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Diapause development and cold hardiness of *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) larvae in Greece

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Abstract. Larval diapause development and termination and some characteristics of cold hardiness in *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) were studied under field conditions in northern Greece. *P. gossypiella* overwintering larvae were sampled at 20 to 30 day intervals and subjected to two photoperiodic regimes at 20°C. In larvae kept under a long-day photoperiod (16L : 8D) diapause development was accelerated compared to those kept under a short-day photoperiod (8L : 16D). There was no difference in response to the two photoperiods after February. Mean number of days to pupation of *P. gossypiella* overwintering larvae decreased progressively through the sampling period, from November to April. Chilling is not a prerequisite but does accelerate diapause development. Supercooling points for *P. gossypiella* overwintering larvae ranged from –14 to –17°C with the majority dying after freezing.

INTRODUCTION

Pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae) is one of the most destructive insects attacking cotton fields world-wide. The feeding of the larvae may reduce lint yields by as much as 60% (Fry et al., 1978) and cause severe economic losses for cotton growers (Henneberry & Naranjo, 1998). The insect overwinters in diapause as full grown larvae within the seeds or in protected places in the soil. Adult emergence in the south-western United States occurs in late March and continues until late August (Henneberry & Naranjo, 1998). In Greece *P. gossypiella* completes 3 to 4 generations per year. Diapause induction is triggered by a combination of shortening photoperiod and low temperature (Henneberry & Naranjo, 1998; Venette et al., 2000).

Diapause and cold hardiness are the basic means by which insects in temperate zones cope with unfavourable environmental conditions (Tauber et al., 1986; Lee, 1991). Photoperiod and temperature are the most common cues involved in the diapause syndrome and cold hardiness of insects (Tauber et al., 1986; Lee, 1991; Irwin et al., 2001). Diapause duration is among others, a critical factor in the survival of insects during unfavourable winter conditions. Diapause is a dynamic process, during which diapause development varies in intensity, and the abiotic or biotic cues necessary to break diapause also change over time (Tauber et al., 1986; Danks, 1987; Irwin et al., 2001; Hodek, 2002).

Seasonal variation in cold hardiness is reported for a number of overwintering insects (Leather et al., 1993; Block, 1995; Danks, 2005). In many cases this variation is associated with the onset and termination of diapause, although environmental triggers might act, independently of the diapause syndrome, on the accumulation of cryoprotectants and other physiological and biochemical changes resulting in increased cold hardiness (Baust, 1982; Denlinger, 1991; Block, 1995; Hodková & Hodek, 2004).

Data on the cold tolerance of *P. gossypiella* overwintering larvae are scarce and studies on diapause development under field conditions are restricted to some specific areas (Beasley, 1997; Henneberry & Jech, 1999; Venette et al., 2000). In this study the diapause development of overwintering larvae of *P. gossypiella* in Greece is reported. In addition, the cold hardiness of overwintering larvae in relation to seasonal changes are also described.

MATERIAL AND METHODS

Insects

Bolls were collected from a heavily infested cotton field after harvest in November, 1999 and kept under field conditions protected from rain and direct sunlight until the following May. The field was located in northern Greece close to the city of Thessaloniki (latitude 40.5°N).

Diapause termination

For the study of diapause development in the field 50 overwintering larvae from bolls, kept under field conditions, were transferred to the laboratory at 20 to 30 days intervals during the winter of 1999–2000. Larvae were placed in Petri dishes with a piece of paper towel and kept either under a long-day (16L : 8D) or a short-day photoperiod (8L : 16D) at 20°C in controlled environment chambers (Precision Scientific, General Electric, Louisville, KY). Pupation was recorded daily and pupae were

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TABLE 1. Mean number of days (± SEM) to pupation for overwintering larvae of *P. gossypiella* sampled during the winter in Greece.

