FURTHER EXPERIMENTS ON THE THERMAL RELATIONS OF THE DIAPAUSE DEVELOPMENT IN THE CABBAGE MOTH PUPA, *BARATHRA BRASSICAE* LINNÉ.

(Lepidoptera : Noctuidae)

By

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I. Introduction

In the cabbage moth pupa, the existence of two physiologically different phases of development has been elucidated. One is morphogenesis, and the other is diapause. These two processes are remarkably different in their temperature relations, and the latter process has been known to require a particularly low range of temperature which is suboptimal for the other process (OTUKA and SANTA, 1956; MASAKI, 1956). At the same time, it has been suggested that the temperature requirement of the diapausing pupae is far from simple; it may be shifted in the latter part of the diapause stage (MASAKI, 1956). Since then, another effort has been made to analyse this sequence of events in more detail by applying similar or other régimes of temperature condition. In the present paper, the results of three series of such experiments will be accounted for; they are (i) intermittent chilling, (ii) daily alternation of low and high temperatures, and (iii) incubation at different temperatures after different lengths of cold exposure.

II. Material and Method

During the first half of October, 1955, about 1000 or more pupae of the cabbage moth were collected on the farm in the University campus at Sapporo. They were stored in a high temperature cabinet

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of 26°C. until subjecting to a variety of experimental conditions. During this storage prior to experiments, dead, parasitized or unhealthy pupae were discarded. No moth emerged from them during the preparation period, showing that the diapause condition was still maintained firmly in these samples at the time of collection. It should be noted here, however, that some pupae of the first generation of the cabbage moth enter diapause and hibernate concurrently with those of the second generation, the proportion doing so being varied dependent on the annual climatic conditions. The present material probably contained the diapausing pupae of both generations, and it was impossible to separate them. Consequently, this heterogeneous constitution of the material might, to some extent, influence the experimental results.

The pupae were divided into batches of forty individuals each, and subjected to the following régimes of temperature from the end of October.

The first is substantially a supplementary series of the experiments described in a previous paper (MASAKI, 1956). Ten batches of pupae were distributed respectively into treatments which are shown in the following table. At the end of each treatment, the batches were experienced a total period of 45 days at 5°C., and they were subsequently kept at 26°C. If any pupae resumed development during an intermittent incubation period between periods of chilling, they were

TABLE 1. The treatments of intermittent chilling. Numerals in brackets indicate the number of days at 26°C. and those out of brackets, the number of days at 5°C.

Batch	Treatment
a	5 (10) 5 (10) 5 (10) 5 (10) 5 (10) 5 (10) 5 (10) 5 (10) 5
b	10 (10) 10 (10) 10 (10) 10 (10) 5
c	15 (10) 15 (10) 15
d	20 (10) 20 (10) 5
e	25 (10) 20
f	30 (10) 15
g	15 (10) 15 (10) 15
h	15 (5) 15 (5) 15
i	15 (1) 15 (1) 15
j	45

removed and checked for emergence as moths.

The next series was conducted to test the effect of daily fluctuation of temperature upon the completion of the diapause; in these tests, a given period of low temperature alternated with a period of high temperature each day. The pupae were sbjected to 5°C. for eight hours and to 26°C. for sixteen hours each day. This treatment was lasted for 45 or 135 days; in the latter case the duration of chilling amounted to 45 days, enabling a direct comparison of the result with that of the control batch which accepted the same amount of chilling continuously. In other two batches, cold exposure was lasted 16 hours daily, the remaining hours being spent at 26°C.; the treatment was again continued for 45 or 67 days, the latter involving a total of 45 days at the low temperature. All five batches of pupae were kept constantly at 26°C. from the end of each treatment.

In the last series of experiments, twelve batches of pupae were kept at 5°C. continuously, and four batches were removed to 15°, 20°, 26° and 30°C., respectively, after each of 28, 43 and 77 days, in order to detect any change in the response of the diapausing pupae to high temperatures.

In all these tests, the initiation of the adult formations was judged by examining the pigmentation of moth through the pupal cuticle. The daily observation was continued for six months, including the periods of treatments. No pupae resumed development during the period kept at 5°C. which is undoubtedly below the developmental threshold.

