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Seasonal variation in generation time, diapause and cold hardiness in a central Ohio population of the flesh fly, *Sarcophaga bullata*

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Abstract. 1. Generation time, diapause phenology and cold tolerance of the flesh fly, *Sarcophaga bullata*, were examined under confined natural conditions in central Ohio. In this locality, the fly can complete a maximum of four generations annually.

2. Very few pupae entered diapause in the first and second generations (May to July in 1988). In the third generation (August) 37% of the pupae entered an overwintering diapause, as did all pupae from the fourth generation (September).

3. The adult eclosion date in the spring and annual generation time can be predicted accurately from degree day data.

4. Cold tolerance of the field-overwintering portion of the population was high. After 30 days under field conditions, diapausing pupae readily survived a 7-day exposure to −17°C. Glycerol appears to be the major cryoprotectant in *S.bullata*, and glycerol concentrations in the field population (95–142 mM) remained high throughout the winter.

5. In contrast, diapausing flies reared under laboratory conditions (20°C, 12:12 LD) were less cold tolerant, and glycerol concentrations were lower (6.9–21.2 mM). Field conditions thus promote the acquisition of high levels of cold tolerance, presumably as a consequence of the accumulation of higher concentrations of glycerol.

6. In spite of differences in the cold tolerance of laboratory and field flies, the supercooling points of the two groups of flies were nearly the same, thus implying that the supercooling point is not a good indicator of cold tolerance.

Key words. Generation time, diapause, cold-hardiness, glycerol, flesh fly, *Sarcophaga bullata*.

Introduction

A fairly comprehensive overview of diapause and cold-hardiness has been generated from studies on flesh flies (Sarcophagidae). This large cosmopolitan group has an overwintering pupal diapause over much of its range (Denlinger, 1981; Kurahashi & Ohtaki, 1989), and even in tropical regions of Africa flesh flies enter diapause during the cool months of the year

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(Denlinger, 1978). Many aspects of environmental and hormonal regulation (Fraenkel & Hsiao, 1968a; Denlinger, 1981; Richards & Saunders, 1987), clock function (Saunders, 1982; Gnagey & Denlinger, 1984), and the genetics of flesh fly diapause (Henrich & Denlinger, 1982; Kurahashi & Ohtaki, 1977) have been investigated, and recent work has provided considerable information about the response of diapausing flesh fly pupae to low temperature (Lee & Denlinger, 1985; Lee *et al.*, 1987). Laboratory studies indicate that the flesh flies are freeze-intolerant insects, and though the pupal supercooling points are quite low (around −22°C), pupae die at temperatures well above their supercooling point. In response to low temperature, glycogen phosphorylase in flesh flies is activated (Chen & Denlinger, 1990), which in turn elevates the haemolymph concentration of glycerol and thus increases cold tolerance (Chen *et al.*, 1987a; Lee *et al.*, 1988). Most work on flesh fly diapause and cold tolerance, however, has been done in the laboratory, and few previous studies have been carried out under more natural conditions. The seasonal incidence of diapause and the phenology of diapause development have been described for a population of *Sarcophaga bullata* reared under field conditions in Illinois (Denlinger, 1972b), and diapause incidence and generation time for two African species have been reported for field conditions (Denlinger, 1978), but we have no information about generation time or cold tolerance for temperate zone populations reared under natural conditions.

In this study conducted in central Ohio, we observed cold tolerance, developmental and diapause phenology in a flesh fly, *S.bullata*, reared in confined conditions in the field throughout the year and compare these results with those obtained from flies reared in the laboratory. We previously calculated the thermal constant threshold and thermal constant, i.e. degree days (DD), for development and generation time in a closely related flesh fly, *Sarcophaga crassipalpis* (Chen *et al.*, 1987b). Are these data reliable predictors of generation time in nature, and can the laboratory data be used to accurately predict adult emergence in the spring? Laboratory experiments have defined the roles of photoperiod and temperature in regulating diapause, but how well does the information correlate with field results? In the laboratory, diapausing pupae clearly have higher cold tolerance than nondiapausing flies, and glycerol, the major cryoprotectant in flesh flies, accumulates in response to low temperature (Lee *et al.*, 1987; Chen *et al.*, 1987b), but the response of pupae to the more severe low temperature conditions that may prevail in the field is unknown. The field observations completed in this study permit us to appraise the validity of our laboratory-derived concepts of diapause and cold hardiness in flesh flies.

