MINI-REVIEW

Photoperiod, diapause and cold-hardiness

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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-
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 dia
pause individuals are generally more cold hardy than non-dia
pause 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 charac
terised 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

**Table 1. Hormonal regulation of parameters related to cold hardiness.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Developmental stage</th>
<th>Treatment</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diap. larva</td>
<td>20-HE</td>
<td>SCP ↑</td>
<td>Tsumuki &amp; Hirai, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JH I (0.01 µg)</td>
<td>SCP ↓</td>
<td>Tsumuki &amp; Kanehisa, 1981</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JH I (1 µg)</td>
<td>glycerol ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-E</td>
<td>glycerol ↓</td>
<td></td>
</tr>
<tr>
<td>Ceruchus piceus</td>
<td>larva (mid winter)</td>
<td>AKH</td>
<td>SCP ↑</td>
<td>Xu et al., 1990</td>
</tr>
<tr>
<td></td>
<td>larva (spring, autumn)</td>
<td>JH I</td>
<td>SCP ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>INA ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SCP ↓</td>
<td></td>
</tr>
<tr>
<td>Tenebrio molitor</td>
<td>larva</td>
<td>JH I</td>
<td>AP ↑</td>
<td>Xu et al., 1992</td>
</tr>
<tr>
<td>Eurosta solidaginis</td>
<td>larva</td>
<td>JH I</td>
<td>SCP ↑</td>
<td>Rojas et al., 1987</td>
</tr>
<tr>
<td></td>
<td>ligated larva</td>
<td>head ligation</td>
<td>SCP ↓</td>
<td>Hamilton et al., 1986</td>
</tr>
<tr>
<td>Sarcophaga crassipalpis</td>
<td>larva</td>
<td>20-HE</td>
<td>SCP ↑</td>
<td>Lee et al., 1988</td>
</tr>
<tr>
<td></td>
<td>pupa</td>
<td>JHA</td>
<td>glycerol ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20-HE (0.1 µg)</td>
<td>glycerol ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20-HE (0.5 µg)</td>
<td>SCP ↑</td>
<td></td>
</tr>
<tr>
<td>Pieris brassicae</td>
<td>non-diap. pupa</td>
<td>JH</td>
<td>glycerol ↑</td>
<td>Pullin &amp; Bale, 1989a</td>
</tr>
<tr>
<td></td>
<td>diap. pupa</td>
<td>JH</td>
<td>sorbitol ↑</td>
<td>Pullin, 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>sorbitol ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20-HE</td>
<td>sorbitol ↓</td>
<td></td>
</tr>
<tr>
<td>ADULT</td>
<td>Aulacophora nigripennis</td>
<td>diap. adult</td>
<td>survival ↓</td>
<td>Watanabe &amp; Tanaka, 1998b, 1999a, 2000</td>
</tr>
<tr>
<td>Pyrrhocoris apterus</td>
<td>non-diap adult</td>
<td>extirp. CC-CA</td>
<td>SCP ↓</td>
<td>Hodkova &amp; Hodek, 1994</td>
</tr>
<tr>
<td></td>
<td>diap. adult</td>
<td>extirp. CA</td>
<td>SCP ↓ (at low T)</td>
<td>Hodkova et al., 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extirp PI</td>
<td>PL restructuring</td>
<td>Hodkova et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SCP ↑</td>
<td>Hodkova, unpubl.</td>
</tr>
</tbody>
</table>

**Legend:**
- 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.
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
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.

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,
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<sub>50</sub> = 28.6 days, Kostal et al., 2001) than in non-diapause (LT<sub>50</sub> = 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<sub>50</sub> 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 transfers 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 (C₁₆:0) and di-unsaturated linoleic acid (C₁₈:2) at the expense of phospholipids with 18-carbon fatty acids (C₁₈), both saturated and unsaturated. Further increase in the proportion of the same molecular species (C₁₆:0/C₁₈:2) and changes in the composition of the phospholipid head group (increase in the level of phosphatidylethanolamine at the expense of phosphatidylincholine) 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 C₁₆:0 and C₁₈:2 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 C₁₈ 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).

Polylols

Although polylols 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 polylols (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 polylol at low temperatures. No accumulation of polylols 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 polylols 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 polylol 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).](image-url)
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).

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, 1989b). 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...
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. brassicae* (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 (C16:0/C18:2) fatty acid pairs (Kostal et al., 2003). It is noteworthy that the more cold tolerant *Drosophila* species from northern regions have higher proportion of C16 fatty acids and lower proportion of C18 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-
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).

