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INTERACTION OF FOOD AND PHOTOPERIOD IN THE TERMINATION OF LARVAL DIAPAUSE IN *CHAOBORUS AMERICANUS* (DIPTERA: CULICIDAE) [1]

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The role of food in the termination of diapause is probably without significance in the many diapausing insects which are inactive and which seldom, if ever, feed. There are, however, certain nematocerous Diptera which overwinter as larvae and are capable of both movement and feeding. Indeed, studies concerning the photoperiodic induction of development in diapausing larvae of the chironomids *Metriocnemus* (Paris and Jenner, 1959) and *Chironomus* (Engelmann and Shappirio, 1965) or of the culicids *Anopheles barberi* (Baker, 1935), *Anopheles bifurcatus* (Vinogradova, 1964), *Anopheles plumbeus* (Vinogradova, 1962), *Aedes triseriatus* (Love and Whelchel, 1955), and *Toxorhinchites rutilus* (McCrary and Jenner, 1965) involved the effects of photoperiod on fed animals only. Studies concerning the overwintering larvae of the culicid *Chaoborus americanus* (Bradshaw, 1969) considered the effects of photoperiod on both fed and starved animals. The assessment of the contribution of food is difficult, if not impossible, in filter feeders or detritus eaters like the chironomids or the culicine and anopheline mosquitoes. Chaoborine and toxorhinchitine mosquitoes, on the other hand, are carnivores. In *Chaoborus,* food and photoperiod have been shown to interact synergistically to effect the termination of larval diapause (Bradshaw, 1969). Furthermore, development in *Chaoborus* is proportional to the number of long days with food. *Chaoborus,* therefore, is conveniently adapted for the study of the food component in the termination of diapause.

Materials and Methods

Animals and general conditions

All experiments in this research involve large yellow larvae of *Chaoborus americanus* Johannson caught in George Pond, a small kettle hole in the center of the University of Michigan's George Reserve, near Pinckney, Michigan, during January and February, 1967 and 1968. The larvae were transferred on the day of capture to maintenance conditions of short day (8L, 16D) and 5° C without food. Large numbers of larvae were kept in open gallon jars under these conditions until the starting date of the experiment at which time they were removed, the large yellow larvae, known to be the fastest responding morphs in the population (Bradshaw, unpublished observations), were sorted from this stock, warmed to 25° C,

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and placed on the experimental conditions outlined below. Food in all cases consisted of laboratory larvae of the mosquito, *Culex pipiens,* provided in saturating amounts unless otherwise stated. All the experiments were run at 25° C under either long day (16L, 8D), or short day (12L, 12D) conditions.

Statistical methods

Standard methods (Snedecor, 1956, Ch. 6, 7) were used to derive regression lines, regression coefficients (r), and the probabilities of correlation (P). Percentage data were also analyzed by computation of the normal variate as outlined by Ractliffe (1967, pages 136–137).

Comparisons of sample means were made either by the standard t test or by a modification of Duncan's Multiple Range Test (Duncan, 1955). Analysis of variance was carried out with raw data only when appropriate as demonstrated by Bartlett's test for homogeneity of variance (Snedecor, 1956, pages 285–289); otherwise, arcsin-per cent-square-root or log transformations were tried until homogeneity of variance was achieved. If the value obtained for F was significant at the 5% level of confidence, then a variation of Duncan's Multiple Range Test was employed to find significant differences between means (D). In the present study, instead of ranking n means according to their magnitudes, Duncan's Studentized value for comparison at the nth rank is used to derive the significant difference (D). This method is more conservative than either the commonly used Least Significant Difference or Duncan's original method but embodies the convenience of the former.

Experimental Results

Effect of various combinations of food and daylength on the large yellow larvae

Large yellow larvae were exposed to short day without food starting 2–2–68 and 3–31–68, to long day without food starting 2–2–68 to 5–11–68 (5 replicates), to short day with food starting 2–2–68 to 5–11–68 (6 replicates), and to long day with food starting 2–2–68 to 5–11–68 (4 replicates). They were exposed to experimental conditions for at least 30 days unless all larvae had either developed or died prior to that time.