Transfer date	Photoperiodic regime (L : D)	
	16 : 8	8 : 16
24 Nov. 99	127 ± 5.38a[1]A	184 ± 11.4aB
28 Dec. 99	110 ± 5.92aA	161 ± 9.5abB
24 Jan. 00	76 ± 5.46bA	121 ± 9.71bB
25 Feb. 00	63 ± 3.61bcA	75 ± 8.82cA
15 Mar. 00	50 ± 7.48cdA	79 ± 9.17cA
1 Apr. 00	37 ± 5.06dA	60 ± 11.06cA
25 Apr. 00	28 ± 3.76dA	53 ± 7.98cB

[1] Means followed by the same latter within a column and by the same capital letter within a row are not significantly different (Tukey HSD, P = 0.05).

placed individually in plastic cells until adult emergence. As there is no morphological criterion for discriminating diapausing larva, pupation after transfer to the laboratory was used as a criterion for the end of diapause development.

Diapause intensity

Overwintering larvae were collected from two major cotton-growing regions of Greece. One in the north of Greece (Thessaloniki) and one 200 km far to the south, in central Greece (Larissa). The two areas are separated by Mount Olympus, the highest mountain in Greece (2.918 m). Both populations were collected in early November before the onset of low temperatures in these areas. Two hundred larvae from each population were placed in 10 Petri dishes (20 per Petri-dish) and kept at 5°C for 15 or 30 days in continuous darkness to avoid any potential influence of photoperiod on diapause development. After this the larvae were maintained at 20°C under a short-day photoperiod (8L : 16D) and the number pupated recorded daily. The mean number of days to pupation was used as a criterion of diapause intensity. The duration of diapause under the specific environmental conditions was used to measure diapause intensity, in the sense of Masaki (2002).

Determination of super cooling point (SCP)

In the winter of 1999, 15 overwintering larvae from the field were transferred to the laboratory at monthly intervals starting from December until May 2000. Each larva was placed individually in a transparent plastic capsule (16 × 7 mm) and immobilised with cotton. A copper constantan thermocouple was attached to the larvae (Digitron 2000T, Kalestead Ltd, UK) to monitor its temperature. The capsule containing the larva and the sensor was placed in a test tube, which was then immersed in a circulating bath (Model 9505, Polyscience) containing a solution of ethylene glycol and water. The cooling rate was set at 1°C/min. The temperature of a larva when its body fluids crystallized and released latent heat was recorded as the SCP of that individual.

Determination of lethal temperature and lethal time

In the winter of 1999–2000 the cold tolerance of overwintering larvae of *P. gossypiella* was determined on three occasions. Larvae were placed in test tubes and then immersed in a circulating bath which was cooled gradually to temperatures rangeing between –3 and –9°C. After reaching a particular temperature the larvae were kept at this temperature for 2 h. Larvae were then left to warm up gradually to ambient and kept in the laboratory at 25°C. Larvae that did not respond to mechanical stimuli a few hours later were considered to be dead. The live larvae were transferred to 20°C (16L : 8D) and mortality assessed daily. The mortality that occurred after exposure to –3, –6, or –9°C for various periods of time was used to estimate lethal time. For determining of Ltime50 and Ltime90 in days, groups of overwintering larvae were exposed for different lengths of time (from 3 h to 15 days depending on the temperature) to a constant temperature. Larvae were transferred to each specific temperature directly from the culture. Upon removal from the low temperature treatment they were kept in the laboratory (25°C) for a few hours and mortality assessed daily after the living larvae were transferred to 20°C, 16L : 8D. In each experiment there was 4 replicates of 10 larvae.

TABLE 2. Anova results of the affect of photoperiod and transfer date on the diapause development of overwintering larvae of *P. gossypiella*.

