POINT OF VIEW

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Physiological relationships between insect diapause and cold tolerance: Coevolution or coincidence?

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Abstract. It is argued that the relationship between diapause and cold tolerance has been obscured by the complexity of these strategies. If cold tolerance is broken down into its component mechanisms a relationship between diapause related metabolic suppression and carbohydrate cryoprotectant synthesis is supported by an increasing number of studies. This link seems to have been overlooked because of the view that temperature is the primary cue for cryoprotectant synthesis. However, some major studies cited in support of the latter view have not rigorously tested the role of diapause. An evolutionary analysis of the relationship suggests that diapause-related carbohydrate synthesis could be a primitive feature within the insects that played an important initial role in their colonisation of colder climates.

INTRODUCTION

This review considers the evolutionary and physiological relationships between the diapause syndrome and the tolerance of insects to cold. Insects are found in a wide range of environments that experience extremes of abiotic factors such as temperature and precipitation and they have been conspicuously successful at colonising all but the marine environment. Consequently it is of interest to know to what extent major adaptive strategies and their integration have contributed to this success.

The diapause state is normally regarded as an adaptation which has enabled insects to survive through periods generally unfavourable to development and/or reproduction. Common examples are survival through periods of temperature below reproductive and developmental thresholds, and periods of drought when normal activity would result in lethal desiccation. However, diapause is not necessarily regarded as part of the strategy which enables the same species to withstand exposure to subzero temperatures or severe desiccation. Additional adaptations appear to be necessary to survive these extremes (sometimes for a very short time, e.g. a minimum temperature).

The colonisation of large areas of the earth by insects seems to have involved both diapause and cold tolerance. Have they evolved completely independently or has a common evolutionary origin played a part in the rapid diversification of the insects and their domination of terrestrial environments?

In previous work evidence has been found both for (Chino, 1957, 1958; Sømme, 1965; Asahina, 1969; Mansingh, 1971, 1974; Pullin & Bale, 1989c,d; Pullin, 1992) and against (Lees, 1955; Salt, 1961; Ring, 1972) a relationship between diapause and cold tolerance. Most recently Denlinger (1991) comprehensively reviewed this subject and concluded that the evidence for a relationship is mixed. In some species there is good evidence for a

relationship and in other species there is good evidence that no relationship exists. In most species, some data exist, but the appropriate experiments testing for a relationship have not been performed.

The problem facing any analysis of this kind arises from the diversity of both diapause and cold-tolerance strategies.

THE DIVERSITY OF DIAPAUSE STRATEGIES

Diversity in diapause is evident at most levels of organisation (see Danks, 1987 for review). Diapause occurs at different life-history stages in different species, although there are definite phylogenetic relationships, for example, most saturniid moths diapause as pupae (Crotch, 1956) and most coccinellid beetles as adults (Hodek, 1973). Diapause progression differs between species as does the degree of metabolic suppression. The endocrinology of diapause appears different in each life-history stage. Very generally, larval and pupal diapause is controlled through low ecdysone and initially high juvenile hormone titres. In contrast, adult diapause is characterised by low juvenile hormone titre (Denlinger, 1985). Even though the underlying mode of control appears to be common to most species there is general agreement that diapause has evolved many times in insects (Danks, 1987).

THE DIVERSITY OF COLD-TOLERANCE STRATEGIES

Within cold tolerance there appears to be a diversity of strategies and a diversity of mechanisms. Traditionally, strategies of cold tolerance have been divided into two alternatives, freeze tolerance (the tolerance of extracellular ice formation) and freeze avoidance /intolerance (the depression of freezing point and supercooling point, decreasing the probability of lethal ice formation). More recently these convenient definitions have been questioned and Bale (1993) further subdivided the freeze avoidance/intolerance category into four sections; freeze avoiding (species which supercool extensively and die only when they freeze), chill tolerant (species that supercool extensively but show some mortality on exposure to temperatures above the supercooling point), chill susceptible (species showing considerable mortality on brief exposure to subzero temperatures above the SCP), and opportunistic (species which suffer mortality at above zero temperatures below their threshold for development). These categories are useful when assessing species cold tolerance in a comparative sense and for predictive purposes, but in an evolutionary context it is not clear whether these are true groups or whether they represent a continuum.

The mechanisms that underlie the strategies are even more diverse, for example: cryoprotectant accumulation, supercooling, production of thermal hysteresis proteins, cold shock responses, management of ice nucleators, management of water, and adjustments of metabolism. Some, such as carbohydrate cryoprotectant accumulation, have been relatively well studied, whilst others, such as management of water and adjustment of metabolism are not well understood. A further complication is that many species appear to use a combination of strategies and the nature of their integration is almost unknown.