III. Results

(i) *Intermittent chilling*

Throughout the present experiments, the total amount of 45 days' cold exposure was employed, because, in the previous experiments, the treatment of this length resulted in about 50% of the pupae to resume development on subsequent incubation, if it was continuously applied; so that a greater fluctuation of the results could be expected by using such a moderate amount of cold treatment than by applying the extremes, in accordance with the variation in the efficiency of various régimes used in the experiments. This may facilitate a more precise interpretation of the results than otherwise. In spite of such a supposition, no clear-cut tendency is indicated in Figure 1, which summarizes the experimental results of intermittent chilling. In all

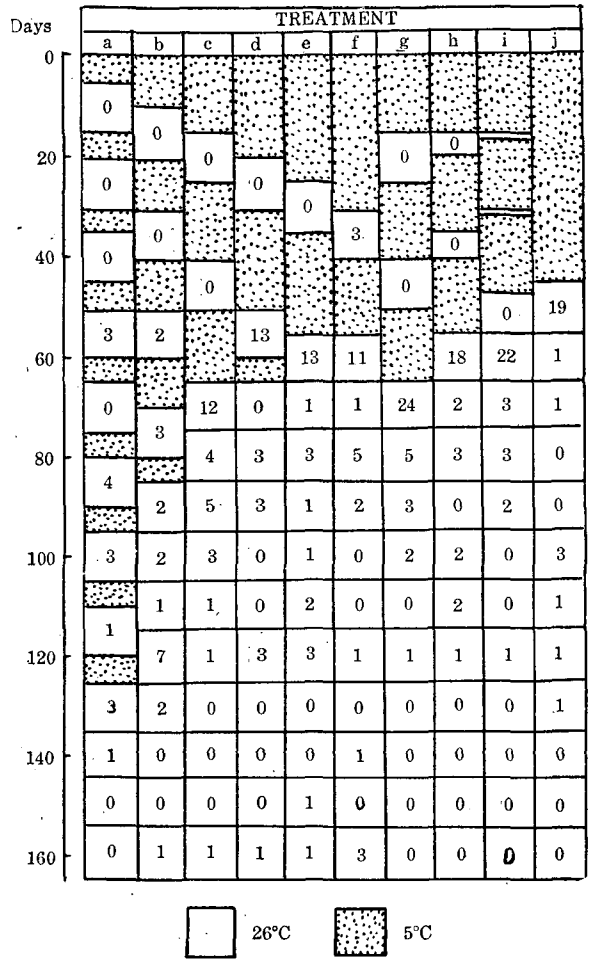

Figure 1. The effect of intermittent cold exposure upon the completion of the diapause in the cabbage moth pupae. Numerals in each square indicate the number of developing pupae in each interval of time which is represented by the scale on the left.

but the two treatments in which one period of chilling lasted only for five or ten days, an apparent peak of moth emergence was observed within twenty days after the onset of continuous incubation at 26°C. This was followed by a more or less continuous but irregular emergence of moths, and the ultimate number of pupae that developed

into the adult during the observation period of six months showed no significant variation among the treatments. A less efficient action of intermittent chilling for completing the diapause, as compared with a continuous one, is indicated only in two batches; when a unit of cold exposure lasted very short, i. e., five or ten days respectively, no prompt and simultaneous resumption of morphogenesis by the pupae was observed. The same tendency has been obtained in the previous experiments much more clearly (MASAKI, 1956). The less apparent results of the present experiments were probably due to the heterogeneity of the material; it might be inferred that the temperature conditions before the time of collection of the pupae had affected and disturbed, to some extent, the stability of the diapause condition in the pupae. As to the absolute length of the pupal stage after the onset of the experiments, it is, however, apparent that the pupal stage was prolonged in proportion to the frequency of intermittent incubation between periods of chilling. The figures in Table 2 indicate the mean length of the pupal stage after the beginning of experiments. Thus, on average, the actual emergence of moths occurred about a month earlier in the batch which was subjected continuously to cold than in the other which was exposed to 5°C. for 5 days repeatedly at 10-days' intervals of high temperature.

TABLE 2. The effect of intermittent chilling upon the length of the pupal stage after the onset of the experiments. For details of the treatments, see Table 1.

Batch	Length of a unit chilling (days)	Length of intermittent incubation at 26°C. (days)	Total number of days at 50°C.	Length of pupal stage (days)	Number of pupae developing during 6 months
a	5	10	45	94.7	14/40
b	10	10	45	109.1	22/40
c	15	10	45	83.1	27/40
d	20	10	45	77.7	23/40
e	25	10	45	79.9	26/40
f	30	10	45	71.2	27/40
g	15	10	45	80.5	36/40
h	15	5	45	72.5	28/40
i	15	1	45	67.0	31/40
j	45	0	45	69.0	27/40

(ii) *Daily alternation of low- and high-temperatures*

The treatments with daily alternating temperatures are modifications of the preceding series; in these treatments a unit of chilling lasted less than 24 hours. However, since the daily rhythm of an environmental condition has a great significance in various biological phenomena, a particular attention should be paid for the effect of daily fluctuating temperatures upon the diapause development. The results are summarized in Table 3. In this table, some conspicuous and interesting features will be noted.

