J. Insect Physiol. Vol. 33, No. 8, pp. 587–592, 1987
Printed in Great Britain.

0022-1910/87 $3.00 + 0.00

ONTOGENETIC PATTERNS OF COLD-HARDINESS AND GLYCEROL PRODUCTION IN *SARCOPHAGA CRASSIPALPIS*

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(*Received* 15 *December* 1986)

Abstract—Developmental patterns of low-temperature tolerance and glycerol production were determined for larval, pupal and adult stages of the flesh fly *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae). Both diapause and non-diapause-destined flies were reared at relatively high temperatures, 20° or 25°C, prior to testing. Cold tolerance was greatest for diapause pupae aged 12–35 days after pupariation. Among non-diapause-destined flies, pupae exhibited a greater level of low temperature tolerance than larvae or adults. Although diapause pupae were more tolerant than non-diapause pupae maximal cold tolerance was not attained in either group until 10 days after pupariation. Non-diapause-destined feeding and wandering larvae had higher glycerol levels than larvae destined for diapause. During the first 6 weeks after pupariation glycerol titres increased steadily in diapause pupae. Rapid loss of glycerol is associated with the termination of pupal diapause.

Key Word Index: Diapause, cold tolerance, Diptera, cryoprotectant

INTRODUCTION

For most insects of the temperate zone activity is restricted to a few months during the summer, while the rigours of winter are circumvented by diapause, a period of developmental arrest. The short days of late summer typically serve as an environmental cue which programme the insect for diapause (cf. Beck, 1980 and Saunders, 1982). However, entry into the physiological state of diapause does not, by itself, assure winter survival. With the onset of winter, insects must cope with environmental temperatures which may plunge far below zero. For those species unable to avoid exposure to low temperature a process of cold-hardening must occur.

Cold-hardiness is the capacity of an organism to survive exposure to low temperature over extended periods lasting weeks or months (Hanec and Beck, 1960). Cold-hardening refers to the biochemical and physiological processes which result in enhanced low-temperature tolerance. The seasonal depression of the temperature at which spontaneous tissue freezing occurs, termed the supercooling point, and the accumulation of antifreeze compounds are generally associated with cold-hardening in insects intolerant of freezing.

Low molecular-weight antifreezes such as glycerol and trehalose function by depressing whole-body supercooling points and haemolymph melting points (Salt, 1961) and may stabilize enzyme function at low temperature (Hochachka and Somero, 1984). Their hydrophilic nature aids in binding water molecules and, thus, may function to prevent desiccation during the winter (Crowe and Clegg, 1973). The environmental factor that commonly triggers cold-hardening is exposure to low temperature (Baust, 1981); however, for some species photoperiodic cues also play a role (Horwath and Duman, 1982; Duman and Horwath, 1983).

Diapausing pupae of *S. crassipalpis* overwinter a few centimetres underground where they may experience sub-zero temperatures for extended periods. This is a freeze-susceptible species which does not tolerate tissue freezing at any stage of development (Lee and Denlinger, 1985). The diapause physiology of *Sarcophaga* has been examined extensively (see reviews by Denlinger, 1981 and Saunders, 1982), however, little information is available on the interrelationships between cold-hardiness and diapause.

To identify and characterize seasonal mechanisms of cold-hardening it is first necessary to understand baseline changes in cold tolerance that are inherent in the developmental programme. In this report we examine ontogenetic changes in cold tolerance for non-diapause and diapause-programmed stages of the flesh fly, *S. crassipalpis*. Secondly, the relationship between developmental patterns of glycerol production and cold-hardiness is defined.

MATERIALS AND METHODS

Insect rearing

The flesh fly, *Sarcophaga crassipalpis* Macquart, was cultured in the laboratory as described by Denlinger (1972). Approximately 80 larvae were reared in each 40 g pack of liver. Adults were reared at 25°C with either a nondiapause-inducing photoperiod (15 h light:9 h dark) or a diapause-inducing photoperiod (12 h light:12 h dark). Larvae and pupae were maintained at 20 or 25°C under the maternal photo-

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phase. Pharate adult development within the puparium was assessed by the criteria of Fraenkel and Hsiao (1968).

Developmental stages

At 20°C, larvae feed for about 7 days after larviposition at which time they leave the food and enter a postfeeding wandering stage for about 5 days. At the termination of wandering the larvae seek a dry site for pupariation. Pupation occurs within 4 days after pupariation. In the absence of diapause pharate-adult development begins immediately and is characterized by the migration of antennal discs on the third day, the appearance of red pigmentation in the eyes on the twelfth and black bristles on day 16. Adult emergence occurs on about day 20. Diapause occurs in the phanerocephalic stage of the pupa and may last more than 120 days at 20°C. Once diapause is terminated the time-course of development is the same as described for the non-diapausing flies.

