

A second diapause in *Wyeomyia smithii*: seasonal incidence and maintenance by photoperiod

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Wyeomyia smithii ordinarily diapauses in the third larval instar, but a second, photoperiodically maintained developmental arrest may occur in the fourth instar. Two years of sampling from a Massachusetts bog revealed that the fourth-instar diapause phenotype is most abundant in the spring after the termination of third-instar diapause, and in the fall when a new overwintering generation of third instars accumulates in the pitcher-plant habitat. Fourth-instar larvae from this population cannot, however, survive the winter. This mortality during winter is apparently balanced by advantages that a second diapause confers upon the mosquito population in the spring.

Fourth-instar diapause may be induced from diapausing third-instar larvae in the laboratory by a brief exposure to long days followed by short days, or by a long-term exposure to short days at 25°C. Continuous exposure to long days readily terminates fourth-instar diapause. The critical photoperiod and number of long days required for the termination of diapause is similar for larvae which diapause in either the third or fourth instar.

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Wyeomyia smithii subit ordinairement sa diapause au cours du troisième stade larvaire, mais peut également subir un arrêt de développement maintenu par photopériode, au cours du quatrième stade. Un échantillonnage de 2 ans, dans une tourbière du Massachusetts, démontre que le phénotype de la diapause de quatrième stade est surtout abondant au printemps, après la diapause de troisième stade, et à l'automne, alors qu'une nouvelle génération d'individus de troisième stade s'accumule dans l'habitat-sarracénie pour y passer l'hiver. Les larves de quatrième stade de cette population ne peuvent cependant pas survivre à l'hiver. Cette mortalité à l'hiver est toutefois compensée par les avantages qu'entraîne une deuxième diapause chez la population de moustiques du printemps.

On peut provoquer en laboratoire une diapause de quatrième stade chez des larves en diapause de troisième stade en les exposant brièvement à des jours longs suivis de jours courts ou en les exposant pour une longue période à des jours courts, à 25°C. Une exposition continue à des jours longs arrête automatiquement la diapause de quatrième stade. La photopériode critique de même que le nombre de jours longs requis pour enrayer la diapause sont les mêmes, qu'il s'agisse de larves en diapause de troisième ou de quatrième stade.

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The pitcher-plant mosquito, *Wyeomyia smithii*, undergoes a seasonal developmental arrest, or diapause, in the third larval instar. This diapause is evoked and maintained by a short-day photoperiod and is averted or terminated in response to long days (Smith and Brust 1971; Bradshaw and Lounibos 1972; Evans and Brust 1972). In our previous communication we noted that the staging of diapause in the penultimate larval instar allowed the mosquito to delay the principal trophic stadium until the spring.

Through an intensive study of the phenology of *W. smithii* larvae in a natural population, we have now discovered that a developmental arrest

may also occur in the trophic, fourth instar, thereby affording an individual mosquito two diapause options in a single season. The present paper elaborates on the seasonal incidence and photoperiodic control of this second diapause and discusses possible reasons for its low-level maintenance in *W. smithii* populations.

Materials and Methods

Field collections of *Wyeomyia smithii* were made in the spring, summer, and fall of 1972 and 1973 from a bog near Forge Village, Massachusetts (42°34' N; 71°30' W). Larvae were sorted from pitcher-plant (*Sarracenia purpurea*) fluid collected with a Sabolt pipette. Though four collections in the spring of 1972 yielded from 22 to 99 larvae, each sample thereafter was represented by at least 200 larvae. Sample sizes averaged 495 larvae per collection in 1972 and 595 per collection in 1973. Third- and fourth-instar larvae were identified by head capsule widths (Bradshaw and Lounibos 1972) and exposed to long (17L:7D) or short (8L:16D or 12L:12D) daylengths