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

### 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).

<table>
<thead>
<tr>
<th>Species (family, order)</th>
<th>Parameter measured</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sarcophaga crassipalpis</strong> (Sarcophag., Dipt.)</td>
<td>glycerol from &lt;10 to &gt;70 mM</td>
<td>Lee et al., 1987</td>
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<tr>
<td><strong>Pieris brassicae</strong> (Pier., Lep.)</td>
<td>sorbitol, 47× increase c-h at 20°</td>
<td>Pullin &amp; Bale, 1989b; Pullin et al., 1991</td>
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<tr>
<td><strong>Leptinotarsa decemlineata</strong> (Chrysomel., Col.)</td>
<td>proline</td>
<td>Lefevere et al., 1989</td>
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<tr>
<td><strong>Cylindrocoptus adspersus</strong> (Curculion., Col.)</td>
<td>trehalose from 32 to 42 µg/mg ww</td>
<td>Rojas et al., 1994</td>
</tr>
<tr>
<td><strong>Achaeaeranea tepidiorum</strong> (Therid., Araneae)</td>
<td>inositol, glycogen (but no increase in glycerol)</td>
<td>Tanaka, 1995, 1996</td>
</tr>
<tr>
<td><strong>Aulacophora nigrigennis</strong> (Chrysomel., Col.)</td>
<td>myo-inositol at 15, 20, 25°</td>
<td>Watanabe &amp; Tanaka, 1998a, b; 1999a, b</td>
</tr>
<tr>
<td><strong>Drosophila triauraria</strong> (Drosophil., Dipt.)</td>
<td>survival 24 h at -8° 100 vs 20%</td>
<td>Goto et al., 1998</td>
</tr>
<tr>
<td><strong>Syrphus ribesii</strong> (Syrph., Dipt.)</td>
<td>SCC, survival</td>
<td>Hart &amp; Bale, 1998</td>
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<tr>
<td><strong>Orius sauteri, O. minutus</strong> (Anthocor., Het.)</td>
<td>survival at 0° LT50: 140d vs 20d</td>
<td>Ito &amp; Nakata, 1998</td>
</tr>
<tr>
<td><strong>Calliphora vicina</strong> (Calliphor., Dipt.)</td>
<td>survival at 0, -4, -8°</td>
<td>Saunders &amp; Hayward, 1998</td>
</tr>
<tr>
<td><strong>Adoxophyes orana</strong> (Tortric., Lep.)</td>
<td>c-h increase at 20° survival at -5°: 37% 30d vs 0% 6d</td>
<td>Milonas &amp; Savopoulou-Soultani, 1999</td>
</tr>
<tr>
<td><strong>Aphidius ervi</strong> (Bracon., Hym.)</td>
<td>SCC, survival at -10°: 50% 10d vs 5d</td>
<td>Langer &amp; Hance, 2000</td>
</tr>
<tr>
<td><strong>Delia antiqua</strong> (Anthem., Dipt.)</td>
<td>survival at -15°: 80 vs 5% trehalose: 16 vs 4 mg/mg ww</td>
<td>Nomura &amp; Ishikava, 2001</td>
</tr>
<tr>
<td><strong>Euseius finlandicus</strong> (Phyto., Acri.)</td>
<td>c-h increase at 20° survival at -4°</td>
<td>Broufas &amp; Koveos, 2001</td>
</tr>
<tr>
<td><strong>Corna (Helix) aspersum</strong> (Helic., Gastropoda)</td>
<td>SCP: 16L: -3°, 8L: -5.2° mOsm/kg: 16L: 287, 8L: 342</td>
<td>Ansart et al., 2001; Ansart &amp; Vernon, 2003</td>
</tr>
<tr>
<td><strong>Graphosoma lineatum</strong> (Pentatom., Het.)</td>
<td>c-h increase from IX to X; survival at -10° 9d: 70 vs 0%; trehalose 0.75 vs 0.1 mg/??</td>
<td>Slachta et al., 2002b</td>
</tr>
<tr>
<td><strong>Chymomyza costata</strong> (Drosophil., Dipt.)</td>
<td>1 h survival at -25°: 65 vs 0% membrane PL remodelling</td>
<td>Kostal et al., 2003</td>
</tr>
</tbody>
</table>

| c-h – cold hardness; 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 diapausing adults at 0°C was seven times longer than in non-diapausing adults at 0°C; LT50 was 140 days vs. 20 days in non-diapausing adults (Ito &
Nakata, 1998), in the braconid A. ervi, the survival of diapause mummies at −10°C was only twice as great (LT<sub>50</sub> : 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-Soutzani, 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 leucothaeniella 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. leucothaeniella, 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 temperature 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.

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