Hexane treatment

Exposure to hexane may be used as a tool to initiate synchronous adult development in large groups of diapausing pupae (Denlinger *et al.*, 1980). In this study, pupae were placed in a glass desiccator and exposed to hexane vapours for 3 h. This treatment terminated diapause in all individuals.

Low temperature survival

Flies were exposed to low temperature in test tubes immersed in a refrigerated bath, Lauda RMT-20. Following exposure to low temperature flies were returned to the standard rearing conditions: only those individuals which successfully completed development and emerged as adults from the puparium were counted as survivors. Per cent emergence was usually based on 30–90 individuals, but never less than 20.

Glycerol determination

Glycerol levels were analyzed using high-performance liquid chromatography (Waters Associates) as described by Baust *et al.* (1983) and Lee *et al.* (1983). For each sample, two individuals were homogenized in 3 ml ethanol in a Teflon-glass tissue homogenizer. The homogenizer was rinsed with 2 ml methanol and the combined homogenate was centrifuged at 2000 *g* for 5 min. The pellet was reextracted with 3 ml methanol and centrifuged twice more. The combined supernatants were passed through a Sep-Pak C_{18} cartridge and evaporated to dryness. Prior to injection the sample was resuspended in 0.5 ml ethanol–water (1:1, v/v) and filtered through a 0.22 μm filter. Glycerol levels were determined using a radially compressed silica column modified with tetraethylenepentamine. Glycerol concentration was expressed in mM units based on fly water contents determined by Adedokun and Denlinger (1985). Each mean value is based on 3 replicate samples.

RESULTS

Ontogeny of cold tolerance

Nondiapause-destined feeding and wandering larvae exhibited minimal levels of cold tolerance as <10% survived to eclosion after 2 h exposure to −10°C (Fig. 1A). Cold-hardiness increased shortly after pupariation reaching a maximal level on day 10 and decreased sharply immediately prior to adult emergence. No adults survived 2 h at −10°C.

The greatest level of cold tolerance for diapause programmed flies was observed in pupae 12–35 days after pupariation (Fig. 1B). During the first 9 days after pupariation, diapausing pupae exposed to −17°C for 1 day were unable to survive until adult emergence, but rapidly increased hardiness by day 12 to 43%. Larvae and adults exhibited limited cold tolerance as compared to the pupal stage.

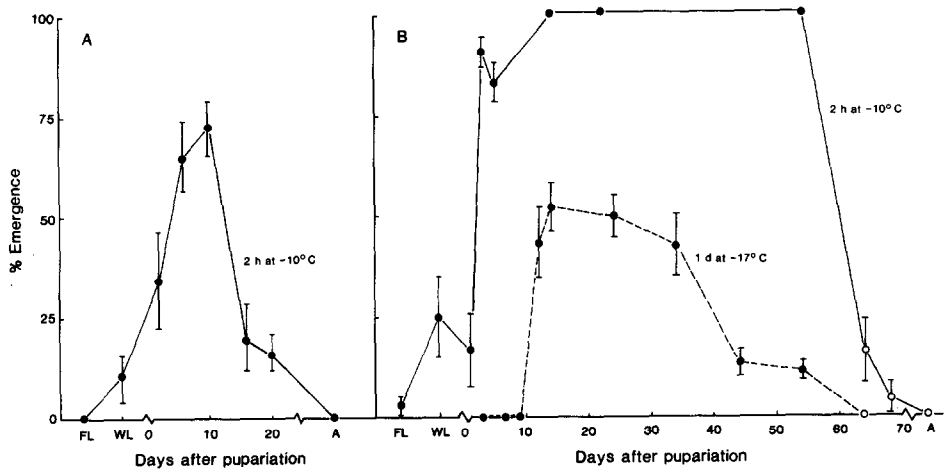

Fig. 1. Ontogeny of cold tolerance for non-diapause (A) and diapause (B) developmental stages of *Sarcophaga crassipalpis*. In Fig. 1B the closed circles represent pre-diapause and diapause stages, while open circles are post-diapause. Non-diapause flies were reared at 15 h light:9 h dark at 20°C, diapause-destined flies at 12 h light:12 h dark at 20°C. Cold tolerance was assessed by determining adult emergence from puparia after exposure to −10°C for 2 h or −17°C for 1 day. Developmental stages are represented by: FL = feeding larvae, WL = wandering larvae and A = adult. Pupal age is defined as days after pupariation.