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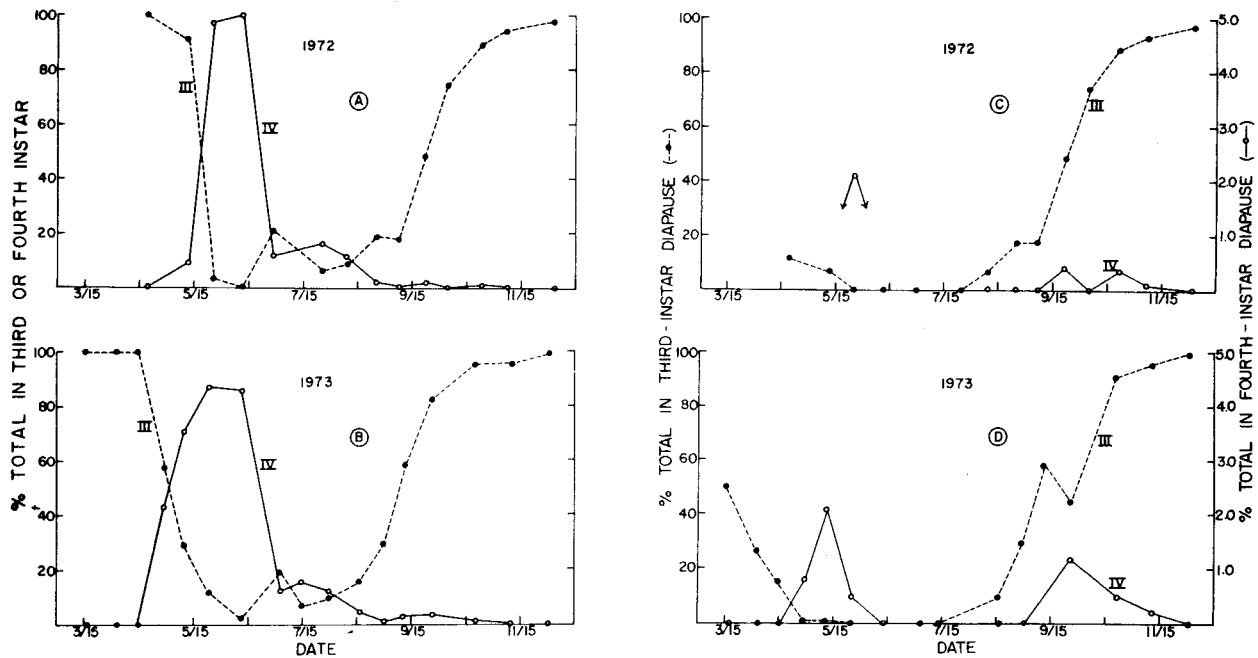


FIG. 1. Seasonal incidence of third- and fourth-instar larvae in field collections from Forge Village, Massachusetts, during (A) 1972 and (B) 1973. Occurrence of each instar is expressed as a percentage of the total collection. (C) and (D) indicate percentages of the total Forge Village collection in third- or fourth-instar diapause in 1972 and 1973.

in incubators kept at $25 \pm 1.5^\circ\text{C}$ except where otherwise noted.

A laboratory colony from this same site was maintained according to our previously described methods, except that lyophilized brine shrimp were fed to larvae, and raisins to adults. An additional laboratory stock of *W. smithii* was colonized from Moore's Meadows, New Jersey ($39^\circ 48' \text{N}$; $74^\circ 35' \text{W}$).

We define diapause in the present report as a developmental arrest in which third-instar larvae fail to molt to the next instar within 20 days at $25 \pm 1^\circ\text{C}$, or fourth instars fail to pupate within 20 or 30 days at that same temperature. In all experiments diapause could be terminated by an appropriate long-day photoperiod. The symbols IV_{50} and P_{50} used in the text refer to the number of days required for 50% of a diapausing third- or fourth-instar sample to molt to the fourth instar or pupal stage, respectively (Bradshaw and Lounibos 1972).

Experimental Results

(1) Phenology of Third and Fourth Instars

At Forge Village we recovered only third-instar larvae from pitchers sampled in March through mid-April (Figs. 1A, B). During late April the proportion of third-instar larvae declined, and fourth instars appeared in our samples. Fourth-instar larvae occurred earlier in 1973 than in 1972, but in both years greater than 85% of the population was in the fourth instar by late May or early June. We observed transient, secondary peaks of third- and fourth-instar larvae during

late June and July; presumably these larvae represent the F_1 progeny of the overwintering generation. The proportion of thirds rose again in August and September to gradually become the most abundant stage inhabiting the pitcher-plant environment.

