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LARVAL SENSITIVE STAGE FOR THE ACTION OF EXTERNAL FACTORS CONTROLLING THE OCCURRENCE OF DIAPAUSE IN THE CABBAGE MOTH PUPA, *BARATHRA BRASSICAE* LINNÉ

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I. Introduction

The action of temperature and photoperiod upon the occurrence of diapause is widespread among many species of insects (cf. LEES, 1955). These factors have been regarded by some authors as "token stimuli", which operate upon insects long before the actual onset of diapause. Thus, the caterpillar of the cabbage moth is highly sensitive to the action of temperature and photoperiod, while diapause supervenes, in effect, only after pupation (MATSUMOTO et al., 1953; OTUKA and SANTA, 1955; UCHIDA and MASAKI, 1953, '54). During this time-lapse between the sensitive period and entrance into diapause, the growth pattern of the pupae must be predestined. This process of determination is more or less irreversible. Once diapause has been induced, any "token stimuli" can no longer exert their influence. The critical stage for the action of the "token stimuli" should be determined at first to analyse the sequence of events involved in the induction process of diapause.

The present investigation has been carried out along this line. A particular attention has been directed to the sensitive period in the larval life of the cabbage moth, during which temperature and photoperiod exert their influence upon the incidence of the pupal diapause. Conspicuous effects of these physical factors have been reported in detail by the authors cited above.

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II. Material and Method

The experiments have been carried out during 1952–55. The material were collected as egg-batches in the field in the University campus at Sapporo during the seasons. In mid-summer or winter, when the egg-batches of the cabbage moth could not be obtained in the field, the stock of chilled diapausing pupae was incubated for emergence at a high temperature, and the eggs obtained from the resulting moths were used. The larvae were fed on various cruciferous vegetables—chiefly varieties of the cabbage. They were kept in petri-dishes of 10 cm. diameter × 3 cm. depth, or in small breeding jars of 10 cm. diameter × 10 cm. depth which were covered with perforated zink gauze. Food plants were renewed each day, and sheets of paper were placed in each container on the bottom, to absorb excessive moisture. A care was taken to avoid the adverse effect of over-crowding of the larval population in a vial; the number of individuals per vial was gradually reduced as the larvae grew larger. When the larvae were full-fed, and took no amount of food, they were removed to another petri-dish on the bottom of which sheets of moist paper were placed. The larvae pupated successfully on the moist paper.

Within a day after pupation, the pupae were removed from each experimental condition, individually kept in a vial and placed at 26°C. for testing the diapause. Daily observation was made of adult emergence from the resulting pupae for three months. The exact length of the pupal stage of each individual was recorded, when the moth emerged during this observation period. The pupal stage of non-diapausing type lasted from 13 to 17 days at 26°C. After the emergence of non-diapausing insects, there was usually no emergence of moths from the remaining pupae during three months, except an interesting anomality which will be stated in a later section. The diapausing and non-diapausing pupae were easily separated even on the seventh day of pupation. At that time, the eyes of the developing moth were already well pigmented in non-diapausing insects, and could be easily seen through the pupal cuticle; while the appearance of diapausing pupae remained unchanged from the second day of pupation.

Using above procedures, the larvae were subjected to a number of combinations of diapause-preventing and diapause-inducing conditions, and the proportion of diapause in the resulting pupae were determined. The detail of each treatment will be noted in the following sections.

III. Experimental

Under field conditions, temperature and photoperiod act concurrently upon the incidence of diapause. Even under experimental conditions, the absolute separation of these factors is impossible. Sometimes, the two factors act in concert with each other, or, at the other times, in opposing direction. But a certain range of temperature excludes the photoperiodic effect almost completely, and, in contrast, the effect of typical short or long photoperiod appears suprisingly stable over a considerably wide range of temperature. For the present purpose, diapause-inducing and diapause-preventing factors will be defined as follows, to exclude complications.

a) Diapause-inducing temperature: a temperature which induces diapause in 100% of the resulting pupae in the absence of light, e.g., 20°C. or below.

b) Diapause-preventing temperature: a temperature which in darkness prevents diapause in 100% of the resulting pupae, e.g., 26°C.

c) Diapause-inducing photoperiod: a photoperiod which induces diapause in 100% of the resulting pupae in the moderate range of temperature between 20°–26°C., e.g., 10–12hr. per day.

d) Diapause-preventing photoperiod: a photoperiod which prevents diapause in 100% of the resulting pupae in the temperature range mentioned above.