As shown in Table I, short day without food failed to elicit any substantial pupation in the large yellow larvae. Either long day without food or short day

Table I

Developmental response of large yellow larvae to various combinations of food and daylength in n replicates of 50 larvae each (mean per cent ± standard deviation)

	Per cent Pupation		n
	After 15 days	After 30 days	
Long day fed	81.0 ± 11.2	89.8 ± 2.7	4
Long day starved	6.0 ± 4.4	6.4 ± 4.1	5
Short day fed	14.7 ± 13.8	16.3 ± 13.4	6
Short day starved	0.0 ± 0.0	0.0 ± 0.0	2

with food elicited some pupation but never more than 20% for the former or 40% for the latter. Long day with food, on the other hand, elicited substantial pupation, even after only 15 days. Thus, while either food or long day photoperiod is necessary for some development, a substantial response is not evoked unless the food and long day stimuli occur simultaneously. It would further appear that 90% of all potential development is realized after only 15 days experimental time. For reasons of convenience, 15 days was therefore chosen as the standard assay period to be used in subsequent experiments.

Effect of daylength on total amount of food consumed

On 4–18–68, two groups of 50 diapausing larvae were placed in $\frac{1}{2}$ oz jars, one larva per jar. One group was placed on long day, the other on short day. Each *Chaoborus* larva was offered twelve *Culex* per day for five days. Thereafter, the food was removed and the *Chaoborus* observed for ten additional days. Development was then scored and the experiment terminated. To increase homogeneity of food particle size, only those mosquito larvae which passed through a 2×2 mm mesh but not a 1×1 mm mesh were used. Each day, the number of mosquito larvae eaten was tabulated, the remainder removed, and twelve fresh larvae added.

Of the 60 mosquitoes offered, each *Chaoborus* larva ate an average of 30 on long day and 32 on short day. On long day, of 45 surviving individuals, the 44 pupating larvae ate an average of 30 mosquitoes while the only non-developing larva ate 36. On short day, of 49 surviving individuals, both the 19 pupating and the 30-non-developing larvae ate an average of 32 mosquitoes. Daylength, therefore, has little or no effect on the amount of food consumed.

Effect of food or long day on sustaining development

The following experiments are designed to determine whether long day alone or food alone following long day with food is stimulatory, neutral, or inhibitory with respect to development induced by long day with food. A series of 10 oz jars was each provided with 50 diapausing larvae. These groups of larvae were then exposed to 0, 1, 2, 3, or 4 long days with food after which time they were placed on short day without food (experiment started 4–18–68 and replicate started 5–11–68), on long day without food (experiments started 4–18—68 and replicate started 5–11–68), or on short day with food (experiment started 5–11–68) for a total of 15 days experimental time after which development was scored and the experiment terminated.

In all experiments, there was a significant correlation between the number of days exposure to long day with food and the amount of subsequent development (Fig. 1). All exposures to one long day with food elicited more development than the control, *i.e.,* 15 short days without food (Fig. 1A), 15 long days without food (Fig. 1B), or 15 short days with food (Fig. 1C). Furthermore, a significant difference was noted between the responses to any two day increment in exposure to long day with food, *i.e.,* 3 or more days exposure to long day with food elicited significantly more development than 1; 4 days elicited more than 2 or 1, *etc.* (Duncan's Multiple Range Test after arcsin transformation: $F = 47.67$; $P < 0.01$; $D = 11.0$).

After subtracting out the control values, there was no significant difference between the amount of development elicited by short day without food, long day without food, or short day with food following long day with food (analysis of variance: $F = 1.2$; $P > 0.25$). It would thus appear that neither long day nor food has any ability to retard or sustain development once it has been initiated.