Even as the new overwintering generation of third instars accumulated from September through November, a low percentage of fourth-instar larvae persisted in the plants (Figs. 1A, B); these individuals decreased in density and were completely absent by December. Moreover, no pupae were ever collected after September, a finding that suggests that final-stage larvae rarely, if ever, reach adulthood in the fall. The absence of an autumnal brood in the seasonal samples indicates that *W. smithii* are ordinarily bivoltine at Forge Village.

(2) Incidence of Third- and Fourth-instar Diapause in the Field

Although early spring collections consisted entirely of third instars, 50 to 90% of these larvae molted during exposure to a short (12L:12D) laboratory daylength at $25 \pm 1^\circ\text{C}$, revealing that most of the overwintering larvae had terminated diapause (Figs. 1C, D). By May, only 0 to 8% of the sampled third-instar larvae remained in a

TABLE 1
Incidence of fourth-instar larvae from field collections of *Wyeomyia smithii* in fall of 1970

Collection site	Date	Total no. larvae	% fourth instar
Baxter Park, Pistaquis Co., Maine (46°00' N)	Sept. 15, 1970	1657	1.4
Cape Breton Highlands, Nova Scotia (46°45' N)	Sept. 20, 1970	7532	2.2
Acadia Park, Hancock Co., Maine (44°15' N)	Sept. 23, 1970	4394	0.1
Moore's Meadows, Burlington Co., New Jersey (40°00' N)	Oct. 23, 1970	2036	0.2
Hidden Lake, Livingston Co., Michigan (42°30' N)	Nov. 14, 1970	1028	0.1

photoperiodically maintained developmental arrest. Diapausing phenotypes were completely absent from late May through late July, but later in the summer third-instar diapause reappeared in the population in concert with the relative increase in numbers of penultimate stage Larvae (Fig. 1).

Field-collected, fourth-instar larvae exposed to long days in the laboratory invariably pupated and completed adult development. By contrast, short days occasionally produced or maintained a developmental arrest in the last instar. This retarding effect of short-day photoperiod was observed only among fourth-instar larvae collected in the spring or fall, at which times the incidence of a fourth-instar diapause ranged from 0.1 to 2.1% of the total collection (Figs. 1C, D).

Thus, while representing a relatively small proportion of the total collection, fourth-instar diapause appeared in the spring when overwintering third instars terminated diapause and molted, or in September and October when a new generation of third-stage larvae accumulated in the population (Figs. 1C, D). In both 1972 and 1973 the relative percentage of fourth-instar diapausing larvae encountered in the spring was greater than in the fall. A peak in occurrence of fourth-instar diapause in September 1973 coincided with a transient decrease in the incidence of diapause among third-instar larvae (Fig. 1D).

In the fall of 1970, we collected large numbers of *W. smithii* from bogs in Nova Scotia, Maine, Michigan, and New Jersey. Diapausing third-instar larvae predominated in these collections, but fourth-instar larvae, ranging in abundance from 0.1 to 2.2%, were also encountered (Table 1).

Fourth-instar larvae collected from Baxter Park (Maine) and Cape Breton (Nova Scotia) were kept at 2–3°C for about 2 weeks and transferred to 12L:12D at $21 \pm 1.5^\circ\text{C}$. Five of 13 (38.5%) final-stage larvae from Baxter Park and

24 of 32 (75.0%) larvae from Cape Breton failed to pupate within 30 days after exposure to this laboratory daylength. These values correspond to an incidence of fourth-instar diapause of 0.54% of the Baxter Park and 1.70% of the Cape Breton populations in mid-September.

(3) Fourth-instar Diapause from Field-collected Third Instars

Third-instar larvae from Forge Village collections were sorted and exposed immediately to short days at several laboratory temperatures. From certain collections, a portion of the third instars molted within 10 days and subsequently arrested development in the fourth instar. Those larvae that remained in the terminal instar for at least 30 days were defined as diapausing.