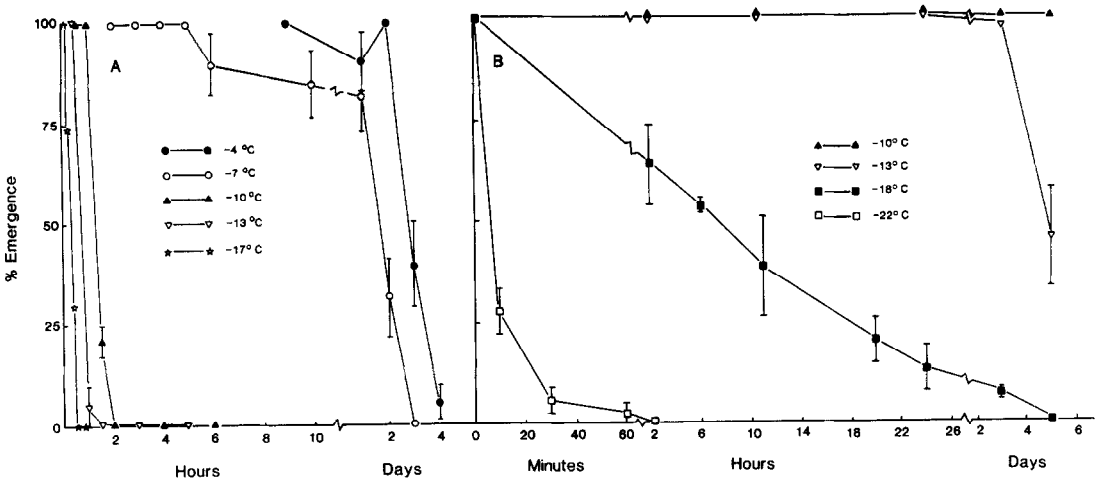

Fig. 2. Comparison of the effect of variable temperature and duration of exposure on cold tolerance of non-diapause (A) and diapause (B) stages of *Sarcophaga crassipalpis*. Non-diapause flies were reared at 15 h light:9 h dark at 25°C and exposed to low temperature during the red-eye stage of pharate-adult development. Diapause pupae were tested 40 days after pupariation.

An overall comparison of cold tolerance for non-diapause and diapause-programmed flies (Fig. 1A vs 1B) reveals: (1) the pupal stage exhibits the greatest level of cold-hardiness, although diapause pupae were more tolerant than nondiapause pupae and (2) maximal levels of pupal cold tolerance were not attained until 10 days after pupariation for both groups.

A comparative study of the limits of cold tolerance for nondiapausing and diapausing flies after exposure to a range of low temperatures for varying intervals are summarized in Fig. 2. Non-diapausing pharate adults in the red-eye stage of development were relatively intolerant of exposure to sub-zero temperatures. Fewer than 5% emerged after 4 days at −4°C, while no flies survived 2 h at −10°C or lower (Fig. 2A). In contrast, 40-diapausing pupae experienced no mortality after exposure to −10°C for 5 days.

Developmental patterns of glycerol production

Glycerol was the only low molecular weight polyhydric alcohol identified in relatively high concentrations (>1 mM) from tissue extracts of *S. crassipalpis*. Both the feeding and wandering phases of non-diapause-destined larvae had substantially higher levels of glycerol than those programmed for diapause (Fig. 3A vs B). Since the high levels of glycerol observed in nondiapause-destined larvae were unexpected, additional replicates of this stage were completed. Results were nearly identical with the data shown in Fig. 3A, thus confirming the observation of high glycerol levels in larvae that are not programmed for pupal diapause. For diapausing pupae glycerol levels rose steadily during the first 44 days after pupariation reaching a maximal value of 71 mM (Fig. 3B). Glycerol titres decreased rapidly at the termination of pupal diapause. A fundamental difference in the general pattern of glycerol prod-