This apparent complexity and lack of systematic pattern (which has been used as evidence to support independent origins of cold tolerance) may result from progress in insect cold tolerance research having largely been made by studying species from extreme environments (e.g. polar, boreal or alpine). Such species are likely to be the most interesting,

TABLE 1. Number of insect species from different climatic zones on which significant cold tolerance research (at least reports of supercooling points related to other aspects of their biology) has been published.

Climate zone	No. of species
Polar/alpine	42
Cold temperate	39
Warm temperate	3
Tropical	2

but they are also likely to express specialised cold tolerance characteristics. An emphasis on such species may have produced a false impression of a high diversity of mechanisms that defies evolutionary analysis. This understandable bias can be expressed quantitatively if we look at the numbers of species from different climates on which significant cold tolerance research has been carried out (Table 1). This includes species on which supercooling points and levels of cryoprotectants have been measured.

AN ALTERNATIVE VIEWPOINT

If each of the mechanisms of cold tolerance are treated separately (instead of considering the strategy as a whole) then the bias of data towards highly cold tolerant species can be eliminated and the possible role of diapause examined in more detail.

The evidence then suggests that there are some mechanisms not always directly linked to diapause, but frequently coincident with it.

The **supercooling capacity** of some non-diapause insects is often cited as evidence for lack of an evolutionary relationship between diapause and cold tolerance. For example, non-diapause pupae of *Pieris brassicae* supercool to –21°C, compared with –25°C in diapause. Some species, such as *Pyrrhocoris apterus*, show a strong relationship between diapause and supercooling capacity (Hodková & Hodek, 1994), whilst others increase supercooling capacity during winter without diapausing (Sømme, 1982).

Cold shock responses are also not necessarily linked to diapause. They are not even always linked to overwintering and the inactive state (Lee et al., 1987). Additionally, these phenomena have limited value when considering survival through extended periods of cold.

Thermal hysteresis proteins have not been studied very extensively in insects and there is only limited evidence that diapause is important in their production (Duman et al., 1991). It is interesting to note that these proteins have been found frequently in the Coleoptera but not in the Diptera or Hymenoptera (Duman et al., 1991). This relative confinement within taxa supports the idea of this mechanism being specialised or advanced.

There is also ample evidence that some invertebrate adaptations to extremes have evolved in the absence of diapause (Hochachka & Somero, 1984). Nevertheless, in general, insects entering some form of dormancy exhibit greater tolerance to low temperature than those that remain active (Lee, 1991). This inevitably involves suppression of metabolism in some form, but may be in direct response to the environment (i.e. quiescence) rather than through hormonal suppression. Cryptobiosis could be seen as an extreme form of dormancy and is well documented among the crustaceans, nematodes and tardigrades (Crowe et al., 1992; Kinchin, 1994).

AN EVOLUTIONARY SCENARIO

Insects first appear as fossils from the Devonian (400 million years BP) and may date from the Silurian. However, the major groups of modern insects did not appear until the

Permian era 250 My BP (Carpenter, 1992). The major radiation probably began in the warm humid climate of the first forests of Pangea before the breakup of the continents and continued after the terminal Permian mass extinction into the Jurassic era, a trend which appears to have continued until the present day in the holometabolous orders (Labandeira & Sepkoski, 1993). The great majority of this diversity still occurs in the humid tropics and little diversification appears to have occurred in cold climates. This favours a model of diversification within warm climates and irregular colonisation of colder climates by restricted taxa.

On the basis of this scenario, one could assume that before the period of radiation the early insects showed either none or only precursors of cold-tolerance traits. Then as they became exposed to changing, often colder climates, whether this was because of continental drift, dispersal of species into higher latitudes or simply changing climate; they must have evolved mechanisms to cope. What were the initial mechanisms that enabled these colonisation events?

The climate during and since the Permian has been very dry at times and therefore many insects will have been exposed to desiccating conditions (Hennig, 1981). Many of today's species in similar conditions enter diapause and if early insects had already evolved diapause they could have been predisposed to survival in colder climates because they were able to survive through short periods of moderate cold. From limited evidence on it's evolution, diapause appears to be a primitive strategy, widespread among the insects and widespread geographically (Danks, 1987). Importantly, there is increasing evidence that diapause is widespread in tropical insects (Denlinger, 1986). Are there any cold tolerance mechanisms which show this same pattern and therefore could be a primitive feature which further assisted the colonisation of colder climates, and crucially, was it in combination with, or as part of the diapause syndrome?