A higher incidence of fourth-instar diapause was observed among third-stage larvae collected in April and early May than later in the spring or summer (Table 2). In a single fall collection made while the new overwintering generation of third-instar larvae accumulated in the population, a relatively high incidence of fourth-instar diapause was initiated in the laboratory. A higher proportion of fourth-instar diapause was observed when a May 1972 collection was exposed to a shorter (8L:16D) photoperiod and lower temperature (Table 2).

(4) Survival of Implanted Diapausing Larvae

The data presented in Fig. 1 demonstrate that while diapausing fourth-stage larvae are present in the Forge Village population in the late spring and early fall, they are absent from winter and early spring collections, suggesting that this diapause stage may be incapable of surviving the winter. To test this hypothesis, diapausing third- and fourth-instar larvae were collected Sept. 11, 1973 and kept on a short daylength at 23°C for 2 months. We then acclimated larvae for 1 week at 15°C and 1 week at 8°C in preparation for transfer to tagged pitcher plants at Forge Village. They were subsequently implanted before the

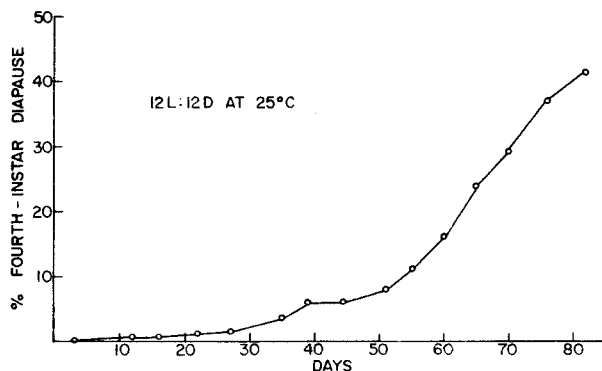


FIG. 2. Induction of fourth-instar diapause through continuous exposure of diapausing third-instar larvae to short days (12L:12D) at $25 \pm 1^\circ\text{C}$. All larvae molting to the fourth instar remained in that stage for 20 days or longer at this photoperiod and temperature.

onset of winter (Dec. 1, 1973) into *Sarracenia* pitchers in densities ranging from 0.5 to 1.4 mosquitoes per milliliter of pitcher fluid. The next spring (April 2, 1974) we recovered 8 out of 57 (14%) implanted third-instar larvae, but none of the 62 (0%) implanted fourth instars survived.

(5) Induction of Fourth-instar Diapause in the Laboratory

On Sept. 11, 1973, 305 third-instar larvae were collected at Forge Village, chilled at 8°C for 8 days, and then exposed to short days at $25 \pm 1^\circ\text{C}$ for 82 days. The cultures were cleaned, fed, and checked at regular intervals for diapausing fourth-instar larvae. The criteria for fourth-instar diapause were satisfied if larvae remained in that instar for over 20 days after the preceding molt and later pupated within 15 days exposure to long days.

During persistent exposure to $25 \pm 1^\circ\text{C}$ and short days, a proportion of larvae molted from the third to the fourth instar (Fig. 2), while otherwise maintaining a state of developmental arrest. The percentage of the experimental population molting to a terminal instar diapause remained below 10% for the first 50 days, then accelerated markedly until the conclusion of the experiment. However, third-instar mortality increased from 5.2% to 32.5% in the final 40 days of the experiment, thereby contributing to the relative increase of fourth-instar diapause among living larvae.

To examine the effects of transient long-day stimulation, we exposed third-instar, diapausing larvae to long days at 16.5, 21, and 25°C , followed by short days at 25°C . These diapausing larvae were collected at Moore's Meadows (MM) on January 1, 1974, and at Forge Village (FV) on April 2, 1974. They were stored at $2-5^\circ\text{C}$ until the initiation of experiments on March 25 (MM) or April 20 (FV), 1974. Fifteen third-instar larvae from Forge Village or 20 from Moore's Meadows were exposed to 0, 1, 2, or 3 long days at $25 \pm 1^\circ\text{C}$; 0, 2, 4, or 6 long days at $21 \pm 1^\circ\text{C}$; or 0, 2, 4, 7, or 8 (MM only) long days at $16.5 \pm 1^\circ\text{C}$. They were then transferred to short days (11L:13D) at $25 \pm 1^\circ\text{C}$ and observed for 20 additional days. Any individuals that molted to the fourth instar were observed for 30 days in that instar. Individuals remaining in the third instar for 20 days or in the fourth for 30 days were considered to be in diapause.