By transferring the batches of larvae *a* to *b* and vice versa, or from *c* to *d* and vice versa at various stages of the larval development, the sensitive stage was tested, except where otherwise stated.

(i) *Temperature*

To facilitate a comparison, experimental results already published is again represented in Figure 1 (Uchida and Masaki, 1953). In this experiment, batches of 20–50 larvae were first kept at a diapause-preventing temperature of 26°C., and at the end of each stadium transferred to a diapause-inducing temperature of 19°C. The results show that, though there was a minor anomality, the proportion of diapausing pupae increased in accordance with the length of diapause-inducing temperature. When the low temperature covered the all larval stages, diapause was almost universal among the resulting pupae. The percentage diapause gradually decreased as the duration of the low temperature was shortened, and finally it reached zero when the

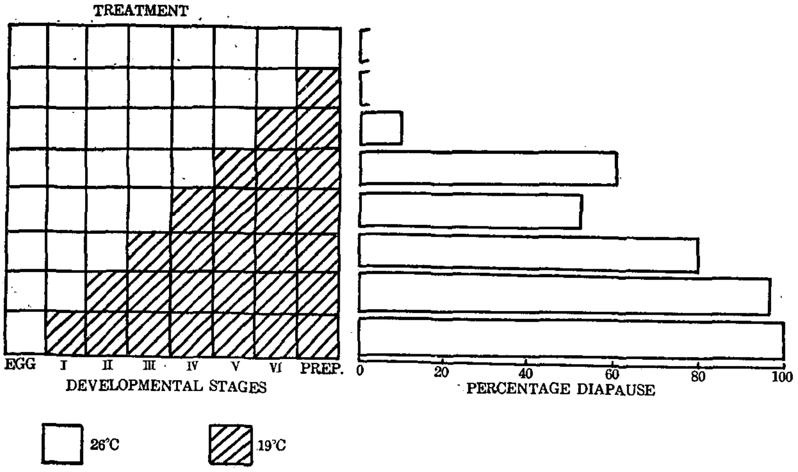

Figure 1. The effect of transferring from a high temperature of 26°C. to a low temperature of 19°C. at the beginning of each consecutive larval instars upon the percentage diapause in the resulting pupae. The larvae were kept in darkness. Each histogram is based on about 20-50 pupae.

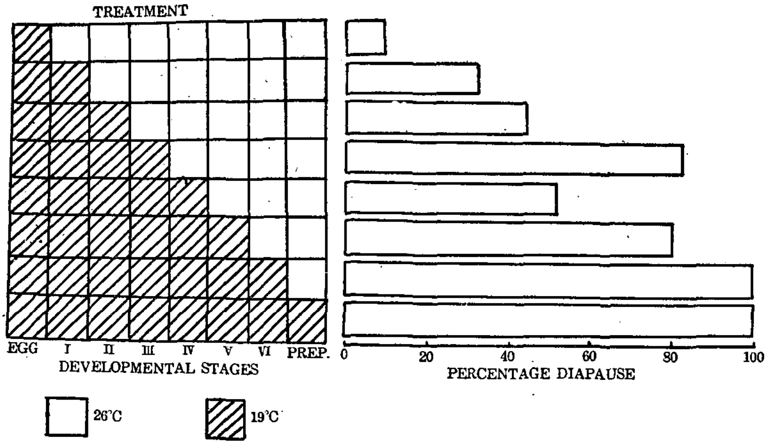

Figure 2. Same as Figure 1, but the direction of temperature change is reversed. Each histogram is based on 30-40 pupae.

low temperature was effected only in the prepupal stage. (Prepupal stage is here defined as the non-feeding period of the last larval instar prior to pupation.) Except for the prepupal stage, thus at all instars the larvae seemed to be sensitive to the action of temperature upon the induction of diapause.

