Cold tolerance in diapausing and non-diapau sing stages of the flesh fly, Sarcophaga crassipalpis

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ABSTRACT. Supercooling points (SCP) and low temperature tolerance were determined for larval, pupal and adult stages of Sarcophaga crassipalpis Macquart (Diptera: Sarcophagidae). No stage tolerates tissue-freezing. Ontogenetic changes in SCP profiles are similar for comparable developmental stages of diapause and non-diapause groups. Feeding larvae have SCPs near -7°C which decrease to -11°C in the postfeeding wandering phase of the final larval instar. The lowest SCPs are recorded for pupae at -23°C. The capacity to survive at -17°C varies with age of the diapausing pupae: 10-day-old pupae are less cold tolerant than pupae that have been in diapause for 45–80 days. Although the SCP of non-diapausing pupae is as low as in diapausing pupae, non-diapausing pupae are extremely sensitive to low temperature exposure and do not survive to adult eclosion when exposed to -17°C for as little as 20 min. The use of hexane to break pupal diapause has no effect on SCPs or low temperature tolerance.

Key words. Cold-hardiness, pupal diapause, supercooling point, flesh flies, Sarcophaga crassipalpis.

Introduction

The supercooling point (SCP) represents the temperature at which spontaneous tissue freezing occurs. Seasonal enhancement of the capacity of body fluids to supercool represents the primary mechanism by which freeze-intolerant species cold-harden. Experimentally, the SCP is determined by cooling at a rate of c. 1°C/min until the exotherm associated with the latent heat of fusion is detected (Salt, 1961). Somme (1982) summarizes factors known to effect the SCP, including contact moisture, feeding stages, water content, cryoprotectant levels and ice nucleating agents.

For freeze-intolerant species the SCP represents the absolute limit of low temperature tolerance. However, its applicability as an indicator of survival under field conditions is less clear. Many freeze-intolerant insects survive long periods at temperatures a few degrees above the SCP as long as tissue freezing does not occur. Other insects die at temperatures above the SCP (Bursell, 1970; Turnock et al., 1983). Flesh flies (Sarcophaga) overwinter as diapausing pupae a few centimetres...
underground (Denlinger, 1981) where they are normally exposed to sub-zero temperatures for many months. In S. crassipalpis Macquart, cold hardiness appears to be a component of the diapause syndrome (Adeodokun & Denlinger, 1984): diapause-destined larvae tolerate exposure to $-10^\circ$C much better than non-diapause-destined larvae, and diapausing pupae are consistently more tolerant of low temperature than non-diapausing pupae.

This conspicuous difference in cold hardiness between diapausing and non-diapausing pupae provides an opportunity to investigate relationships between the SCP, diapause and cold tolerance. In this report we describe relative levels of cold tolerance for larval, pupal and adult stages of S. crassipalpis reared under diapause (short photophase) and non-diapause (long photophase) conditions. The experiments described here assess the validity of using SCPs as a measure of cold-hardiness, and we evaluate the effect of hexane, a tool used to break diapause (Denlinger et al., 1980), on SCPs and cold tolerance.

Materials and Methods

Insect rearing

A colony of S. crassipalpis originating from Champaign County, Illinois, was maintained in the laboratory as described by Denlinger (1972a). Adults were kept at 25°C with a daily light:dark cycle of either LD 15:9 h or LD 12:12 h. To avert pupal diapause, larvae collected from LD 15:9 h adults were reared at LD 15:9 h and 20°C. Groups with a high incidence of pupal diapause (>95%) were collected from LD 12:12 h adults and reared as larvae at LD 12:12 h and 20°C. Diapausing and non-diapausing pupae, as well as pharate adults, were held at 20°C. The developmental status of each pupae was checked by removing the anterior portion of the puparium to look for signs of antenna formation and the pigmentation characteristic of pharate adult development (Fraenkel & Hsiao, 1966).