Source	df	MS	F	P
Photoperiod	1	72319.5	64.45	< 0.000
Transfer date	6	75404.7	67.20	< 0.000
Ph. * Tr. date	6	2645.6	2.36	0.031
Error	244	1122.2		

Statistical analysis

Mean developmental time to pupation and adult emergence of overwintering *P. gossypiella* larvae were estimated for each sampling date and photoperiodic regime. A two-way analysis of variance (ANOVA) was deployed to discriminate any effect of sampling date and photoperiod on diapause development, measured as the mean time required for pupation and adult emergence. The t-test was used to compare means for the two photoperiods at each sampling date. The effect of temperature and location on diapause intensity measured as the mean time to pupation was estimated using ANOVA (SPSS, 2000). The influence of sampling date and sex on SCP was determined by ANOVA. Probit analysis was used to estimate the lower lethal temperature of each experimental group and the length of exposure needed to kill a given percentage of the population at each temperature. Chi-square test was used to compare the survival of larvae during the overwintering period.

RESULTS

Diapause termination

The duration in days to pupation of overwintering *P. gossypiella* larvae was significantly reduced within the

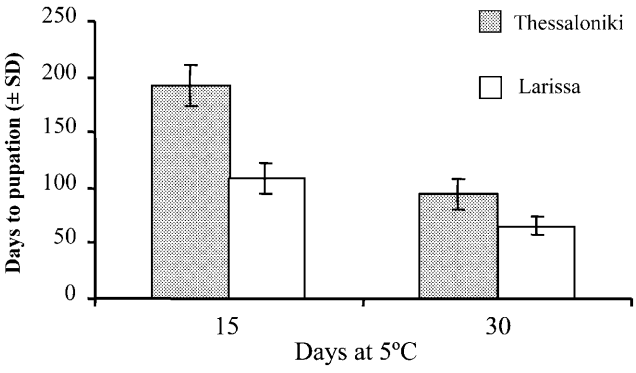

Fig. 1. Mean number of days to pupation of overwintering *P. gossypiella* larvae from two locations kept at 20°C and a photoperiod of 8L : 16D, after exposure to 5°C for 15 and 30 days, respectively.

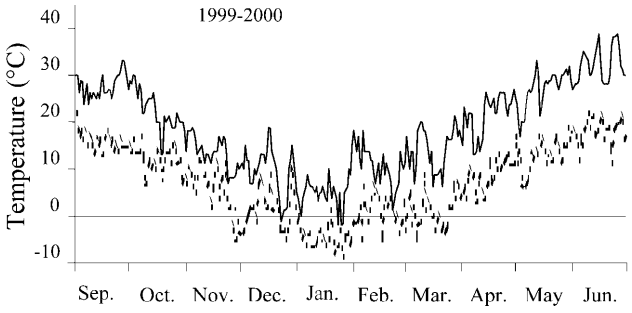

Fig. 2. Daily minimum and maximum air temperatures from September to June, 1999–2000.

sampling period, November to April, when they experienced either a long-day ($F_{6,126}$ = 52.05; P < 0.05) or short-day photoperiod ($F_{6,118}$ = 28.65; P < 0.05) (Table 1). Overwintering *P. gossypiella* larvae pupated after 127 days when transferred to the laboratory on 24th November and maintained at a photoperiod of 16L : 8D, whereas those transferred on 1st April pupated after 37 days. The larvae that were transferred to the laboratory on November 24th and maintained at 20°C under a photoperiod of 8L : 16D pupated after 184 days and those transferred on April 1st after 60 days. Mean number of days to pupation gradually declined from autumn to spring at both photoperiods. There was no affect of transfer date on larval development after 25th February. The difference in mean number of days to pupation was significant between the two photoperiodic regimes up to 24th January. Overwintering *P. gossypiella* larvae required more days for pupation under the short than under long-day photoperiods. Photoperiod and sampling date both significantly influenced the diapause development of *P. gossypiella* larvae (Table 2). The interaction between these factors was also significant for diapause development in terms of the mean number of days required for overwintering *P. gossypiella* larvae to pupate (Table 2).

