintering larvae. *Appl. Entomol. Zool*. **28**: 417–421.

GOTO M., TAKAHASHI K. & SUZUKI C. 1993b: Ecological study on the barnyard grass stem borer, Enosima leucotaeniella (Lepidoptera: Pyralidae) IX. Effect of temperature on carbohydrate contents in diapausing and early post-diapausing larvae. *Appl. Entomol. Zool*. **28**: 433–437.

GOTO S.G., YOSHIDA K.M. & KIMURA M.T. 1998: Accumulation of *Hsp 70* mRNA under environmental stresses in diapausing and nondiapausing adults of Drosophila triauraria. *J. Insect Physiol.* **44**: 1009–1015.

HAMILTON M.D., ROJAS R.R. & BAUST J.G. 1986: Juvenile hormone: Modulation of cryoprotectant synthesis in Eurosta solidaginis by a component of the endocrine system. *J. Insect Physiol*. **32**: 971–979.

HANC Z. & NEDVED O. 1999: Chill injury at alternating temperatures in Orchesella cincta (Collembola: Entomobryidae) and Pyrrhocoris apterus (Heteroptera: Pyrrhocoridae). *Eur. J. Entomol.* **96**: 165–168.

HART A.J. & BALE J.S. 1998: Factors affecting the freeze tolerance of the hoverfly Syrphus ribesii (Diptera: Syrphidae). *J. Insect Physiol.* **44**: 21–29.

HAZEL J.R. 1997: Thermal adaptations in biological membranes: Beyond homeoviscous adaptation. In Bittar E.E. & Willis J.S. (eds): *Advances in Molecular and Cell Biology: Thermobiology*. *Vol. 19*. Jai Press, London, pp. 57–101.

HODKOVA M. & HODEK I. 1994: Control of diapause and supercooling by the retrocerebral complex in Pyrrhocoris apterus. *Entomol. Exp. Appl.* **70**: 237–245.

HODKOVA M. & HODEK I. 1997: Temperature regulation of supercooling and gut nucleation in relation to diapause of Pyrrhocoris apterus (Heteroptera). *Cryobiology* **34**: 70–79.

HODKOVA M., BERKOVA P. & ZAHRADNICKOVA 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.

HODKOVA M., SIMEK P., ZAHRADNICKOVA H. & NOVAKOVA O. 1999: Seasonal changes in the phospholipid composition in thoracic muscles of a heteropteran, Pyrrhocoris apterus. *Insect Biochem. Molec. Biol.* **29**: 367–376.

HODKOVA M., SOMME L., HANZAL R., BRUNNHOFER V. & HODEK I. 1992: The effect of allatectomy and photoperiod on the supercooling point in Pyrrhocoris apterus adults. *Physiol. Entomol*. **17**: 165–168.

HOFFMANN A.A., SORENSEN J.G. & LOESCHKE V. 2003: Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**: 175–216.

HORWATH K.L. & DUMAN J.G. 1983: Preparatory adaptations for winter survival in the cold hardy beetles, Dendroides canadensis and D. concolor. *J. Comp. Physiol.* **151**: 225–232.

ITO K. & NAKATA T. 1998: Diapause and survival in winter in two species of predatory bugs, Orius sauteri and O. minutus. *Entomol. Exp. Appl.* **89**: 271–276.

KIMURA M.T., AWASAKI T., OHTSU T. & SHIMADA K. 1992: Seasonal changes in glycogen and trehalose content in relation to winter survival of four temperate species of Drosophila. *J. Insect Physiol.* **38**: 871–875.

KOSTAL V. & SIMEK P. 2000: Overwintering strategy in Pyrrhocoris apterus (Heteroptera): the relations between life-cycle, chill tolerance and physiological adjustments. *J. Insect Physiol.* **46**: 1321–1329.