In another series of experiments, which was conducted later, batches of 30–40 larvae were transferred from the diapause-inducing temperature to the diapause-preventing one at various stages. The results are illustrated in Figure 2. The general tendency was as similar as the preceding experiment. Again, the percentage diapause increased in proportion to the duration of the low temperature during the larval stage, irrespective of the reversed direction of the transferrance. From these facts, it can be concluded that all larval instars are more or less sensitive to the diapause-controlling action of temperature in the absence of photoperiodic effect. Whereas temperature fails to alter the pattern of development—diapause or non-diapause—just after pupation. A critical period for the action of temperature must therefore lie at any stage of the final larval instar. In Figures 1 and 2, it will be noted that the effect of temperature suddenly drops from the fifth instar to the last, and that it has no effect in the prepupal stage. The latter might be due to too short period of that stage (about 2–3 days at 26°C. and 4–5 days at 19°C.) to establish the diapause-inducing action of the low temperature; or due to the insensitivity of this stage to temperatures. At any rate, some critical change might occur during the final larval stage in regard to the determination of diapause. This supposition was tested by the following experiments.

For the determination of the sensitivity of the larva during a definitely short period, an extreme condition, which will complete its effect within a relatively brief period, should be necessary. By using a temperature as low as 15°C., which acts intensely upon the insect to induce diapause, our present need was satisfied. The treatments are shown in left diagrams of the Figure 3. Under these experimental conditions, particularly adverse moisture conditions in a low temperature cabinet, the vigour of the larvae seemed to be reduced, causing a high mortality before pupation. Although only a small number of pupae was obtained by such reasons, the results in Figure 3 indicate clearly a marked depression of the sensitivity to the low temperature in the final larval stadium. Diapause was almost universal among the

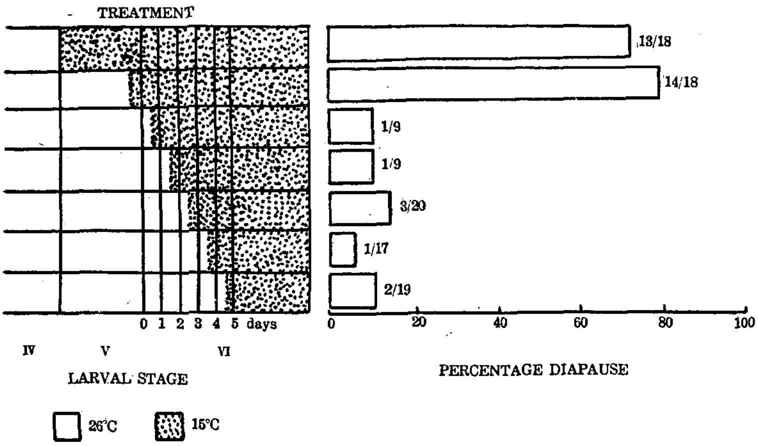

Figure 3. The critical period for the diapause-inducing action of low temperature. Numerals on the right of each histogram represents the number of diapausing pupae per total pupae.

resulting pupae, when the larvae were transferred from 26°C. to 15°C. at the beginning of the fifth instar. The same was also true even when the larvae were subjected to the low temperature from the moulting sleep at the end of penultimate instar. After the ecdysis and initiation of feeding, the effect of low temperature rapidly decreased. Thus, when the larvae were removed 0–1 day after the moulting from the high temperature (26°C.) to the low, the latter no longer exerted its effect to induce diapause. Up to the fifth day of the final instar, other batches of larvae were transferred each day from the high temperature to the cold cabinet. The results show no substantial fluctuation in the percentage diapause among these batches; a small number of pupae entered diapause throughout these treatments.

In the light of this experiment just mentioned, the disappearance of temperature effect in the last larval stage seemed to be due to the insensitivity of the insect to the action of this factor. The validity of the present reseults is considerably reduced by the high larval mortality in the experiment. The conclusion must be deferred to much more extensive experiments in future. Nevertheless, even with such a reservation, a remarkable similarity may well be pointed out between the results by Way and Hopkins (1950) and those herein presented. Way and Hopkins found in the tomato moth, *Diataraxia* *oleracea*, that the photoperiod is particularly operative during the moulting sleep at the end of penultimate instar; and that the effect is almost completely dissipated after the moulting. This is just comparable to the results illustrated in Figure 3, where, however, temperature is the operative factor for controlling diapause. Further, the cabbage moth and the tomato moth differ each other in that the latter is sensitive to photoperiod only during a very short period around the final larval moult, while the former is sensitive during almost entire larval stage before the critical period. A more detaild discussion will be given in a later section.