Figure 3 shows that the incidence of fourth-instar diapause was inversely proportional to the

TABLE 2
Induction of fourth-instar diapause from field-collected, third-instar *Wyeomyia smithii* from Forge Village, Massachusetts

Date of collection	Daylength L:D at 25°C	% third instars molting to fourth	Fraction of resulting fourths in diapause	%
1972				
Apr. 19	12:12	88.5	7/43	16.3
May 11	12:12	92.5	6/48	12.5
May 11	8:16 ^a	90.0	8/27	29.6
July 26	12:12	100.0	0/11	0.0
Aug. 8	12:12	20.0	0/10	0.0
Aug. 26	12:12	8.0	0/4	0.0
1973				
Apr. 15	8:16 ^b	84.0	5/22	22.7
May 23	8:16 ^b	100.0	1/21	4.8
June 12	8:16 ^b	100.0	0/8	0.0
July 2	8:16 ^b	100.0	0/37	0.0
Sept. 26	12:12	44.0	12/41	29.3

^a 22°C .
^b 23.5°C .

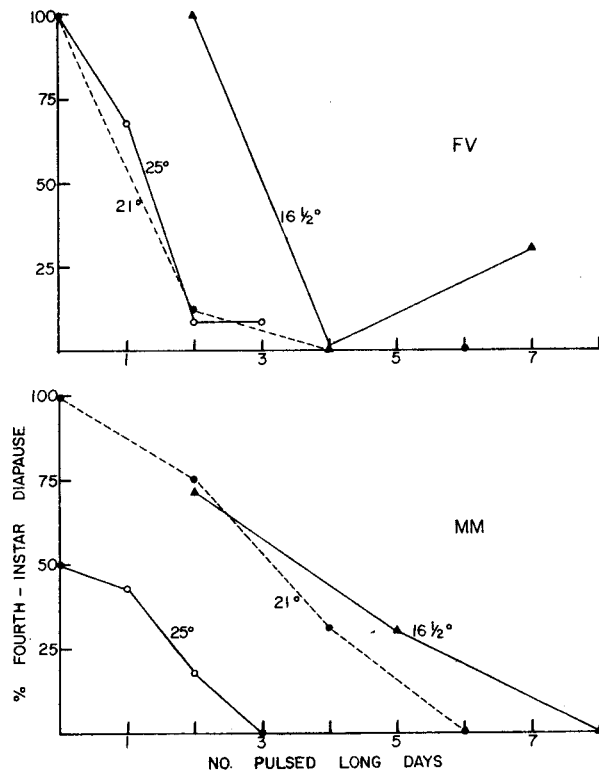


FIG. 3. Percentage of fourth-instar larvae entering diapause in response to long days pulsed to diapausing third-instar larvae. Long days consisted of a 15-h photopause for Forge Village (FV) and a 16-h photopause for Moore's Meadows (MM). Percentage fourth-instar diapause was calculated from the proportion of larvae which molted to the fourth instar that diapaused at that stage.

number of long days experienced during the previous instar. A given number of long-day pulses generally elicited a greater percentage of fourth-instar diapause at lower temperatures than at higher ones.

(6) Termination of Fourth-instar Diapause

A laboratory colony of *W. smithii* from Moore's Meadows produced 24 diapausing fourth instars when reared on 10L:14D at $25 \pm 1^\circ\text{C}$. Half of these were kept on 10L:14D and half transferred to 17L:7D at 23°C . All fourths exposed to the longer daylength pupated within 13 days, but only 1 of 12 short-day larvae developed within 25 days after the initiation of the experiment.