KOSTAL V. & SLACHTA M. 2001: Variation in cold hardiness during overwintering in Pyrrhocoris apterus (Heteroptera: Pyrrhocoridae). *Acta Soc. Zool. Bohem.* **65**: 239–245.

KOSTAL V., SLACHTA M. & SIMEK P. 2001: Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of Pyrrhocoris apterus (Heteroptera). *Comp. Bioch. Physiol. (B)* **130**: 365–374.

KOSTAL V., BERKOVA P. & SIMEK P. 2003: Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of Chymomyza costata (Drosophilidae). *Comp. Bioch. Physiol. (B)* **135**: 407–419.

LANGER A. & HANCE T. 2000: Overwintering strategies and cold hardiness of two aphid parasitoid species (Hymenoptera: Braconidae: Aphidiinae). *J. Insect Physiol.* **46**: 671–676.

LEE R.E. JR., CHEN C.-P., MEACHAM M.H. & DENLINGER D.L. 1987: Ontogenetic patterns of cold-hardiness and glycerol production in Sarcophaga crassipalpis. *J. Insect Physiol.* **33**: 587–592.

LEE R.E. JR., DENLINGER D.L. & CHEN C.P. 1988: Insect cold-hardiness and diapause: Regulatory relationships. In Sehnal F., Zabza A. & Denlinger D.L. (eds): *Endocrinological Frontiers in Insect Ecology*. Wroclaw Tech. Univ., Wroclaw, pp. 243–262.

LEFEVERE K.S., KOOPMANSCHAP A.B. & DE KORT C.A.D. 1989: Changes in the concentrations of metabolites in haemolymph during and after diapause in female Colorado potato beetle, Leptinotarsa decemlineata. *J. Insect Physiol.* **35**: 121–128.

LEOPOLD R.A. 1991: Cryopreservation of insect germplasm: cells, tissues and organisms. In Lee Jr.R.E. & Denlinger D.L. (eds): *Insects at Low Temperature*. Chapman & Hall, New York, pp. 174–198.

LEWIS R.N.A.H., MANNOCK D.A., MCELHANEY R.N., TURNE D.C. & GRUNER S.M. 1989: Effect of fatty acyl chain length and structure on the lamellar gel to liquid-crystalline and lamellar to reverse hexagonal phase transition of aqueous phosphatidyletanolamine dispersions. *Biochemistry* **28**: 541–548.

MERIVEE E. 1978: *Cold-Hardiness in Insects.* Acad. Sci. Estonian SSR. Tallin, 178 pp. (In Estonian, with 23 pp. of English summary.)

MILONAS P.G. & SAVOPOULOU-SOULTANI M. 1999: Cold hardiness in diapause and non-diapause larvae of the summer fruit tortrix, Adoxophyes orana (Lepidoptera: Tortricidae). *Eur. J. Entomol*. **96**: 183–187.

MOREAU R., OLIVER D., GORDOUX L. & DUTRIEU J. 1981: Carbohydrate metabolism in Pieris brassicae L. (Lepidoptera: variations during normal and diapausing development. *Comp. Biochem. Physiol. (B)* **68**: 95–99.

NEDVED O., HODKOVA M., BRUNNHOFER V. & HODEK I. 1995: Simultaneous measurement of low temperature survival and supercooling in a sample of insects. *Cryo-Letters* **7**: 108–112.

NEDVED O. & WINDSOR D. 1994: Supercooling ability, fat and water contents in a diapausing tropical beetle, Stenotarsus rotundus (Coleoptera: Endomychidae). *Eur. J. Entomol.* **91**: 307–312.

NOMURA M. & ISHIKAWA Y. 2001: Dynamic changes in cold hardiness, high temperature tolerance and trehalose content in the onion maggot, Delia antiqua (Diptera: Anthomyiidae), associated with the summer and winter diapause. *Appl. Entomol. Zool.* **36**: 443–449.