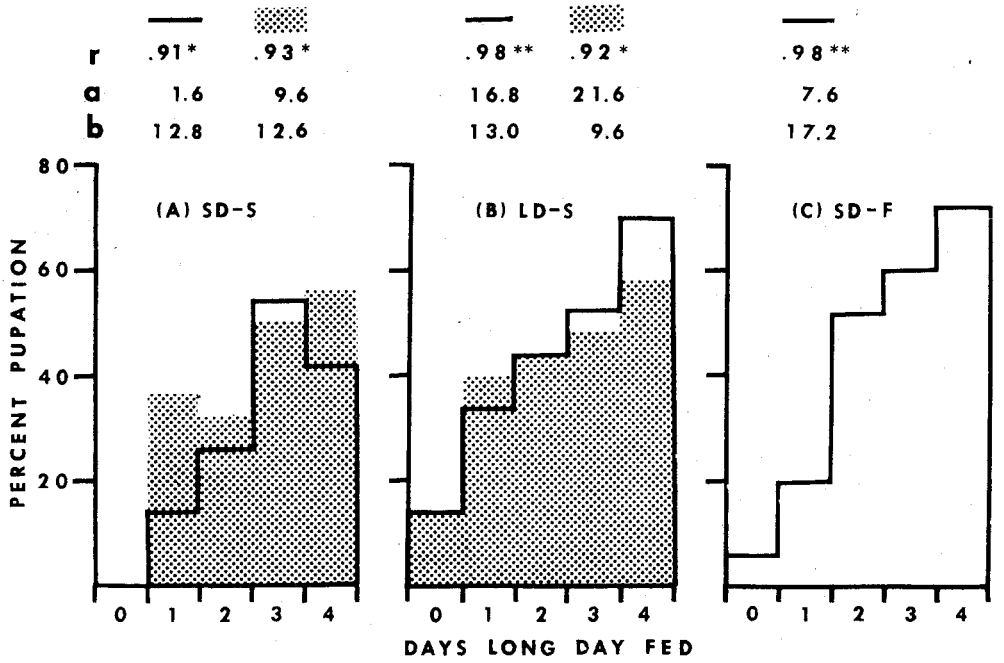

FIGURE 1. Ability of long day and food to sustain development. Diapausing larvae were exposed to long day with food for 0–4 days after which they were placed on short day and starved (A), long day and starved (B), or short day and fed (C), in experiments starting 4–18–68 (solid line) or 5–11–68 (shaded outline). Abbreviations are: *r*, coefficient of correlation between days long day fed and and per cent pupation; *, significant correlation; ** highly significant correlation; *a* and *b*, constants in the regression equation: % pupation $= a + b$ (days long day fed); SD, short day; LD, long day; F, fed; S, starved.

Persistent effects of long day

This experiment was designed to test whether previous exposure to long day without food would enhance the developmental response of larvae subsequently exposed to short day with food. Diapausing larvae were placed in a series of 10 oz jars, 50 animals to a jar. They were then exposed to long day without food for 0 (control), 1, 2, 3, 4, 5, or 6 days after which they were transferred to short day and provided an excess of food for a total experimental time of 15 days after which development was scored and the experiment was terminated. The initial experiment starting 5–11–68 was repeated with fresh animals starting 6–1–68.

Unlike the response to long day with food (Fig. 1), there was no correlation between the duration of exposure to long day without food and the amount of development elicited (Fig. 2, solid line) (after subtracting out the control values: $r = 0.14$; $P > 0.85$ for 10 df). While there were no significant differences among responses elicited by 1, 2, 3, 4, 5, and 6 days exposure to long day without food,

2 and 6 days exposure elicited a significantly greater amount of pupation than did the control (Duncan's Multiple Range Test after arcsin transformation: $F = 5.00$; $P < 0.05$; $D = 12.8\%$). These results suggest the photoperiodic information may be accumulated to some extent by *C. americanus* but that the synergistic interaction of food and photoperiod requires both parameters simultaneously rather than sequentially.