(ii) *Photoperiod*

The experiments pertaining the action of photoperiod were conducted at temperatures between 20°–26°C. The larvae were reared in a light-proof wooden box, or in a dark room. As a source of light, a 10 W. tubular fluorescent lamp was used, and the daily length of illumination was regulated by means of a time switch. In the first experiment, a diapause-inducing photoperiod covered two consecutive larval instars. Three batches of larvae were subjected to a short photoperiod of 12 hr. during the 1st–2nd, 3rd–4th, and 5th–6th instars,

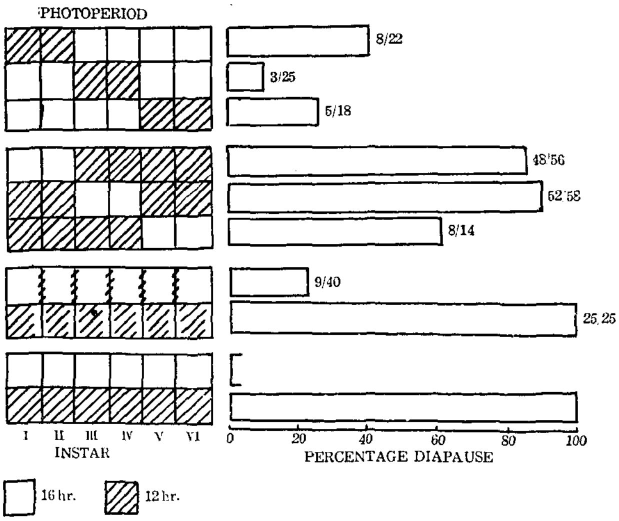

Figure 4. The effect of photoperiod at various stages of the larval development upon the percentage diapause in the cabbage moth pupae. Numerals on the right of each histogram indicate the number of pupae.

respectively, and during the other instars kept under a long photoperiod of 16hr. The results are illustrated in histograms in Figure 4. An inspection of this figure indicates no limited sensitive stage for the action of diapause-inducing short-photoperiod. Fluctuation in the percentage diapause among the batches shows no significant tendency. The short photoperiod, covering only two consecutive larval instars, resulted in relatively low percentages of diapause in the batches of pupae, as compared with the results obtained in the following experiments.

In the next, the treatments are just contrary to the preceding tests. Two consecutive larval instars were here accepted diapause-preventing long-photoperiod, and the other instars were spent under the short-photoperiod. The results show that the sensitivity to the diapause-preventing photoperiod was not limited to any particular instar. There was a considerbale reduction in percentage diapause when the larvae were treated with long photoperiod during the last two larval stages, as compared with the other two treatments. Such a reduction may not, however, be substantial. In general, the occurrence of diapause in these three batches was much more pronounced as compared with the preceding series of tests. This pronounced incidence of diapause is apparently caused by longer duration of short-day treatment. In both series of experiments, the duration of photoperiodic action was more substantial than the developmental stage of the larvae in determining the percentage diapause in the resulting pupae. In other words, there was no particular stage in the larval development, in which the cabbage moth was most highly sensitive to photoperiod. The larvae retained their sensitivity to light throughout the entire feeding stage.

The caterpillar of the cabbage moth, like other immature insects, undergoes cyclical changes in physiological activities which are marked by the occurrence of an ecdysis. This physiological cycle is primarily caused by the larval endocrine system controlling growth and moulting (WIGGLESWORTH, 1954). Since diapause is an arrest of morphogenetic activity, in most cases, and is closely correlated with the neurosecretory system (WILLIAMS, 1946), it seems natural to expect that the sensitivity to diapause-inducing stimuli may be changed during this endocrine secretory cycle. This was tested by applying a particular photoperiod to the caterpillar of the cabbage moth during each moulting sleep. In one batch, as shown in Figure 4, a diapause-inducing photoperiod was given only in each moulting sleep, covering the end of a given instar to the beginning of the next. During the feeding stage in each instar, the larvae were kept under long, diapause-preventing photoperiod. The reversed treatment was applied to another batch of larvae; the larvae were kept under diapause-inducing photoperiod except at each moulting sleep in which a long photoperiod was given. In practice, a moulting sleep did not last more than a day in every instar, so that in these traeatments a short or long photoperiod was interrupted by one antagonistic photoperiod at the end of each instar. The larvae entering a moulting sleep during light period were removed to the opposite treatment, and returned again after experiencing a unit cycle of the photoperiod. As illustrated in Figure 4, the results show no particular significance of moulting sleep in accepting the photoperiodic action. The diapause-inducing photoperiod covering each moulting sleep resulted in a small number of pupae entering diapause. Also, the diapause-preventing photoperiod was failed to overcome the diapause-inducing effect of a short photoperiod, when it acted only in each moulting sleep. The control experiments, in which all larval stages were exposed uniformly to either of a long or short photoperiod, are shown on the bottom in Figure 4. All larvae reared under a short photoperiod entered diapause after pupation, while the opposite treatment gave exclusively non-diapausing pupae.