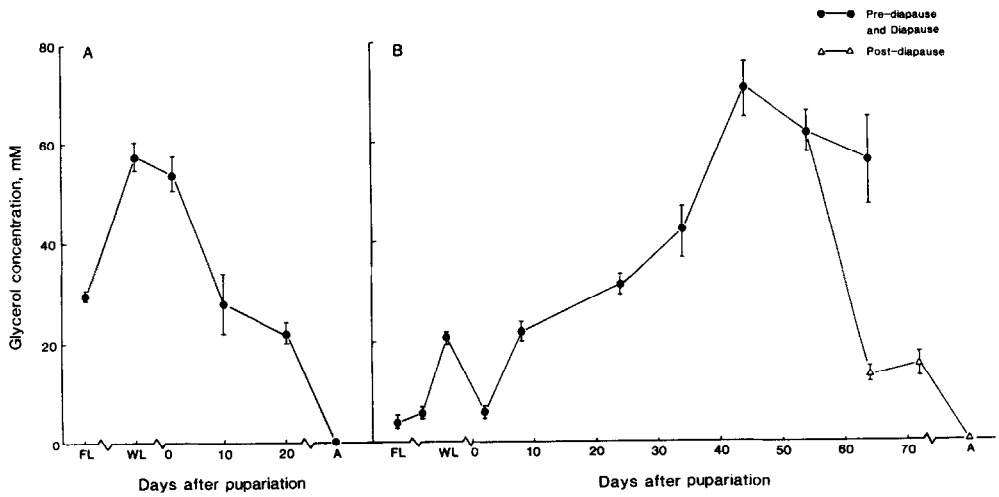

Fig. 3. Glycerol concentration in non-diapause (A) and diapause (B) developmental stages of *Sarcophaga crassipalpis*. Rearing conditions and abbreviations are described in Fig. 1.

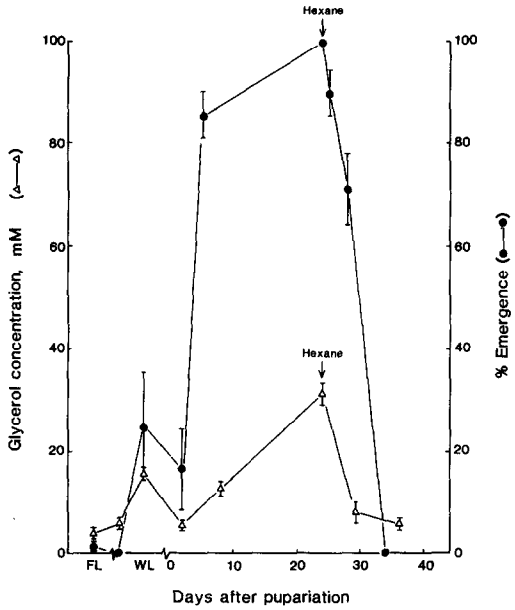

Fig. 4. Effect of diapause termination after hexane application on glycerol concentration and cold tolerance of 20-day old diapause pupae. Cold tolerance was assessed by determining adult emergence from puparia after exposure to −10°C for 2 h. All stages were reared at 12 h light:12 h dark and 20°C except for adults reared at 25°C.

uction was evident between the groups: for non-diapause-destined flies maximal levels were observed in wandering larvae, whereas, under the diapause programme it was during late pupal diapause that the highest levels were measured (Fig. 3).

Hexane-induced termination of pupal diapause was followed by marked reductions in cold-hardiness and glycerol levels (Fig. 4). These data are consistant with the data of Figs 1 and 3 in which diapause was terminated spontaneously.

DISCUSSION

The diapause pupa is the overwintering form of *S. crassipalpis*, and, as such, the development of cold tolerance in this stage is of particular importance. In an earlier study we provided evidence for a two-step mechanism of cold-hardening in diapause pupae (Lee and Denlinger, 1985). Supercooling points for wandering larvae are near −11°C. This value decreases to −23°C during the first 10 days after pupariation. Although a reduction in the supercooling point is necessary for enhanced cold tolerance in a freeze-susceptible species, it alone is not sufficient to explain the process of cold-hardening in diapause pupae. Only for pupae aged 20 days or older does the lower lethal temperature approach the supercooling point.

Previously we had postulated a second unknown mechanism of cold-hardening related to the aging of diapause pupae (Lee and Denlinger, 1985). At least a partial explanation for such a mechanism is suggested by the dynamics of glycerol synthesis early in pupal diapause (Fig. 3B). From day 8 to day 44 glycerol levels increase more than 5-fold from 13 to 71 mM. During this same interval the proportion of flies emerging after exposure to −10°C for 2 h increased from <20% to >80% (Fig. 1B).

It is well known that the addition of low-molecular-weight polyols and sugars to water increases its supercooling capacity, however, in the present study changes in whole-body supercooling points were unrelated to patterns of glycerol production. In both non-diapause and diapause-destined flies the transition from larvae to pupae is characterized by an abrupt decrease in the supercooling point from −7°C to −23°C (Lee and Denlinger, 1985). However, during this same interval glycerol levels fall, not rise, in both groups. Furthermore, the supercooling point for diapause pupae remains constant from 20–110 days after pupariation (Lee and Denlinger, 1985) even though glycerol levels more than double from day 20 to day 40 (Fig. 3B).