The most widely reported mechanism of cold tolerance in temperate regions is carbohydrate accumulation, usually assumed to be for cryoprotection. There is also some evidence that this strategy is used in desiccation tolerance (Ring & Danks, 1994), although this comes mainly from work on other invertebrates (Hochachka & Somero, 1984). Carbohydrate accumulation therefore appears to be a candidate for a primitive cold tolerance mechanism and its relationship with diapause merits closer examination.

DIAPAUSE AND CARBOHYDRATE SYNTHESIS

Good evidence for a relationship between diapause and carbohydrate synthesis comes from a significant number of species where the diapause state is necessary for carbohydrate accumulation. This relationship was first noted by Chino (1957, 1958) in *Bombyx mori*, and has recently been studied in some detail in *P. brassicae*, a temperate insect (Pullin & Bale, 1989c,d; Pullin et al., 1991; Pullin, 1992). In the latter the concentration of sorbitol in the whole body and in the haemolymph is closely correlated with the level of diapause-induced metabolic suppression measured in terms of phosphorus metabolism by nmr (Pullin et al., 1991) and by respirometry (Pullin, 1992). Metabolic suppression is widespread as part of diapause and may be a primitive feature of insect biochemistry.

The above relationship has probably not been so clearly recognised because of the prevailing view that temperature acts as the trigger for carbohydrate/cryoprotectant accumulation (e.g. Baust, 1982). But this view is based on a small number of species and in many

of these the role of diapause has been ignored, rather than tested. The species *Eurosta solidaginis* is a good example. This species has been used as a model for study of temperature induced cryoprotectant accumulation, yet despite references to diapause in early work on the species biology (Uhler, 1951), its role in cryoprotectant accumulation has been largely ignored (but see Sømme, 1964). Studies on other species show temperature-related induction of carbohydrate synthesis without stating whether the insect was in diapause or not, or without testing the effect of temperature in both diapause and non-diapause individuals.

Very few studies are thorough enough to discount a relationship between diapause and carbohydrate accumulation. A study by Gehrken (1985) on *Ips acuminatus* has shown a relationship between diapause induction and accumulation of ethylene glycol, followed by a partial decoupling of diapause and cryoprotectant levels during the postdiapause development stage. One example of an apparent complete lack of relationship comes from the work of Kukal et al. (1989) on the arctic moth, *Gynaephora groenlandica*. This species is highly specialised to severe arctic conditions and shows many advanced cold-tolerance strategies and is perhaps not a surprising exception to the norm. Nevertheless any exceptions have to be accounted for.

So how might the strategy of cryoprotectant synthesis have first evolved? One way of approaching this question is to assume that the scenario of origin in warm climates and movement to cold ones might be reflected in the changes in cold-tolerance strategy over the current climatic gradient from the tropics to the polar regions. In other words changes that have occurred over time should be paralleled to some extent by changes that we see now over space. This leads us logically to look for primitive cold-tolerance mechanisms or their precursors in tropical insects.

Recent work by Pullin & Wolda (1993) tested the hypothesis that polyol accumulation may have first evolved as a result of metabolic suppression associated with tropical diapause. They used the fungus beetle *Stenotarsus rotundus* which has a 10-month diapause during which the adults aggregate at the base of palm trees. This diapause lasts through the wettest and then the driest months of the year from June to March (Wolda & Denlinger, 1984). Samples were collected from Barro Colorado Island, Panama, and analysed for polyol accumulation. The data revealed an accumulation of glycerol (from 1.2 to 4.4 mg/g dry weight) which corresponds to a period of metabolic suppression during the rainy season. Glycerol then decreases during December at the beginning of the dry season. Since there is no evidence that *S. rotundus* experiences either cold or desiccation stress during the rainy season, it seems unlikely that this pattern is an adaptation to cold or desiccation. Yoder et al. (1992) found that aggregation during diapause in this species promotes water conservation when subjected to extreme desiccating conditions (0% relative humidity) in the laboratory, but they provide no evidence that desiccation stress is experienced in the field during the period of elevated glycerol levels.

Unless the glycerol is accumulating for an unrecognised reason, the data support the hypothesis of accumulation as a byproduct of metabolic suppression. Against this, Chen et al. (1990) found no evidence of glycerol accumulation in tropical flesh flies, but only in response to short term cold exposure. More direct evidence is needed but there is further circumstantial evidence that also supports this hypothesis.

The accumulation of potential cryoprotectants during overwintering has been reported in many and diverse species of insects and other invertebrates (Sømme, 1982). The

common polyols which act as cryoprotectants, such as glycerol and sorbitol, may accumulate in high concentrations in some insects. But in many temperate species their accumulation is less dramatic and there is little evidence (practical or theoretical) for a role in supercooling-point depression. For example, despite the accumulation of 0.04 M sorbitol during diapause in *P. brassicae* the SCP does not change (Pullin et al., 1991). This is in agreement with predictions based on the colligative properties of these substances (Zachariassen, 1985). However, there is some evidence that such low concentrations may afford protection from chill injury above the SCP (Chen et al., 1987; Pullin et al., 1991). The mechanism for this protection may involve the stabilisation of soluble proteins and lipid bilayers (Crowe et al., 1987; Carpenter & Crowe, 1988).