From these experiments, a conclusion can be reached that the cabbage moth is more or less sensitive to diapause-inducing or -preventing effects of photoperiod throughout the entire larval stage.

(iii) *Anomality in the duration of diapause*

Throughout five years of rearing the cabbage moth under the laboratory conditions, the diapausing and non-diapausing pupae were fairly distinct, and no intermediate type had been observed. The non-diapause pupae normally complete metamorphosis into the adult in about a fortnight at 26°C., and the spread of variation in length of the pupal stage is always small. It ranges from 13 to 17 days, and any specimen seldom falls out of this range. When the larvae are bred under diapause-inducing conditions, the resulting pupae show no sign of development for over six months. On the other hand, under diapause-preventing conditions, all the resulting pupae give rise to adults at a fairly uniform rate. If intermediate conditions prevail during the larval stage, the pupae can be separated precisely into two categories–diapause and non-diapause types. This distinct separation of the two growth pattern of the pupae is an ecological or physiological characteristic of the Hokkaido populations of the cabbage moth, which are differentiated by the absence of aestival diapause from the Honshu populations (MASAKI, 1956). In most of the diapausing pupae that were obtained in the experiments stated in the preceding subsections, the diapause condition persisted for a very long period of time at 26° C. Two pupae, however, resumed development in a relatively short time. The length of their pupal stage was 41 and 42 days, respectively. A more remarkable occurrence of untimely emergence of moths was observed from a batch of pupae which had been bred in July to August under an artificial day-length of 14.5 hr. in the larval stage. Of total thirty-six pupae, eight were of non-diapause type whose pupal stage ranged from 15 to 17 days. The remaining pupae, which were thought to be diapausing pupae, were kept at the room temperature of 18–26° C. after the emergence of non-diapausing pupae. All of them unexpectedly emerged as moths before winter. The exact length of individual pupal stage was recorded, that is presented in Table 1. In the same season, a similar untimely emergence was also observed in another batch of pupae. In this case, the larvae were transferred from diapause-inducing conditions to diapause-preventing ones, and the resulting pupae were kept constantly at 26°C. The frequency distribution of the pupal stage is presented in the third column of Table 1.

TABLE 1. Frequency distribution of abnormal length of the diapausing pupal stage.

Length of pupal stage in days	The length of photoperiod in the larval stage	
	14.5 hr.	From 11 to 16 hr.
	Temperature in the pupal stage	
	18°-20°C.	26°C.
21-30	0	4
31-40	0	5
41-50	7	3
51-60	14	5
61-70	5	6
71-80	1	1
81-90	1	1
Total	28	25

On the other hand, in the batch which was transferred conversely from a long photoperiod to a short one, the untimely emergence of moths was observed in only four cases out of seventeen.

Before the season in 1954, the untimely emergence from diapausing pupae was seldom observed in a number of breeding tests, and again in the season, 1955, only a very few cases of this occurred. These anomalous development of diapause pupae resembles that of the aestival-diapause pupae of the southern local populations of the cabbage moth. One may emphasize that the Hokkaido populations (at least in Sapporo) have also a disposition to enter aestival diapause as well as the southern populations. But this could not be accepted for certain reasons. First, the seasonal emergence of the cabbage moth under the field conditions at Sapporo hardly suggests the existence of aestival diapause in the summer generation. The bait-trap data of the Hokkaido Agricultural Experiment Station, that were analysed by MATSUMOTO (1956), show that the first and second mode of the cabbage moth emergence are normally at the end of June and the end of August, respectviely. The two peaks of moth emergence are thus separated from each other by intervening two months, which are just comparable to the time required to complete a life cycle of non-diapausing type. It may be inferred with a considerable accuracy that, at least under prevailing climatic conditions at Sapporo, the cabbage moth alternates diapausing and non-diapausing generations each year, without intervention of any aestival dormancy. Indeed, most of the experimental results hitherto published indicate no evidence of aestival diapause at Sapporo. On the other hand, aestival diapause occurs with a considerable regularity among the pupae of southern populations as a result of the response to long photoperiod and high temperature during the larval stage. It can be concluded that the untimely emergence of moths from diapausing pupae seems to be an anomalous phenomenon in the Sapporo population of the cabbage moth. The cause of such an anomality is quite unknown. In fact, it could not be determined whether an anomalous condition upsets the presistence of diapause, thus accelerating the moth emergence, or whether it retards the normal course of non-diapausing development.