The environmental stimulus responsible for the induction and accumulation of polyols varies among species (Steele, 1981). In some insects exposure to low temperature directly triggers the accumulation of polyhydric alcohols and is independent of the diapause programme (Baust and Miller, 1972; Wood and Nordin, 1976; Ring, 1980; Duman and Horwath, 1983; Nordin *et al.*, 1984). However, polyol accumulation in the goldenrod gall fly, *Eurosta solidaginis*, is more complex: maximal rates of sorbitol synthesis occur at 0–5°C, while glycerol accumulation is independent of temperature (Baust and Lee, 1982; Rojas *et al.*, 1983; Storey, 1982). At the other extreme, glycerol and sorbitol production are firmly linked to the diapause state and accumulation occurs at high constant temperatures (Tsumuki and Kanehisa, 1978).

The complexity of the regulatory process for antifreeze accumulation is evidenced by a synergistic interaction between diapause and low-temperature acclimation. Transfer of diapausing pupae of *Pieris bassicae* from +23°C to +4°C further elevates trehalose levels (Moreau *et al.*, 1981). A similar pattern of change has been found for glycerol and sorbitol in larvae of *Isia isabella* (Mansingh and Smallman, 1972) and for glycerol in the silkworm, *Bombyx mori* (Ziegler and Wyatt, 1975).

Glycerol is the primary polyol synthesized by *S. crassipalpis* (Fig. 3). Although the specific patterns of production differ between diapause and nondiapause groups, it is clear that this species is able to accumulate glycerol even when reared continuously at high temperatures, 20 or 25°C. The fact that the greatest levels of accumulation were found in diapausing pupae suggest that glycerol accumulation is one facet of the overall diapause syndrome for this species (Fig. 3B).

Glycogen catabolism provides the carbon source for the synthesis of sorbitol and glycerol (Mansingh and Smallman, 1972; Ziegler and Wyatt, 1975; Storey *et al.*, 1981). The termination of diapause is characterized by the rapid disappearance of polyols and their resynthesis to glycogen (Mansingh and Smallman, 1972; Steele, 1981).

A similar pattern of interconversion between glycogen and glycerol is likely for diapause pupae of *S. crassipalpis*. Adedokun and Denlinger (1985) found that glycogen decreases to its lowest level in mid-diapause and then rises during pharate-adult devel-

opment: the present study identified complementary changes in glycerol titres (Fig. 3B). During the first 6 weeks of pupal diapause glycerol levels increased steadily followed by an abrupt decrease at the end of diapause.

The ontogeny of cold tolerance correlates closely with the pattern of glycerol synthesis identified in this study: stages with high levels of glycerol exhibited greater cold tolerance. Pharate adults and adult flies are relatively intolerant of the cold and contain little glycerol (Figs 1 and 3). During the first few days after pupariation diapausing pupae are relatively intolerant of low-temperature exposure and correspondingly have low levels of glycerol (Figs 1B and 3B). Cold tolerance increases concomitantly with glycerol titres during the first 30–40 days of pupal diapause. The abrupt decrease in cold tolerance after hexane-induced termination of diapause was closely matched with a loss in glycerol (Fig. 4). In one instance, however, the link between enchanced cold tolerance and the accumulation of glycerol is broken: nondiapause larvae accumulate substantial amounts of glycerol (Fig. 3), but are less cold tolerant than diapause-destined larvae which do not accumulate glycerol (Adedokun and Denlinger, 1984).

The enhancement of supercooling capacity to −23°C appears to be a property intrinsic to entering the pupal stage for both nondiapause and diapause-programmed flies (Lee and Denlinger, 1985). We suggested that this capacity may represent a "preadaptation" which facilitated the dispersal of *Sarcophaga* from tropical to temperate regions (Lee and Denlinger, 1985). In this study we find that nondiapause-destined wandering larvae and pupae contain high levels of glycerol (Fig. 3A). Though the reason for this is not apparent, the capacity for even nondiapausing flies to accumulate glycerol may have conferred enhanced low-temperature tolerance which, in turn, promoted dispersal to colder climates.

Acknowledgements—This research was supported in part by a National Science Foundation grant, DCB-8517875, to R.E.L. and grant no. 8300051 from USDA-CRGO to D.L.D.

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