A list of freeze susceptible species (for which we know both SCP and carbohydrate accumulation) from different climatic zones supports the idea that the carbohydrate accumulation is at levels which are ineffective at SCP depression except in insects from cold temperate and polar zones which experience temperatures below –30°C (Table 2). In these zones there appears to be a ten-fold enhancement of carbohydrate levels and this may truly provide cryoprotection by supercooling point depression.

TABLE 2. Comparison of cryoprotectant accumulation and SCP in freeze avoiding insects from different climate zones.

Taxon	Climate	SCP	Cryoprotectant	Reference
Stenotarsus rotundus	Tropical	–13°C*	Glycerol (0.016M)	Pullin and Wolda (1993) *Nedvěd & Windsor (1994)
Pieris brassicae	Warm Temperate	–24°C	Sorbitol (0.04M)	Pullin et al. (1991)
Aglais urticae	Warm Temperate	–22°C	Glycerol (0.1M)	Pullin & Bale (1989a,b)
Inachis io	Warm Temperate	–21°C	Glycerol (0.09M)	Pullin & Bale (1989a,b)
Leguminovora glycinivorella	Warm Temperate	–25°C	Trehalose (0.1M)	Shimada et al. (1984)
Sarcophaga crassipalpis	Cold Temperate	–23°C	Glycerol (0.01M)	Lee et al. (1987)
Ips acuminatus	Cold Temperate	–24°C	Ethylene Glycol (1M)	Gehrken (1985)
Megachile relativa	Cold Temperate	–42°C	Glycerol (1.5M)	Krunic & Salt (1971)
Megachile rotundata	Cold Temperate	–43°C	Glycerol (0.75M)	Krunic & Salt (1971)
Epiblema scudderiana	Cold Temperate	–38°C	Glycerol (2M)	Rickards et al. (1987)
Rhabdophaga sp.	Polar	–62°C	Glycerol (5M)	Ring (1981)

The evolutionary scenario this suggests is that diapause induced metabolic suppression resulted in accumulation of low concentrations of carbohydrates which may have initially been selectively neutral. Selection for enhanced carbohydrate accumulation may have first occurred in response to drought stress in tropical regions and has more recently been selected for its cryoprotectant function in cold temperate and polar climates, allowing relatively rapid colonisation of these regions, perhaps by successive waves of insects as a result of independent evolutionary events. The latter may explain to some extent the different cryoprotectants used in different taxa. Advanced characteristics such as

cryoprotectant accumulation by enzyme activation in response to low temperature, in the absence of diapause, as seen in *G. groenlandica* may have evolved subsequently in response to the extreme climate in polar regions.

DISCUSSION

The potential of this evolutionary hypothesis is that it begins to place cold hardiness strategies within a framework which has some predictive value. We would expect advanced/derived cold-tolerance strategies to appear much more commonly in colder climates and also to be taxonomically more confined since they evolved after the major adaptive radiation of the insects. The strategy of freeze tolerance and associated mechanisms such as synthesis of ice-nucleating proteins are less widespread and probably represent derived features.

Freeze tolerance does not appear to be a very common strategy when considered across all environments, but it is more common in climates which experience temperatures below –30°C. It is however difficult to know whether this strategy evolves as a result of populations being pushed up against a barrier of very low temperatures and individuals in those populations eventually evolving freeze tolerance, or if other factors in warmer climates predispose species to freeze tolerance which they then exploit by colonising cold climates. The shift from a freeze avoiding strategy of substantial supercooling (typically to –30°C) to a freeze tolerant strategy of limited supercooling (SCP above –10°C) is a difficult evolutionary pathway to envisage (but see Horwath & Duman, 1984). The alternative is that an adaptation such as desiccation tolerance predisposes the insect to freeze tolerance without it experiencing subzero temperatures and therefore developing substantial supercooling ability. Dispersal into colder climates may then result in freezing at relatively high subzero temperatures, survival of a few individuals and subsequent strengthening of the adaptation through selection.

However, further analysis is limited by our knowledge of what a cold hardiness strategy is. The relative importance of cryoprotectants, ice nucleators and thermal hysteresis proteins are all difficult to assess and more fundamental adjustments in metabolism and water relations may be of greater importance, but they are harder to measure and quantify. The coevolution and physiological integration of these mechanisms awaits further research.

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