Developmental stages

At 20°C, larvae of S. crassipalpis feed for c. 7 days and then, as fully grown third instar larvae, leave the food and enter a postfeeding wandering phase which lasts c. 5 days. At the onset of the wandering phase, larvae evacuate their gut and seek a dry site for pupariation. Within 4 days of pupariation, the fly has pupated within the puparium and everted its head to complete the transformation to a phaenocerephalic pupa. If diapause intercedes, development is halted at this stage. If development progresses without diapause, pharate adult development is initiated immediately; migration of antennal discs is conspicuous 3 days after pupation. Red pigmentation can be detected in the eyes by day 12, and black bristles are apparent on day 16. Adults emerge c. day 20. Diapause can be maintained at 20°C for over 120 days, but many pupae break diapause spontaneously after 80 days. Once diapause is terminated, the developmental time-table for pharate adult development is identical to that observed in non-diapausing flies.

Hexane treatment

To initiate adult development synchronously in large groups of 20-day-old diapausing pupae, intact puparia were submerged in a hexane bath for 45 min as described by Denlinger et al. (1980). This procedure elicits development in almost 100% of diapause pupae. After the hexane bath, the anterior cap of each puparium was removed to inspect the pupae. Any dead individuals were eliminated from experimental samples.

Supercooling point

The SCPs were determined by positioning a 30 gauge copper-constantan thermocouple in contact with the insect cuticle. A cooling rate of c. 1°C/min was maintained using a Neslab RE-8DD low temperature bath. The SCP was recorded as the lowest temperature recorded prior to the release of the latent heat of fusion as body water freezes.

Low temperature survival

An insulated styrofoam box containing 95% ethanol was fitted with a test tube rack and placed in a freezer. When the temperature of the bath stabilized at $-17^\circ$C, glass test tubes containing fly samples were submerged for various periods of time. Following exposure to $-17^\circ$C,
the pupae were held at 25°C and their developmental fate recorded.

**Results**

Tissue freezing was lethal for larval, pupal and adult stages of flies reared under either long photophase (non-diapause) or short photophase (diapause). Freezing for only a few minutes at the temperature equal to the SCP value was lethal for all individuals regardless of stage or diapause status.

Ontogenetic changes in SCP values for the diapause group are summarized in Fig. 1. Feeding larvae had the highest values near -7°C. Concomitantly with the cessation of feeding SCPs decreased to -11°C for wandering larvae. After pupariation, the SCPs decreased precipitously and continued to do so during the first 10 days of diapause, reaching a supercooling limit near -23°C. Values remained at this level for at least 110 days, the duration of pupal diapause. Newly emerged adults had a mean SCP of -11.0±0.6°C.

A comparison of the long and short photophase groups (Fig. 1 v. Fig. 2) revealed a close correlation in SCP values for each developmental stage. Feeding larvae had the highest values, decreasing as the wandering phase began and dropping sharply to a lower limit of -23°C after pupariation. As was previously observed in diapause pupae, the pupal-adult transition was characterized by an increase in the SCP to -8.9±1.2°C.

To test the effect of hexane treatment on supercooling capacity, SCPs were determined for diapausing pupae at varying intervals after hexane treatment (Fig. 3). The first morphological signs of adult development, the migration of the antennal discs, can be detected 3 days after hexane treatment. However, for the first 12 days post-hexane treatment SCPs remained unchanged near -23°C, a value virtually identical to the supercooling limit for untreated diapausing pupae (Fig. 1). Beginning on day 12, during the red-eye stage of pharate adult development, SCPs began to increase, reaching -12°C 3 days later, as development progressed to the black-bristle stage. The same pattern of SCP change was observed during pharate adult development for untreated diapausing and non-diapausing groups (Figs. 1 and 2).