OHTSU T., KIMURA M.T. & HORI S.H. 1995: The influence of eclosion timing on winter survival and triacylglycerol accumulation in four temperate species of Drosophila. *Physiol. Entomol.* **20**: 248–252.

OHTSU T., KIMURA M.T. & KATAGIRI C. 1998: How Drosophila species acquire cold tolerance: Qualitative change of phospholipids. *Eur. J. Biochem.* **252**: 608–611.

PULLIN A.S. 1992: Diapause metabolism and changes in carbohydrates related to cryoprotection in Pieris brassicae. *J. Insect Physiol*. **38**: 319–327.

PULLIN A.S. & BALE J.S. 1989a: Effects of ecdysone, juvenile hormone and haemolymph transfer on cryoprotectant metabolism in diapausing and non-diapausing pupae of Pieris brassicae. *J. Insect Physiol*. **35**: 911–918.

PULLIN A.S. & BALE J.S. 1989b: Influence of diapause and temperature on cryoprotectant synthesis and cold hardiness in pupae of Pieris brassicae. *Comp. Biochem. Physiol. (A)* **94**: 499–503.

PULLIN A.S., BALE J.S. & FONTAINE X.L.R. 1991: Physiological aspects of diapause and cold tolerance during overwintering in Pieris brassicae. *Physiol. Entomol.* **16**: 447–456.

PULLIN A.S. & WOLDA H. 1993: Glycerol and glucose accumulation during diapause in a tropical beetle. *Physiol. Entomol.* **18**: 75–78.

RAABE M. 1982: *Insect Neurohormones*. Plenum, New York & London, 352 pp.

RING R.A. & DANKS H.V. 1994: Desiccation and cryoprotection: overlapping adaptations. *Cryo-Letters* **15**: 181–190.

RING R.A. & DANKS H.V. 1998: The role of trehalose in cold-hardiness and desiccation. *Cryo-Letters* **19**: 275–282.

RING R.A. & RIEGERT P.W. 1991: Tribute to R.W. Salt. In Lee Jr. R.E. & Denlinger D.L. (eds): *Insects at Low Temperature*. Chapman & Hall, New York, pp. 3–16.

ROJAS R.R., HAMILTON M.D. & BAUST J.G. 1987: Juvenile hormone modulation of insect cold hardening: Ice nucleating activity. *Cryobiology* **24**: 465–472.

ROJAS R.R., CHARLET L.D. & LEOPOLD R.A. 1994: Biochemistry and physiology of overwintering in the mature larva of the sunflower stem weevil, Cylindrocopturus adspersus (Coleoptera: Curculionidae) in the Northern Great Plains. *J. Insect Physiol.* **40**: 201–205.

SAUNDERS D.S. & HAYWARD S.A.L. 1998: Geographical and diapause-related cold tolerance in the blow fly, Calliphora vicina. *J. Insect Physiol.* **44**: 541–551.

SHIMADA K. & RIIHIMAYA A. 1990: Cold-induced freezing tolerance in diapausing and non-diapausing larvae of Chymomyza costata (Diptera: Drosophilidae) with accumulation of trehalose and proline. *Cryo-Letters* **11**: 243–250.

SLACHTA M., BERKOVA P., VAMBERA J. & KOSTAL V. 2002a: Physiology of cold-acclimation in non-diapausing adults of Pyrrhocoris apterus (Heteroptera). *Eur. J. Entomol.* **99**: 181–187.

SLACHTA M., VAMBERA J., ZAHRADNICKOVA H. & KOSTAL V. 2002b: Entering diapause is a prerequisite for successful cold-acclimation in adult Graphosoma lineatum (Heteroptera: Pentatomidae). *J. Insect Physiol.* **48**: 1031–1039.

TANAKA K. 1995: Seasonal change in glycogen and inositol/sorbitol contents of the house spider, Achaearanea tepidariorum (Araneae: Theridiidae). *Comp. Biochem. Physiol. (B)* **110**: 539–545.