TABLE 3. The effect of daily alternation of low and high temperatures upon the completion of the diapause.

Treatment	Length of treatment in days	Total amount of cold exposure in days	Length of pupal stage after the onset of test (in days)	Number of pupae developing during 6 months of observation
8 hr. at 5°C. and 16 hr. at 26°C./day	135 45	45 15	144.1 127.2	13/40 16/40
16 hr. at 5°C. and 8 hr. at 26°C./day	67 45	ca 45 30	84.3 92.3	32/40 30/40
24 hr. at 5°C./day	45	45	69.0	28/40

Firstly, the number of pupae developing during the six months' observation differs significantly among the treatments. Daily exposure of 8 hours to cold was less effective for the initiation of adult formation than 16-hours' or 24-hours' chilling per day, irrespective of whether the total amount of chilling was identical (i. e., 45 days) or not. There was, on the other hand, no significant difference between the cold exposure of 16 hours daily and the continuous one, again irrespective of the difference in the total amount of cold storage. Secondly, the mean length of pupal stage after the onset of the treatment varied considerably depending upon the temperature régimes used. It was the shortest when the pupae were constantly chilled for 45 days; about ten to twenty days longer than this when 16-hours' daily exposure was applied. A remarkable retardation of the diapause development was observed in the batches received 8-hours' daily chilling; in these cases, the mean length of the pupal stage was about two times greater than that of the control batch. Such was also the case even if the total length of cold exposure was identical between the two régimes of temperature. Thirdly, it may well be pointed out that the length

of the treatment was of minor importance for completing the diapause, at least so far as the daily fluctuation of temperatures lasted from 45 to 135 days in the 8-hour chilling, and from 45 to 67 days in the 16-hour chilling. This may indicate the low efficiency of fluctuating temperature upon terminating the diapause; or in such a pattern of treatment, the effect of low temperature for the completion of diapause may not be accumulated within the mechanism of diapause development. Finally, there was a marked difference among the treatments in the frequency distribution pattern of the adult emergence. This is clearly indicated in Figure 2, which illustrates

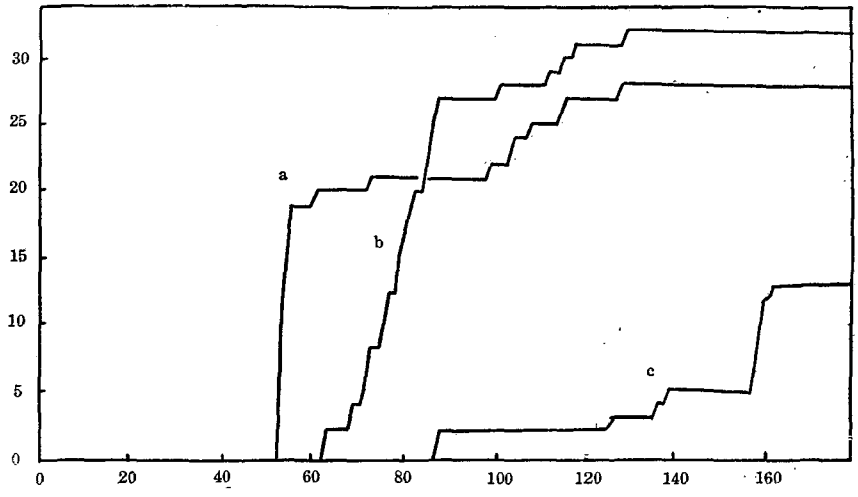

Figure 2. The effect of the daily alternation of low- and high-temperatures upon the emergence of moths from diapausing pupae. Ordinate: number of pupae resuming development. Abscissa: time in days after the onset of experimental conditions. *a*, continuously exposed to 5°C. for 45 days; *b*, exposed to 5°C. 16 hr./day for 67 days; *c*, exposed to 5°C. 8 hr./day for 135 days.

the cumulative number of pupae resuming development during six months of observation. In any case, a peak of adult emergence was not observed, whenever the batches of pupae underwent alternation of the two temperatures. In contrast, an apparent peak of moth emergence appeared in about a fortnight after removing from the continuous cold storage. Among other things, a very sporadical distribution of the moth emergence over a prolonged period of observation may also illustrate a poor effect of the alternating

temperatures for promoting the resumption of morphogenesis.

All these facts, stated above, lead to a conclusion that the effect of a low temperature upon the completion of the diapause is greatly suppressed by daily alternation with a high temperature, which is substantially optimal for only the rapid morphogenetic process.