Materials and Methods

Fly culture. A colony of the flesh fly, *Sarcophaga bullata*, collected in Franklin County, Ohio, was maintained in the laboratory for 2 years under nondiapausing conditions (LD 15:9, 25°C) before the initiation of this experiment. Under short-day conditions (LD 12:12, 20°C) 96.8% ($N = 277$) of the laboratory flies entered pupal diapause. Larvae and adults were maintained in the field (northern Franklin County) using the rearing methods previously described for laboratory flies (Denlinger, 1972a), and pupae were buried 5−8 cm underground. Flies were additionally protected by a large screened cage to prevent disturbance from other animals. Field experiments were carried out between August 1987 and May 1989. Generation time was defined as the interval from larviposition to larviposition of the next generation, and diapause incidence was determined by removing the anterior portion of the puparium and evaluating the developmental status of the pupa, as described by Fraenkel & Hsiao (1968b).

Temperature record and predictions of development time. Temperature data were obtained from the weather station of the Ohio State University, located a few kilometres from the field site. Spring emergence and generation time were predicted using thermal constant threshold and thermal constant (degree days) data obtained from a closely related temperate zone species, *S.crassipalpis*, reared under constant temperature conditions in the laboratory (Chen *et al.*, 1987b). Developmental time for the nondiapausing generations was estimated from the DD requirement for a whole generation, using a thermal constant of 12°C, the thermal constant for a whole generation. The prediction of spring emergence was based on the DD requirement

for development from the initiation of pharate adult development to adult eclosion, using the thermal constant for that interval (10°C).

Cold tolerance. Overwintering pupae were sampled monthly to evaluate seasonal changes in cold tolerance and glycerol concentrations. An insulated styrofoam box containing 95% ethanol was fitted with a test tube rack and placed in a freezer. When the bath temperature stabilized at −17°C, glass test tubes (10×1.5 cm) containing fly samples were submerged for various periods of time. Following exposure to −17°C, pupae were held at 25°C, and their developmental fate as well as emergence percentage were recorded. Each temperature treatment consisted of three replicates of fifteen pupae.

Cryoprotectant determination. Low molecular weight polyols were analysed by high-performance liquid chromatography (Waters Associates) as described previously (Lee *et al.*, 1983; Chen *et al.*, 1987a). Glycerol concentrations were expressed in mM units based on water-content data for the corresponding developmental stage of the same species.

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

Results

Generation time and seasonal change of diapause incidence

A maximum of four generations were completed for *S.bullata* under natural conditions between May 1988 and May 1989 (Fig. 1). The overwintering generation initiated pharate adult development late in March (first signs of development were detected in 15% of the flies on 25 March, 1988, and in all flies by 7 April) and emerged in mid-May. The overwintering flies deposited their first batch of larvae in late May and none of them entered diapause as pupae. Generation time was 39 days. Two additional generations were completed between early July and early September, requiring 30 and 31 days

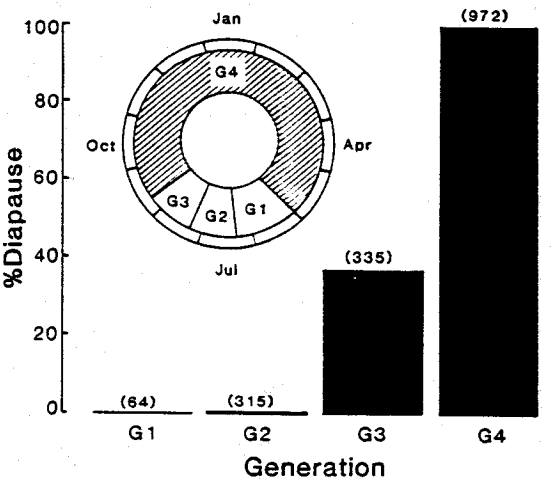

Fig. 1. Generation time and diapause incidence (May 1988 to May 1989) of the field flies reared under confined natural conditions in central Ohio. Numbers in parentheses are sample sizes.

respectively. Very few flies (0.3%) of the second generation entered diapause, but 37.3% of the third generation entered diapause for the winter. Larvae of the fourth generation, which were deposited in early September, pupariated in late September, and all entered diapause and emerged as adults the following spring (generation time, 265 days).

Predictions of spring emergence and the annual generation number

The spring emergence time of the overwintering generation could be accurately predicted based on our previous thermal constant threshold and thermal constant data for a congeneric species, *S.crassipalpis* (Chen *et al.*, 1987b). For nondiapausing flies, a total of 191.3 DD is required for development from pupariation to adult emergence, and the interval from pupariation to the onset of adult development utilizes 49.6% of the total interval, thus the remainder (96.4 DD) should predict the DD requirement for the interval between termination of diapause (onset of pharate adult development) and eclosion. Based on field cumulative average soil temperatures, adult emergence was thus predicted for 12–13 May and the adult flies actually emerged on 11 May (50% emergence).