Persistent effects of food

The following experiments reciprocate the ones above and are designed (1) to test whether trophic information is stored by *C. americanus* and (2) to substantiate the inference that the synergistic interaction of food and long day requires simultaneous input. For this purpose, diapausing larvae were placed in a series of 10 oz jars, 50 animals to a jar. They were then exposed to short day with an excess of food for 0 (control), 1, 2, 3, 4, 5, or 6 days after which time the food was removed and the *Chaoborus* larvae transferred to long day conditions without food for a total experimental time of 15 days. The initial experiment starting 4–18–68 was repeated in duplicate, both of the latter experiments starting 5–11–68.

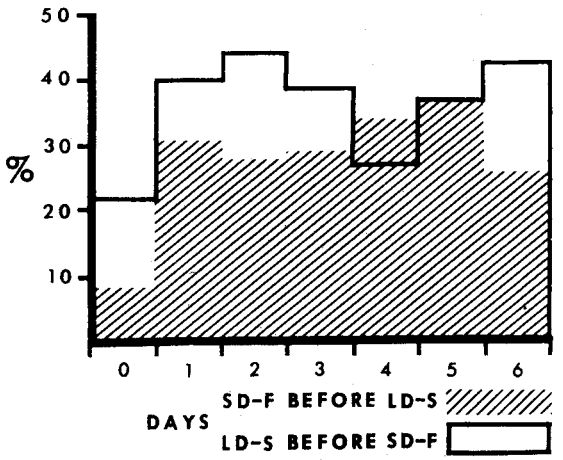

FIGURE 2. Developmental response of larvae on short day with food after 0–6 days exposure to long day without food (average of two replicates) and of larvae on long day without food after 0–6 days exposure to short day with food (average of three replicates); ordinate, per cent pupation; other abbreviations as in Figure 1.

As in the case of long day followed by food, there was no correlation between the duration of exposure to food and the amount of development elicited (Fig 2, diagonal shading) (after subtracting out the control values: $r = 0.07$; $P > 0.99$). On the other hand, all of the experimental values were significantly greater than the controls, even though they were not significantly different from each other (Duncan's Multiple Range Test after arcsin transformation: $F = 3.03$; $P < 0.05$; $D = 13.2\%$). These results confirm the concept that the synergistic response to food and long day requires simultaneous rather than sequential input of these factors.

Inspection of the larval mortality (Fig. 3A, solid dots) and pupal survivorship (Fig. 3B) after 15 days experimental time reveals that both are highly significantly

correlated with the number of days feeding on short day. This observation indicates that food is stored—as long as 15 days—and may be called upon to support larval life or adult development.

The following experiments, designed to further elucidate the long-term effects of food, employed the duplicate experiments started on 5–11–68 as described above. At the end of 16 days total experimental time, the duplicates were split into two groups of 7 experimental populations each, having received from 0 to 6 days prior feeding. The sample size in each experimental population then consisted of that number of larvae alive and undeveloped at the end of 16 days. One group was given a one day food pulse, the other a four day food pulse, both under long day conditions. After pulse, the larvae remained on long day without food until a total of 31 days experimental time had elapsed. Larval mortality, pupal survivorship, and amount of development were then scored and the experiment terminated.