(iii) *The effect of different incubation temperatures after different lengths of chilling*

In the previous experiments, it has been suggested that the temperature requirement of the cabbage moth pupae changes from a lower range of temperature to a high, in a later stage of diapause development (MASAKI, 1956). If it be true, the reaction of the pupa to a given temperature might be different at various stages of low temperature treatment. Such a possibility may be confirmed by the experiment which will be stated below.

In Figure 3, the number of pupae which resumed development at various temperaturers between 15° and 30°C. after different lengths of chilling is illustrated on the time axis. The general trend is the same as the results already described elsewhere; the pupae began to develop sporadically over a prolonged period when the initial chilling was insufficient; the initiation of development was accelerated and concentrated in a relatively brief period, and the peak of moth emergence appeared when an adequate amount of cold treatment was applied. This was true at all four temperatures, 15°, 20°, 26° and 30° C., that were used as incubation temperatures. However, further important features should be pointed out.

The data shown in Figure 3 could be summarized as Table 4. In all three sets of experiments, the minimum time for the resumption of development was always decreased at higher temperatures, although such a tendency was somewhat ambiguous when the preceding cold treatment was short. On average, however, the length of pupal stage after removing from cold storage was not necessarily inversely proportional to incubation temperature. After cold exposure for 28 days, the rate of the initiation of adult formation was accelerated from 1.87 to 3.66 by rising temperature from 15° to 20°C. About the same amount of increase in temperature from 20° to 26°C. resulted in no significant acceleration of the resumption of morphogenesis. By further increase in temperature, the rate was remarkably lowered; it decreased from 4.05 at 26°C. to 2.47 at 30°C. This rather unusual rela-

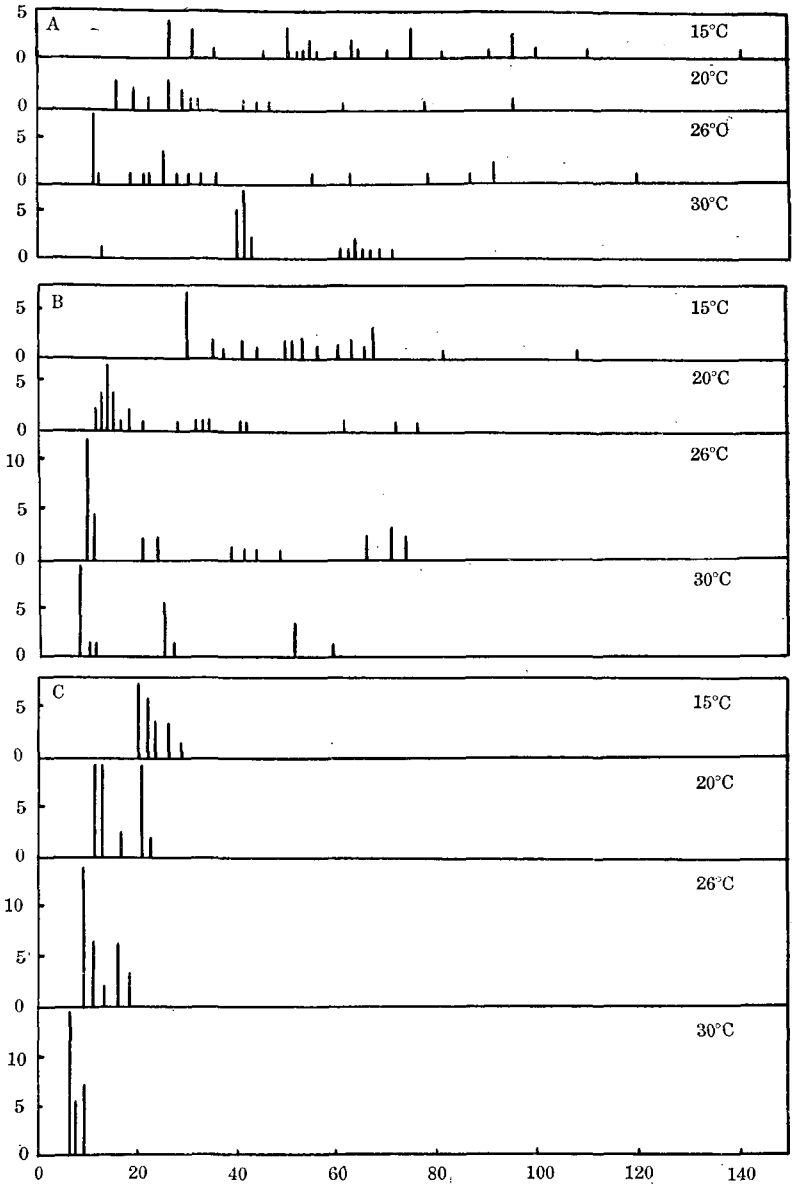

Figure 3. The effect of incubation temperature and the length of previous cold exposure upon the resumption of morphogenesis in the diapausing cabbage moth pupae. Ordinate: number of developing pupae. Abscissa: time in days after removing from cold exposure.