In a more extensive experiment to confirm the effect of photoperiod on diapause termination, we exposed diapausing third- and fourth-stage larvae from Forge Village to long and short days at 25°C . The sample sizes were 50 thirds for the long-day experiments and 25 thirds or fourths

for both long and short daylengths. The cultures were fed and checked daily for fourth-stage larvae and pupae. As shown in Fig. 4A, long days elicited 50% molting of thirds to fourths after 9–10 days (IV_{50}) and 50% pupation among diapausing fourths after 12–13 days. Short days failed to evoke development among either third- or fourth-instar larvae. The shapes of the response curves are similar for termination of both third- and fourth-instar diapause.

To determine the number of long days needed to terminate fourth-instar diapause, third-instar larvae obtained from field collections in the fall of 1972 were first exposed to continuous short days at $25 \pm 1^\circ\text{C}$. Larvae which molted and entered fourth-instar diapause were pooled and subsequently divided into six culture dishes of 30 each. Successive cultures were pulsed with 1 through 6 long days at $23 \pm 1.5^\circ\text{C}$ and then returned to short-day incubators, where diapause termination was assessed as percentage pupation within 20 days.

Three long days were required to achieve 50% termination (T_{50}), and exposure to 4 long days produced a saturated response (Fig. 4B). The response of diapausing fourth-instar larvae to pulsed long days at 23°C was virtually identical with the response of diapausing third-instar larvae to pulsed long days at that temperature, as reported earlier (Bradshaw and Lounibos 1972).

Among diapausing third-instar larvae, the rate with which dormancy is terminated is slowest near the critical daylength (Bradshaw and Lounibos 1972). To determine if this is also true of diapausing fourth-instar larvae, aliquots of 50 individuals of this sort were exposed to four daylengths from 14.5 through 16 h of light per day. The time to 50% pupation was observed and plotted versus daylength. P_{50} increased with decreasing daylength and was most prolonged near the critical daylength, as in the above mentioned experiments on diapausing third-stage larvae (Fig. 4C).

Discussion

Experiments reported here indicate that fourth-instar diapause occurs early within that stadium, as is the case in third-instar diapause (Bradshaw and Lounibos 1972). Although adult differentiation in mosquitoes begins in the fourth instar (Clements 1963), the second diapause in *W. smithii* precedes the initiation of major,

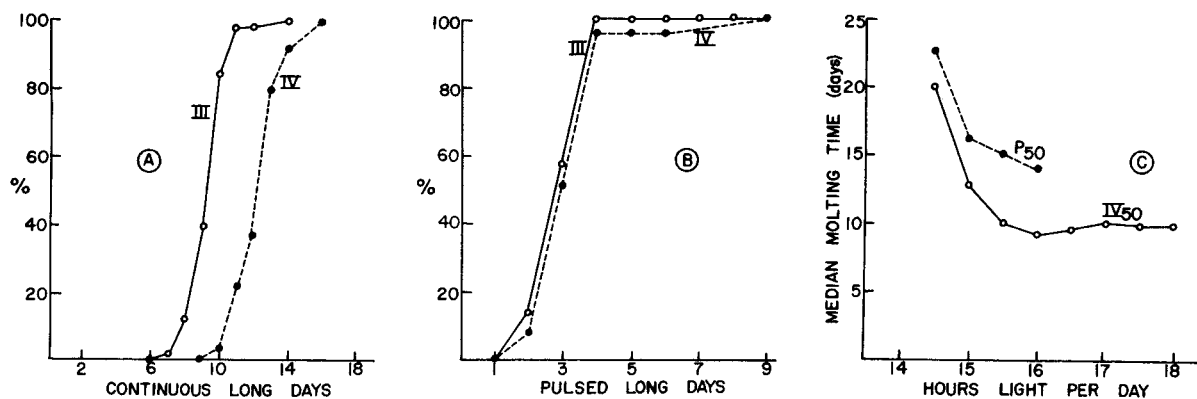


FIG. 4. Diapause termination responses of third- and fourth-instar larvae. (A) The percentages of the two groups molting or pupating in response to continuous exposure to 17L:7D at $25 \pm 1^\circ\text{C}$. (B) Successive cultures of diapausing larvae were exposed to 1 through 9 long days (17L:7D) at $23 \pm 1.5^\circ\text{C}$ and returned to 12L:12D at the same temperature, and percentage molting within 20 days was assessed; B (III) redrawn from Bradshaw and Lounibos (1972). (C) The rate of response of third- (IV₅₀) and fourth- (P₅₀) instar larvae to diapause-terminating daylengths at $23 \pm 1.5^\circ\text{C}$.