A second series of experiments was performed to determine whether the SCP was a
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FIG. 2. Ontogenetic profile of supercooling point values (SCP) for Sarcophaga crassipalpis reared at LD 15:9 h and 20°C (non-diapause programme). (±SEM, n=eight to ten individuals).

valid indicator of low temperature tolerance. The relative survival of diapausing pupae of different ages ranging from 10 to 80 days was determined after exposure to −17°C for 1 or 7 days (Fig. 4). Survival to adult eclosion was high (>85%) for pupae of 45–80 days in age. For younger pupae, low temperature exposure to −17°C produced high mortality: only 25% of 10-day pupae survived 7 days of exposure to −17°C. 10-day-old pupae exposed to −17°C for 3 days had a survival rate of 53% which falls between the survival rates for 1 and 7 days of exposure to −17°C (Fig. 4). The relationship indicates that the rate of survival for 10-day pupae at −17°C is a function of the duration of exposure.

Non-diapasing pupae were extremely sensitive to low temperature exposure compared with

FIG. 3. Supercooling point values at varying intervals after hexane application to diapausing pupae of Sarcophaga crassipalpis. The letters represent the onset of stages in pharate adult development: A = presence of antennal discs, R = red-eye stage and B = black-bristle stage. (±SEM, n=eight to ten individuals).

FIG. 4. Survival to the adult stage for diapausing pupae of varying ages exposed to −17°C for 1 or 7 days. Each point represents relative survival for a group of fifteen individuals.

FIG. 5. Survival to completion of pharate adult development and adult eclosion for non-diapasing pupae exposed to −17°C for varying periods of time. Each point represents relative survival for a group of fifteen individuals.
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FIG. 6. Survival to completion of pharate adult development and adult eclosion after 1 day of exposure to −17°C at different intervals after hexane application. Each point represents the relative survival for a group of fifteen individuals.

diapause (Fig. 5). 10 h of exposure to −17°C was sufficient to prevent any individual surviving beyond the pharate adult stage. Between 2 and 4 h the proportion of flies surviving to the pharate adult stage dropped below 50%, although none of these individuals was able to emerge from the puparium. Remarkably, a 20 min exposure of pupae to low temperature prevented adult eclosion, despite the fact that tissue freezing does not occur until −23°C in these individuals.

To determine the cold tolerance of diapausing pupae after treatment with hexane, pupae were held at −17°C for 1 day at varying intervals after hexane application (Fig. 6). Most pupae transferred to −17°C within 14 h of hexane treatment survived to become pharate adults and successfully eclosed. From 14 to 24 h post-hexane treatment the eclosion rate declined to zero, though 40% of the individuals survived to complete pharate adult development. No pupae subjected to −17°C for 4 or more days after hexane treatment were capable of completing pharate adult development.

Discussion

In freeze-intolerant species the SCP is generally regarded as the lower limit of survival and, as such, is commonly used as a convenient measure of insect cold-hardiness (Somme, 1982). In S. crassipalpis diapausing pupae ranging in age from 10 to 110 days have SCPs which remain constant near −23°C (Fig. 1). However, these uniformly low SCPs do not correspond to uniform levels of cold-hardiness throughout the duration of pupal diapause. Exposure of 10-day pupae to −17°C for 7 days produces substantially greater mortality, near 75%, as compared to pupae aged 20–80 days which experienced less than 15% mortality (Fig. 4). Only during the latter half of pupal diapause does the relatively low SCP of −23°C correspond with a period of enhanced low temperature tolerance.

Two distinct processes related to cold-hardening occur within 10 days of pupariation (Figs. 1 and 4). Wandering larvae, the stage which immediately precedes pupariation (Figs. 1 and 4). Wandering larvae, the stage which immediately precedes pupariation, have relatively high SCPs, suggesting that they contain an unidentified agent or agents which serve as efficient 'seeds' for heterogeneous ice nucleation within the body tissues. In order to increase cold-hardiness by extending the capacity for the supercooling of body fluids, these agents must be removed or their nucleating effect 'masked' during the first 10 days of pupal diapause. Similar processes have been reported in various species (Baust & Morrissey, 1975; Somme, 1982; Baust & Zachariassen, 1983). This reduction in the SCP values, though required for increased cold tolerance in a freeze-intolerant
species, is insufficient to explain the total process of cold-hardening in *S. crassipalpis*.