III. Discussion

The stage at which diapause supervenes is fairly defined for each species of insects. When a diapause is facultative, the stage at which the external factors are effective on the occurrence of diapause is also characteristic of the species. And these two stages are more or less separated in time. This is a characteristic feature of diapause, resembling the process of "determination" in morphogenesis. A number of examples of this is illustrated in the comprehensive review by LEES (1955). In the cabbage moth, the present experiments show that the larva is more or less sensitive to both photoperiod and temperature throughout the entire growth stage, while the actual manifestation of the effect of these physical factors are delayed until after pupation—the diapause supervenes only in the pupal stage. In this respect, the cabbage moth resembles the saturniid, *Antheraea pernyi*, which is also sensitive to photoperiod throughout the larval life (TANAKA, 1950–51). Evidence suggests that there is a critical period in the late stage of the larval life, as to the sensitivity of the cabbage moth to temperature. The determination process must of course precede the actual occurrence of diapause. It is reasonable, therefore, to suppose the existance of the critical period between the time of pupation and the beginning of the insensitive period. In the cabbage moth, the time-lapse between these two points is rather short. A similar but more definite insensitive period was found in the last larval instar of the tomato moth (WAY and HOPKINS, 1950). It is, however, unlikely that all the effects of external condition which have been accumulated during the preceding stages are first set in motion in this brief period. In this connection, suggestive observations were made by SANTA and O-TUKA (1955) on the spermatogenesis in the cabbage moth larvae which had been reared under diapause-inducing or diapause-preventing conditions, respectively. The differentiation of the male sex cells was retarded in the diapause-predestined larva, and no spermatozoa could not be found even on pupation; while in the larva reared under diapause-preventing conditions, the prepupal testes already contained some spermatozoa. Yet remarkably, the size of the gonads was much smaller under diapause-inducing conditions as early as at the fourth moult than under the diapause-preventing conditions. The delayed spermatogenesis in the diapausing insect has also been confirmed by the present writer (unpublished). A similar example was found in *Chilo suppressalis* by FUKAYA (1951), and in *Pyrausta nubilalis* by PARKER and THOMPSON (1927). These facts may indicate that the diapause-inducing factors have already impressed their effects upon the fundamental physiological state of the insects. In the cases of the cabbage moth, it is notable that the larva retains the ability to respond to the opposing conditions until the beginning of the last larval instar while its spermatogenesis is being promoted by diapause-preventing conditions. A precise conclusion must await further analyses, in which histological and endocrinological events may be correlated with diapause-controlling physical factors.

IV. Summary

The present paper deals with the experiments which were carried out to determine the larval sensitive stage for the action of temperature and photoperiod upon the occurrence of the pupal diapause in the cabbage moth, *Barathra brassicae* LINNÉ.

In the experiments, batches of larvae were transferred from a low temperature to a high or vice versa, and from a long photoperiod to a short or vice versa, at various stages of larval development. The percentage diapause among the resulting pupae was examined in each batch by recording the exact length of individual pupal stage. The results show that there is no limited sensitive stage for the action of these two external stimuli during the entire length of the larval stage, except for the final larval instar. In every case, the percentage diapause was approximately proportional to the length of the diapause-inducing treatments in the larval period.

The sensitivity to low temperature as a factor inducing the diapause is, however, markedly reduced after the ecdysis from the fifth to sixth instars. During the prepupal stage the external conditions no longer exerted their effects upon the occurrence of diapause in the resulting pupae. In some experiments, untimely emergence of moths from diapause pupae was observed, and yet the real cause has never been acertained.

V. Acknowledgement

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