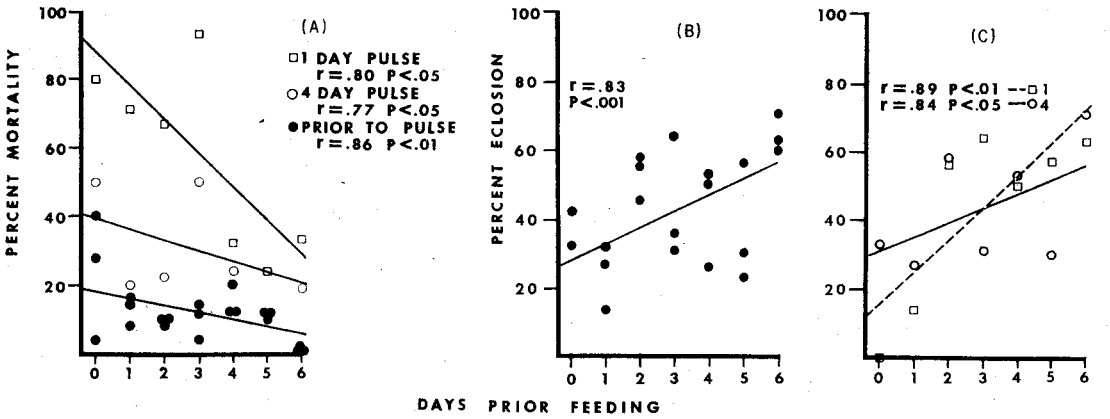

FIGURE 3. Larval mortality (A) and pupal survivorship (B–C) after 0–6 days exposure to food on short day followed by continuous long day without food. Percentages after 15 days experimental time are shown by solid dots ●. On day 16, duplicate experimental groups were provided a one day (□) or a four day (○) food pulse on long day. The food was removed after this time and the larvae again placed on long day without food. Per cent larval mortality and per cent eclosion were scored on the 31st day, using the number of living, undeveloped larvae on day 16 as the sample size.

Among both the one day and four day food pulsed larvae, there was a significant correlation between prior feeding ten to thirty days beforehand and larval mortality (Fig. 3A) or pupal survivorship (Fig. 3C). Development in response to a one day or a four day food pulse also appeared to be proportional to the number of days prior feeding when per cent response was calculated on the basis of the number of larvae alive at the initiation of the food pulse experiments (*i.e.*, the 16th day). But, if percentage development is calculated on the total number of this sample size surviving until the 31st day (n = number of living larvae + number of pupae), then development was no longer proportional to the number of days prior feeding ($r = 0.47$; $P > 0.05$). It would thus appear that food stored from remote feeding is capable of maintaining larval life or sustaining adult development but does not contribute to the termination of diapause in *C. americanus*.

Discussion

Food very clearly is accumulated and stored by the larvae; the effects of this stored food are readily seen contributing to larval survivorship (Fig. 3A) and to adult development (Fig. 3B, C). Yet, these same nutritional reserves do not enable the larvae to respond developmentally to long day in the absence of continued feeding. The apparent inconsistency is easily explained by ascribing a dual role to food in the developmental process. First, food is functioning as an energy source; second, food, or feeding, is acting as a physiological trigger to initiate development. Without long day photoperiod, food still increases the nutritional plane of the larvae but is unable to trigger substantial development. Thus, larvae exposed to food on short day and then exposed to long day without food do not show an increasing response related directly to the number of days prior feeding, nor do they show a food independent synergistic response to long day without food after a certain "critical number" of days prior feeding. Moreover, when careful comparison of food intake is made, it becomes clear that the above failure to respond to feeding on short day is not due to a short day repression or long day stimulation of feeding.

The simplest nutrition-independent explanation of food as a physiological trigger would be stimulation of a neuroendocrine reflex by food as an environmental cue. This model is not novel in endocrine control of development but is paralleled by the control of moulting in the bugs *Rhodnius* (Wigglesworth, 1933) and *Cimex* (Kemper, 1931). In *Rhodnius*, the moulting stimulus is initiated by stretch receptors on the intersegmental muscles of the abdomen which is sizeably distended after a blood meal (Wigglesworth, 1934). Undoubtedly, *Rhodnius* derives nutrition from these blood meals since (1) the moult occurs within a constant time interval after a normal blood meal, regardless of the duration of the fasting period prior to the meal and (2) the animal may continually consume small amounts of blood and yet refrain from moulting indefinitely. The important point is that the induction of moulting by food is effected by independently of these nutritional benefits.