Clearly, these ice nucleating substances differ from the ice nucleating agents which are specifically synthesized during cold-hardening in freeze-tolerant forms and function to induce nucleation at relatively high sub-zero temperatures (Zachariassen & Hammel, 1976; Duman & Patterson, 1978; Lee et al., 1981). Rather, in freeze-intolerant species such as *S. crassipalpis* these agents probably function in some other capacity.

Although SCPs remain constant during pupal diapause, the presence of a second cold-hardening process is evident by the progressive enhancement of cold tolerance with the ageing of the diapausing pupae, but the nature of this second mechanism is unknown.

For non-diapausing pupae the SCP is a poor indicator of low temperature tolerance. Adedokun & Denlinger (1984) observed a similar difference in cold tolerance between diapause- and non-diapause-destined third instar larvae of *S. crassipalpis*. Diapause-destined larvae are more successful in pupariating after exposure to −10°C than non-diapause-destined larvae of the same age. Since in the present study both diapause- and non-diapause-destined larvae have SCPs near −10°C, the SCP is of little value in assessing the extent of cold tolerance in this developmental stage.

Organic solvents are capable of breaking diapause in a variety of insects (Pepper, 1937; Nishiitsutsuji-Uwo & Nishimura, 1972; Zdarek & Denlinger, 1975). Previous work with *S. crassipalpis* demonstrated that topical application of hexane broke pupal diapause and produced no apparent deleterious effects (Denlinger et al., 1980). Shortly after hexane treatment, a pulse of juvenile hormone is released and the ecdysteroid titres rise (Walker & Denlinger, 1980). These hormonal events initiate pharate adult development, and within 3 days migration of the antennal discs is readily visible through the cuticle. However, our results suggest that hexane treatment does not alter development processes related to cold-hardiness.

Under natural conditions at 40°N, overwintering flesh fly pupae initiate pharate adult development in late winter. Antennal discs are not visible in the field until early April, the red-eye stage is reached in late April, and adults emerge in mid-May (Denlinger, 1972b). From comparisons with our developmental profile acquired in laboratory experiments, we would predict that the overwintering pupae would retain a high level of cold tolerance until early April, and SCPs would remain low until early May when exposure to low temperature is no longer likely.

In both diapausing and non-diapausing flies the transition from the larval to the pupal stage is characterized by the presence of two step-functions within the SCP profile. Presumably, each step corresponds to the presence of a distinct class of ice nucleating agents of which the most efficient nucleator within the organism will determine the limit of supercooling (Salt, 1966; Zachariassen, 1982).

The first step is evidenced by a minor decrease in the SCP from −7°C for feeding larvae to −11°C for postfeeding, wandering larvae (Figs. 1 and 2). The influence of gut contents on the SCP has been commonly reported (Salt, 1961; Block et al., 1978). Ring (1972) observed a similar decrease in SCPs with the cessation of feeding in larval blowflies, *Lucilia sericata*. Since wandering larvae purge their gut within 12 h of the cessation of feeding, this step in the profile may represent the removal of one class of highly efficient nucleating agents within the gut. A second class of nucleating agents is responsible for the plateau in SCPs at −10°C. These are removed or 'masked' shortly after pupariation. Since the resulting enhancement of supercooling capacity to −23°C occurs in both diapausing and non-diapausing pupae, it appears to be a phenomenon intrinsic to the pupal stage. As such, it may represent a 'preadaptation' which facilitated the evolution of cold-hardiness and the dispersal of *Sarcophaga* from tropical to temperate regions (Rohdendorf, 1974). The capacity for extended supercooling of body fluids is required for increased cold tolerance in a freeze-intolerant species. Having already acquired the capacity for extensive supercooling the flesh fly pupa was the likely developmental stage to acquire additional physiological and biochemical mechanisms to enhance low temperature tolerance.

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