Diapause intensity

Diapause intensity, measured as the mean number of days to pupation, was significantly affected by location ($F_{1,25}$ = 4.82; P = 0.038) and the time for which larvae were kept at a particular temperature ($F_{1,25}$ = 7.58; P = 0.01) (Fig. 1). Larvae from the area of Thessaloniki required more days to pupate than those from Larissa after they were kept for either 15 or 30 days at 5°C. When larvae from both areas were kept for 30 days at 5°C the mean number of days to pupation declined (Fig. 1).

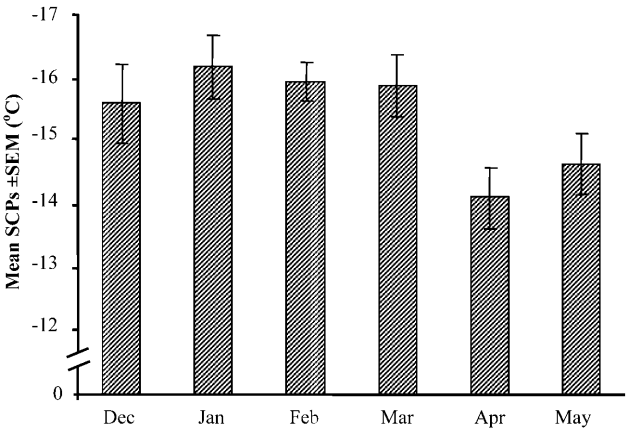

Fig. 3. Seasonal variation in supercooling point of overwintering larvae of *P. gossypiella*.

Cold hardiness

Supercooling point

Fig. 2 shows the daily minimum and maximum air temperatures recorded at the field site during the winter of 1999–2000. The seasonal variation in SCP is shown in Fig. 3. The mean SCP of larvae from the field ranged from –14 to –16.4°C and was similar for males and females ($F_{1,81}$ = 2.7; P = 0.104). Within the experimental period variation among sampling dates was not significant ($F_{5,81}$ = 2.09; P = 0.075).

Pre-freeze mortality: Lethal temperature, lethal time

The Ltemp50 and Ltemp90 for overwintering *P. gossypiella* larvae in November, February and May are shown in Table 3. In November the Ltemp50 was significantly higher than in February and May. Accordingly, at each temperature mortality increased with exposure time. There was a substantial reduction in the Ltime50 and 90 values at the lower temperatures though the differences were not all significant (Table 4). Ltime50 and Ltime 90 were significantly shorter at –9°C than at –6°C and –3°C.

DISCUSSION

In the present study information on diapause termination and cold hardiness of overwintering *P. gossypiella* larvae in Greece is reported which should enhance our understanding of this species phenology. Diapause development in overwintering *P. gossypiella* larvae in Greece seems to be complete by mid winter (mid February). Larvae collected in the field in February take fewer days to pupate than those transferred to the laboratory in November and December. The fact that larvae collected at different times during winter pupated under both pho-

TABLE 3. $Ltemp_{50, 90}$ of overwintering larvae of *P. gossypiella* recorded during the winter of 1999–2000 in northern Greece.

Date	N	Slope	SE	LT_{50} (95%CI)	LT_{90} (95%CI)	X^2
November	119	–0.21	0.08	–6.69 [–7.71–(–4.05)]	–12.5 [–26.6–(–4.1)]	1.47
February	119	–0.28	0.11	–9.2 [–14.55–(–8.26)]	–13.7 [–32.9–(–11)]	3.65
May	120	–0.17	0.08	–7.4 [–10.36–(–5.09)]	–14.6 [–64.3–(–11)]	2.56

TABLE 4. $Ltime_{50, 90}$ of overwintering larvae of *P. gossypiella* recorded during the winter of 1999–2000 in northern Greece.