If food in *Chaoborus* is also acting via a neuroendocrine reflex independently of nutritional benefits, then not only should the persistent effects of food stimulation be of short duration as seen above (Fig. 2), but should also be of the same order of magnitude as the persistent effects of long day stimulation. The response of animals exposed to 1 to 6 long days without food before exposure to short day with food bears out this argument (Fig. 2, solid line).

A paradox remains both in the experiment exposing animals to short day with food before long day without food, and in the experiment exposing animals to long day without food before short day with food: some or all experimental animals respond significantly more than do the controls, even though both food and long day only interact synergistically when they occur simultaneously This problem may be clarified if one ascribes a physiological latency to both food and long day. Such a latency is not especially new since diapause in many insects may be cued by stimuli perceived in previous instars or even the previous generation. Thus, in the present case, while the experimental design implies that diapausing larvae are receiving 1–6 days feeding prior to long day, a lag in the larvae themselves gives the physiological impression that food is being perceived from 2–7 days. In all the experimentals, but not in the controls, there will then be one day's overlap during

which the larvae receive both the latent food stimulus and the newly impressed long day stimulus; hence, one day's worth of synergistic response.

Essentially, this model defines the period of physiological latency as that time during which synergistic interaction of food with long day is taking place. Thus, the actual duration of food latency is the number of days on long day with food necessary to induce development equal to the average development resulting from 1–6 days feeding on short day before exposure to long day without food. The latter value is 29% development for the experiment starting 4–18–68 and 32% development for the experiment starting 5–11–68. The regression equations for development as a function of days long day fed before long day starved are (for experiments started 4–18–68 and 5–11–68, Fig. 1B):

$$4\text{–}18\text{: \% development} = 17 + 13.0 \text{ (days long day fed)}$$
$$5\text{–}11\text{: \% development} = 22 + 9.6 \text{ (days long day fed)}$$

Solving the equations for the parameter (days long day fed), given 29% and 32% development, the latent period of food is 0.9 days for both 4–18 and 5–11. Similarly, the latent period of long day is found from the average pupation induced by 1–6 days long day without food before exposure to short day with food in experiments started 5–11–68 and 6–1–68, 42% and 34% development, respectively. The regression equation for the only estimate of inductive capacity of long day with food before exposure to short day with food on 4–18 (Fig. 1C) is:

$$\text{\% development} = 8 + 17.2 \text{ (days long day fed)}$$

yielding latent periods of 1.5 and 2.0 days. These latter values should be regarded only as estimates since the pertinent experiments were not started concurrently and since this source of error was compounded because the experiments were in progress during that time of year when the effects of long day were declining and those of food increasing (Bradshaw, 1969). Nonetheless, it can be concluded that the latent period of food probably does not exceed that of long day. The latent periods of these two inductive cues thus appear to be similar and completely consistent with the concept of nutritional independence of food in the inductive process. It should be noted that nothing here is meant to imply that food does not make a substantial, long-term contribution to the developing pupa or adult (Fig. 3); the important point to be made again is that this nutritional use of food is independent of the use of food as an environmental signal.

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Summary

1. Food and long day photoperiod interact synergistically to effect the termination of larval diapause but the input of food and long day must be simultaneous rather than sequential for the synergistic effect to take place.

2. Daylength does not affect food consumption.

3. Neither food alone nor long day alone is capable of augmenting development initiated by long day with food; likewise, short day without food does not appear to retard development once it has been initiated.

4. Food is nutritionally capable of affecting larval and pupal survivorship for up to several weeks but its capacity to interact synergistically with long day to induce development persists for only a day.

5. The capacity of long day to interact synergistically with food to induce development persists for $1\frac{1}{2}$ to 2 days.

6. Food, therefore, is probably acting via some neuroendocrine reflex as an environmental cue independently of its nutritional contribution to the overwintering larva or to the resultant pupa and adult.

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