The DD for completion of a nondiapausing generation of *S.crassipalpis* is 369 (Chen *et al.*, 1987b). In our field experiment, the total DD for the interval from spring emergence (11 May) to the end of the third generation (24 August) is 1064, which should permit completion of three non-diapausing generations with 355 DD each. This result is thus fairly close to the predicted value. The field observations and calculated predictions indicate that *S.bullata* can complete up to four generations a year in central Ohio, but a portion of the population, those that enter diapause in July or August, may complete only two or three generations.

Cold tolerance and glycerol production of field flies

Daily temperatures fluctuate dramatically throughout the year, and during our experimental season ambient air temperature ranged from a winter minimum of −20°C to a summer maximum of 40°C. Cold tolerance and glycerol concentrations of the overwintering pupae were examined at different times throughout the winter season. Early in diapause (before November) fewer than 50% of the pupae survived a 1 or 4 day exposure to −17°C. Pupae sampled from November to February (45−160 days in diapause) appeared to be the most cold tolerant: more than 60% survived a 1 or 4 day exposure to −17°C (Fig. 2A). By late March, diapause was broken and the post-diapause pharate adults were unable to tolerate a 2 h exposure to −17°C (0% survival), but they were still capable of tolerating a 4 h exposure to −10°C (55% and 43% survival for early and late pharate adults, respectively).

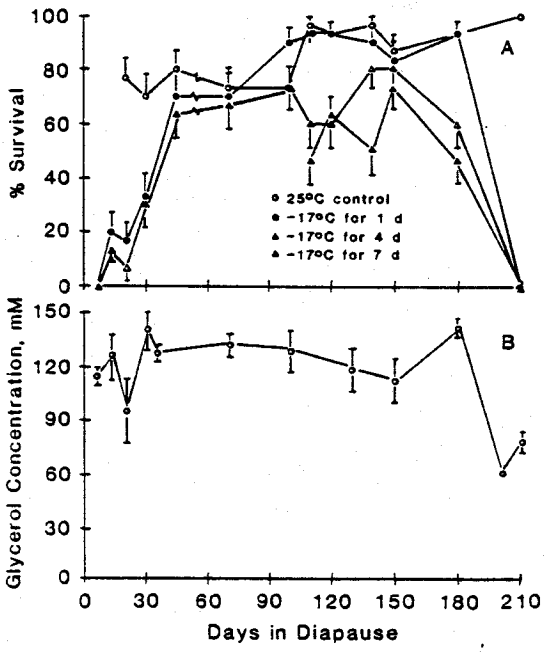

Fig. 2. Cold tolerance (A) and glycerol concentrations (B) of field-reared diapausing pupae. Throughout the winter, samples of pupae were exposed to −17°C for various days, and then held in the laboratory at 25°C. In pupae younger than 45 days, per cent survival was based on the number of flies developing to the black bristle stage (late pharate adult), and thereafter survival was recorded as the percentage of flies that successfully emerged. $\bar{X} \pm SD\ (\sqrt{pq/N})$, per cent survival to adults ($N = 45$) and $\bar{X} \pm SE$, mM glycerol (three replicates of two each).

As in *S.crassipalpis* (Lee *et al.*, 1987), glycerol appears to be the major low molecular weight cryoprotectant in this species. Field diapausing pupae had a relatively constant high level of glycerol (around 120 mM) during the whole diapause season, from late September to early March (Fig. 2B). Glycerol concentrations decreased rapidly (61−78 mM) in March when diapause was broken and pharate adult development began. This marked decrease in glycerol correlated with a reduction in cold-hardiness.

Cold tolerance and glycerol production in laboratory reared pupae

Pupae reared in the laboratory at LD 12:12, 20°C remained in diapause about 150 days, and were less cold tolerant than pupae reared in natural conditions (Fig. 3A). Even in mid-diapause fewer than 30% of the diapausing pupae from the laboratory survived a 4-day exposure to −17°C. Again, the pupae were less cold tolerant in early and late diapause. Likewise, glycerol levels were much lower for laboratory reared diapausing pupae (Fig. 3B) than in the pupae reared outside (Fig. 2B).

Cold tolerance of nondiapausing pupae

To examine survival ability of nondiapausing pupae under winter conditions, nondiapausing pupae (3 days after pupariation) were transferred from the laboratory (LD 15:9, 25°C) to our outside experimental site on 7 January 1988. A 2-day exposure to the prevailing winter con-

Table 1. Supercooling points for overwintering pupae in diapause and nondiapausing laboratory-reared pupae of *S.bullata* $\bar{X} \pm$ SE of five replicates. Significant differences were found for the means by one-way ANOVA test ($F = 18.48$, $P < 0.001$) analysed by GLM Procedure, SAS System.