TANAKA K. 1996: Seasonal and latitudinal variation in supercooling ability of the house spider, Achaearanea tepidariorum (Araneae: Theridiidae). *Func. Ecol*. **10**: 185–192.

TANAKA S. 2000: The role of moisture in the control of diapause, mating and aggregation in a tropical insect. *Entomol. Sci.* **3**: 147–155.

TSUMUKI H. & HIRAI M. 1999: Effects of JH and ecdysone on endogenous ice nucleus production in larvae of the rice stem borer, Chilo suppressalis (Lepidoptera: Pyralidae). *Appl. Entomol. Zool*. **34**: 119–121.

TSUMUKI H. & KANEHISA K. 1980: Effect of low temperature on glycerol and trehalose concentration in haemolymph of the rice stem borer, Chilo suppressalis (Lepidoptera: Pyralidae). *Jap. J. Appl. Entomol. Zool*. **24**: 189–193.

TSUMUKI H. & KANEHISA K. 1981: Effect of JH and ecdysone on glycerol and carbohydrate contents in diapausing larvae of the rice stem borer, Chilo suppressalis (Lepidoptera: Pyralidae). *Appl. Entomol. Zool*. **16**: 7–15.

WATANABE M. & TANAKA K. 1997: Photoperiodic control of adult diapause, cold hardiness and inositol accumulation in a beetle, Aulacophora nigripennis (Coleoptera: Chrysomelidae). *Zool. Sci.* **14**: 233–237.

WATANABE M. & TANAKA K. 1998a: Adult diapause and cold hardiness in Aulacophora nigripennis (Coleoptera: Chrysomelidae). *J. Insect Physiol*. **44**: 1103–1110.

WATANABE M. & TANAKA K. 1998b: Effect of juvenile hormone analogs on diapause termination and myo-inositol content in Aulacophora nigripennis adults (Coleoptera: Chrysomelidae). *Appl. Entomol. Zool*. **33**: 259–262.

WATANABE M. & TANAKA K. 1999a: Cold tolerance strategy of the freeze-intolerant chrysomelid, Aulacophora nigripennis (Coleoptera: Chrysomelidae), in warm temperate regions. *Eur. J. Entomol.* **96**: 175–181.

WATANABE M. & TANAKA K. 1999b: Seasonal change of the thermal response in relation to *myo*-inositol metabolism in adults of Aulacophora nigripennis (Coleoptera: Chrysomelidae). *J. Insect Physiol.* **45**: 167–172.

WATANABE M. & TANAKA K. 2000: Hormonal control of diapause and overwintering traits in a leaf beetle, Aulacophora nigripennis. *Physiol. Entomol*. **25**: 337–345.

XU L. & DUMAN J.G. 1991: Involvement of juvenile hormone in the induction of antifreeze protein production by the fat body of larvae of the beetle Dendroides canadensis. *J. Exper. Zool*. **258**: 288–293.

XU L., NEVEN L.G. & DUMAN J.G. 1990: Hormonal control of hemolymph lipoprotein ice nucleators in overwintering freeze-susceptible larvae of the stag beetle Ceruchus piceus: adipokinetic hormone and juvenile hormone. *J. Comp. Physiol. (B)* **160**: 51–59.

XU L., DUMAN J.G., WU D.W. & GOODMAN W.G. 1992: A role for juvenile hormone in the induction of antifreeze protein production by the fat body in the beetle Tenebrio molitor. *Comp. Biochem. Physiol. (B)* **101**: 105–109.

ZACHARIASSEN K.E. 1985: Physiology of cold tolerance in insects. *Physiol. Rev*. **65**: 799–832.

ZACHARIASSEN K.E. & LUNDHEIM R. 1992: The endocrine control of insect cold hardiness. *Zool. Jb. Physiol*. **96**: 183–196.

Received January 9, 2004; revised April 20, 2004; accepted May 10, 2004