visible metamorphic events. Moreover, the termination and reinitiation of dormancy observed in the spring as third-stage larvae molt to dormant fourths is not a simple "stationary" molt as observed in certain diapausing Lepidoptera (see Chippendale and Reddy 1972). Rather, larvae diapausing in the fourth stage are one molt closer to adulthood since the number of mosquito instars is relatively inflexible.

Similar T_{50} 's and rates of development near the critical daylength (Figs. 4A, C) suggest that the mechanism which controls the termination of diapause in the two instars is conservative. The P_{50} for diapause termination lags IV_{50} by 3 days; since T_{50} is similar for the termination of both third- and fourth-instar diapause (Fig. 4B), this delay is attributable to the extra time needed for completion of the longer post-diapause period in the fourth instar.

In the summer months the fourth-instar diapausing phenotype is notably absent from field populations but reappears in September, long after the earlier instars have been exposed to the waning daylengths that contribute to third-instar diapause. Apparently a few larvae "slip" or bypass diapause in the penultimate stadium and arrest development in the fourth instar.

Winter and early spring samples (Figs. 1A, B), as well as the implant experiments, indicate that *W. smithii* cannot overwinter as a fourth-instar larva at this latitude. Larvae entering this dormant stage in the fall commit an ecological suicide. Nonetheless, this form persists at a low frequency of 0.1 to 1.7% among autumnal samples from as far north as Nova Scotia. The

relatively high vernal incidence of a second diapause temporally coincides with the last cases of a photoperiodically maintained third-instar diapause in the field (Figs. 1C, D). The spring appearance of the second-diapause form may prevent the premature emergence of adults, thereby providing protection against unseasonably late freezes after third-instar diapause has terminated.

Polymorphisms in larval diapause that temporally space species populations are known in other insects. The spruce budworm in eastern Canada normally diapauses in the second larval instar, but a secondary diapause in the fourth instar occurs in about 5% of the population (Harvey 1961). A Carolina population of the dragonfly *Tetragoneuria cynosura* is principally univoltine, but 5–10% are semivoltine (Lutz and Jenner 1964). By contrast, *Anax imperator* in England is principally semivoltine and a few individuals are univoltine (Corbet 1956). Both dragonfly populations achieve temporal infra-population spacing by each maintaining an early- and a late-nymphal diapause. An unprecedented flexibility in the staging of diapause is observed in pine moths of the genus *Dendrolimus*, which may arrest development in any larval instar (Geispits 1965). The Cecropia moth, *Hyalophora cecropia*, exhibits a bimodal emergence pattern in nature, where about 8% of the population consistently emerges 2–3 weeks earlier than the rest (Sternburg and Waldbauer 1969). The basis for this genetically determined polymorphism stems from the termination of diapause rather than from climatic effects on the

period of post-diapause development (Waldbauer and Sternburg 1973). The larvae of the phantom midge, *Chaoborus americanus*, maintain a polymorphism for response time to the trophic and photoperiodic stimuli that terminate diapause. A fast-responding morph predominates in years that follow mild springs, and a conservative phenotype is more common after springs in which the population encounters harsh conditions after an initial thaw (Bradshaw 1973).

The data in Fig. 3 show that exposure of third-instar larvae to incomplete diapause-terminating stimuli may promote a high incidence of fourth-instar diapause. We believe that early spring day lengths are intermediate between long and short, and that vernal temperatures are intermediate between those preventing and those promoting rapid development. The early vernal environment thus provides mostly ambiguous cues. The initiation of a second diapause represents a cautious response to these stimuli. The initiation of fourth-instar diapause in the spring may simply retard a proportion of the overwintering population as a fail-safe mechanism until clement weather is assured. We suspect that a genetic polymorphism may underlie this second diapause form, since a fourth-instar diapause stock has been selected for in a laboratory colony from Moore's Meadows (Bradshaw, unpublished).

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