Sampling data	Days after pupariation	Supercooling point (°C)
Diapausing pupae (field conditions)		
13 September 1987	6	−20.7±0.1
22 September 1987	13	−21.2±0.1
12 February 1988	150	−21.9±0.1
Nondiapausing pupae (laboratory conditions)	10	−21.9±0.1

ditions killed almost 80% of the nondiapausing pupae (Fig. 4B). During this 2-day interval daily soil temperature ranged from −4°C to −10°C (Fig. 4A). Winter air minimum temperatures in central Ohio often drop below −15°C (data not shown), thus it is unlikely that any flesh flies could survive winter without entering diapause.

Supercooling point

SCPs were determined for pupae from both the field and laboratory (Table 1). SCPs ranged from −20.7 to −21.9°C for field reared pupae at different ages. Though these slight differences

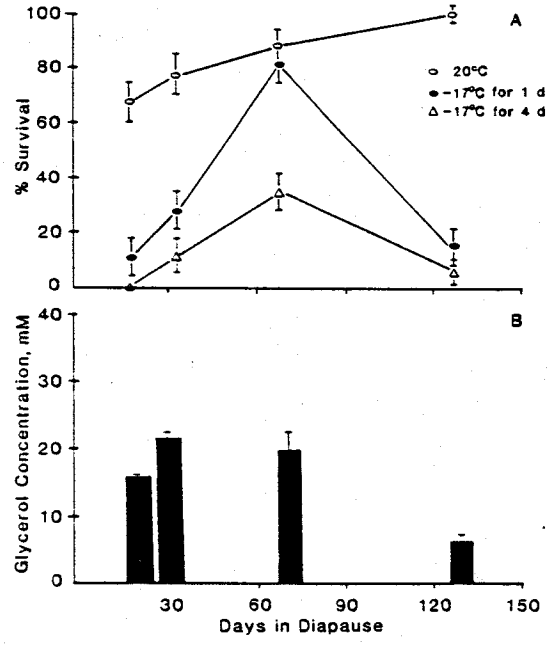

Fig. 3. Cold tolerance (A) and glycerol concentrations (B) of laboratory-reared diapausing pupae (LD 12:12, 20°C). Samples were exposed to −17°C for 1 or 4 days and then held at 25°C. $\bar{X} \pm$ SD ($\sqrt{pq/N}$), per cent survival to adult emergence ($N = 45$) and $\bar{X} \pm$ SE, mM glycerol (three replicates of two each).

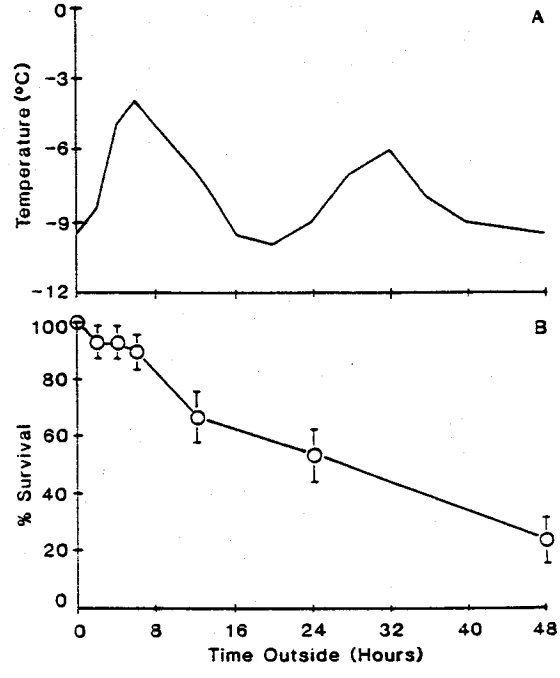

Fig. 4. Nondiapausing pupae (3 days after pupariation) reared in the laboratory (LD 15:9, 25°C) were transferred to the field experimental site on 7 January 1988 to examine survivability under winter conditions. (A) Soil temperatures (2–3 cm deep) recorded at the experimental site and (B) survival ($\bar{X} \pm$ SD, $\sqrt{pq/N}$) to adult emergence ($N = 45$) after various periods of exposure to field conditions.

are statistically significant, the differences are probably not biologically important. The SCP of nondiapausing laboratory-reared pupae (−21.9°C) was very similar to values for the field-reared flies.