Temp. (°C)	N	Slope	SE	LT_{50} (95%CI) (days)	LT_{90} (95%) (days)	X^2
–3	75	2.02	0.76	8.54 (4.18–13.21)	36.69 (19.16–2187.1)	2.11
–6	100	1.84	0.59	2.36 (0.78–3.37)	11.74 (7.26–73.9)	1.52
–9	75	2.55	0.82	0.13 (–0.08–0.19)	0.38 (0.30–0.68)	0.18

toperiodic regimes suggests that low temperature is not a prerequisite for diapause development and termination, although they might promote diapause development (Tauber et al., 1986). Despite the fact that diapause development ended in early February, the first pupation in the field occurred at the end of April. Low temperatures that prevail at that time suppress larval development, which is resumed in spring. This phenomenon is reported for other species of insects in the same general area of northern Greece (Milonas & Savopoulou-Soultani, 2004). It is the means by which this insect synchronises its spring emergence as suggested for other species (Tauber et al., 1986; Fantinou et al., 1998).

Photoperiod is known to influence diapause development in many insects that live in temperate areas (Tauber et al., 1986). In our case diapause development was accelerated under long-days. Exposure to short-days kept the larvae collected in November and January in diapause. Short days prevail at that time. By responding to short days diapause development is decreased and high temperatures early in winter do not terminate diapause.

Diapause intensity is a physiological trait measured as the duration of diapause under specific conditions (Masaki, 2002).

Duration of diapause affects the time of its termination. In *P. gossypiella*, diapause intensity determines the time of diapause termination in the field. Tauber et al. (1986) report that in most temperate zone insects, which undergo an autumnal-hibernal diapause, the specific stimulus responsible for diapause termination is unknown. The sensitivity these insects show to inducing and maintenance factors diminishes progressively and diapause terminates spontaneously during winter. The time of diapause termination depends on its intensity, which is predetermined for each species, as well as for each strain of a species (Tauber et al., 1986). In this study the time for which they were exposed to 5°C increased diapause development at each location. Furthermore, the difference in diapause intensity between the two areas possibly reflects the geographic variation in diapause shown by this species (Gomi, 1997). The two sites are major cotton growing areas in Greece, but the growing period is much shorter in Thessaloniki due to the shorter period of favourable temperatures for the growth of cotton. Thus, the difference in diapause intensity reflects the adaptation of the species to each locality.

Overwintering *P. gossypiella* larvae are very cold hardy. Supercooling points were between –14.6 to –17.1°C and did not differ significantly over the sampling period. Overwintering insects cope with subzero temperatures in one of two ways (Block, 1995). They are either freeze-tolerant or freeze-intolerant. The majority of *P. gossypiella* larvae died after freezing, indicating that they cannot tolerate freezing. On all three occasions significant mortality occurred at temperatures above the SCP, which indicates chilling injury.

Prefreeze mortality also occurred in overwintering larvae of *P. gossypiella*. Mortality at temperatures above the SCP depend on the temperature and duration of exposure. In all cases the presence of chilling injury is documented. Many studies have shown that supercooling point is not a reliable index of insect cold hardiness as it does not take into account the mortality caused at sub-zero temperatures above the insect's supercooling point (Bennett & Lee, 1989; Bale, 1993). As pointed out by Renault et al. (2002) deleterious effects (including death) can be produced by prolonged exposures to lethal low temperatures even if the insect does not freeze. Finally, during long-term exposure to low temperatures, insects may die from starvation. This is well illustrated by the ability of diapausing larvae collected from the field in the USA of withstanding 5°C for 60 d after which 90% mortality occurred (Venette et al., 2000).

The difference in Ltemp50 of field maintained larvae between November and February accords with the finding that diapause development is completed in February. The end of diapause development is often accompanied by several changes in the composition of haemolymph. Levels of glycerol and other cryoprotectants are often lower after diapause development is completed (Pullin, 1996).

Our results suggest that *P. gossypiella* is well able to survive the winter conditions in the cotton growing area of northern Greece and that the intensity of diapause in the southern population is weaker.

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