Discussion

In this study of a population of *S.bullata* from central Ohio, adults from the overwintering generation emerged on 11 May. Remarkably, this is identical to the median emergence date observed in an Illinois population of the same species examined under field conditions in 1971 (Denlinger, 1972b). In spite of the short daylengths that prevail at that time of the year, progeny produced by the overwintering adults failed to enter diapause. This absence of diapause in the first generation is due to the expression of a maternal effect that prevents diapause: progeny of females who have gone through pupal diapause (or been exposed to short daylength during their embryonic and larval life) lack the capacity to enter diapause, even under strong diapause-inducing conditions of short daylength and low temperature (Henrich & Denlinger, 1982; Rockey *et al.*, 1989). After the intervention of one nondiapausing generation, the flies will again respond to short daylength by entering diapause (Henrich & Denlinger, 1982). While the critical photoperiod has not been determined for the Ohio population of *S.bullata* (40°0′N), two populations from similar latitudes (Urbana, Illinois, 40°15′N and St Louis, Missouri, 38°34′N), had critical photoperiods of 13.5 h at 25°C (Denlinger, 1972a). The maternal effect thus prevents the first spring generation from misinterpreting the short daylength of May as a signal for diapause initiation. Under our field conditions, we would anticipate that daylengths prevailing during the photosensitive embryonic and larval periods (Denlinger, 1971) of the second generation would be interpreted as long day and indeed only 0.3% entered diapause in July (second generation). The shortening daylength in August elicited a 37.3% diapause incidence in the third generation, and all individuals entered diapause in the fourth generation (September). Thus the overwintering flies were derived primarily from the third and fourth generations. Diapausing pupae of *S.bullata* initiate development in response to the rise of temperature in early spring (Denlinger, 1972a, b). Though they are capable of initiating development as early as January, the low temperatures at that time of year prevent the onset of development (Denlinger, 1972b). As in the Illinois study, in central Ohio we detected the first morphological signs of pharate adult development in late March.

Previous data on the thermal constant threshold and degree day requirements for *S.crassipalpis*, a closely related species collected in Urbana, Illinois (Chen *et al.*, 1987b), proved useful in predicting the time of spring emergence and the annual generation number. Predicted spring emergence occurred within 1 day of the observed emergence, and degree day accumulation during the summer accurately predicted the completion of a maximum of four annual generations.

No information is available for generation turn-over of flesh flies at other temperate zone sites. But, the three or four generations completed by *S.bullata* in central Ohio contrasts to the seven generations per year completed by flesh flies in the tropical highlands of Nairobi, Kenya (Denlinger, 1978). The hot summer months in central Ohio yielded generation times of 30−39 days; in the East African study generation time for nondiapausing flies ranged from 41 to 62 days. While the tropical flies also have the capacity to enter pupal diapause, only a small portion of the population does so during the cool months (July and August). In contrast, the entire population of *S.bullata* in Ohio overwinters in diapause. And, in this study, we have demonstrated that diapause is essential for overwinter survival: nondiapausing pupae are indeed killed when subjected to winter conditions.

We previously demonstrated in laboratory experiments that diapausing flesh fly pupae are considerably more cold tolerant than nondiapausing flies (Adedokun & Denlinger, 1984; Lee *et al.*, 1987). Under our field conditions, cold tolerance (the ability to survive −17°C) increased progressively during the first 1.5 months of diapause and remained high until the onset of pharate adult development in the spring. Glycerol, the major cryoprotectant used by flesh flies (Lee *et al.*, 1987), was present in high concentrations under the field conditions. The concentration remained around 120 mM throughout the season. In the laboratory at

20°C, the highest concentrations we observed in *S.bullata* were around 20 mM. Thus, natural conditions promoted a much higher accumulation of glycerol, presumably due to the lower temperatures that prevailed. Low temperature activates glycogen phosphorylase (Chen & Denlinger, 1990) and leads to the subsequent production of glycerol (Ziegler *et al.*, 1979).

We previously reported that the supercooling point is not a good indicator of cold tolerance in flesh flies (Lee & Denlinger, 1985), and similar observations have now been reported for several species (Bale, 1987; Bennett & Lee, 1989). In this study we found that diapausing pupae of various ages and nondiapausing pupae all had supercooling points around −21°C. In spite of similarity of the supercooling points at these different stages, cold tolerance differed greatly.

The seasonal changes we observed in the field are thus readily understandable in light of our laboratory-based experiments on diapause induction, termination and cold-hardiness. The most striking difference we noted in the field flies was the dramatic elevation of glycerol, compared to data observed in our laboratory-reared flies. Natural conditions, perhaps both the lower temperatures as well as the diurnal temperature cycle, greatly enhanced this response.

Acknowledgments

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