TABLE 4. The effect of incubation temperature upon the resumption of development at various stages of cold storage at 5°C.

Initial period of cold exposure in days	Incubation temperature in °C.	Time in days required for the pigmentation of adult eyes after removing from the cold storage		Incubation period required for 50% development (in days)	Number of developing pupae during 6 months
		Minimum	Average		
28	15	27	60.7	53.5	31/40
"	20	15	38.4	27.3	21/40
"	26	11	34.8	24.3	23/40
"	30	12	49.3	40.5	23/40
43	15	30	50.9	47.0	27/40
"	20	12	24.5	14.5	30/40
"	26	10	29.6	10.8	32/40
"	30	8	22.4	11.0	22/40
77	15	21	24.0	22.3	24/40
"	20	11	15.4	12.5	28/40
"	26	9	11.6	9.0	38/40
"	30	6	6.8	6.0	28/40

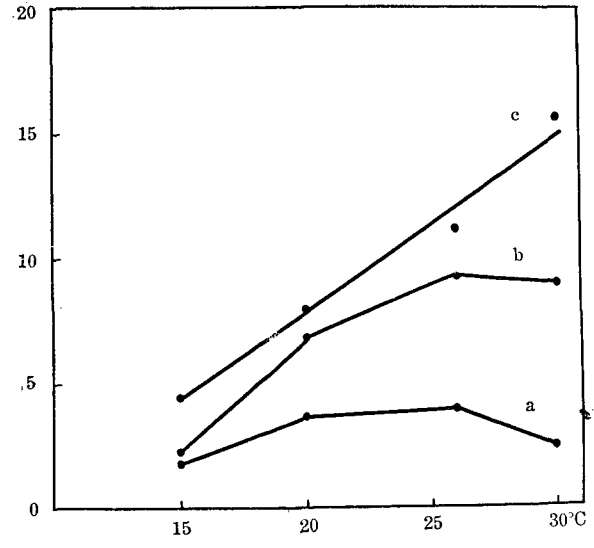

Figure 4. The effect of temperature on the resumption of development of the diapausing cabbage moth pupae after different lengths of cold storage. Ordinate: rate of the resumption of development (1/Time required for the pigmentation of the adult eyes under the pupal cuticle×100).

Abscissa: temperature in centigrade degrees. *a*, kept at 5°C. for 28 days; *b*, for 43 days; and *c*, for 77 days. The time required for development of 50% of the total emergents was used for calculating the rate.

tionship disappeared after cold exposure of 43 days; but the normal temperature-velocity relation was not still encountered. Between 20° and 30°C., the acceleration of moth emergence by rising temperature was obscure. The accelerated response by rising temperature, which is normally found in morphogenetic processes, was regained by the pupae after 77 days of cold storage. These changes in the response of the pupae to temperatures are clearly illustrated in Figure 4, in which the rate (1/Time $\times 100$) of the initiation of the development is plotted against temperature.

IV. Discussion

When the diapausing pupae of the cabbage moth are constantly placed at a high temperature just after pupation, a few of them occasionally transform into the adult after a very prolonged of time. According to the theory of "diapause development" set forth by ANDREWARTHA (1952), this may prove a very slow rate of diapause development at such a high temperature. On the basis of present experiments, the action of high temperature upon the diapausing pupae seems not to be limited to decrease the rate of diapause development. It probably does something more than a mere retardation. The present experiments show that the efficiency of an optimal low temperature for completing diapause was greatly reduced by daily alternation with a high temperature. In this case, daily period of high temperature apparently suppressed the action of low temperature favouring the completion of diapause, because by such a treatment only a low level of diapause termination was attained in the sample, even if the total period of chilling amounted to 45 days, which otherwise resulted in many pupae to resume development.

What is the true nature of the diapause development is still obscure, though recently VAN DER KLOOT (1955) has found that cholinergic substance is synthesized and accumulated in the brain of the cecropia pupa during diapause. And WILLIAMS (1956) has suggested that the interaction of two antagonistic reactions with different temperature coefficients possibly concern with the process terminating diapause. He has further snggested that one of these reactions favours, and the other opposes the activation of the neurosecretory cells which are throught to be the centre controlling the mechanism of diapause. For the present purpose, it is of particular interest that the alternation of low and high temperatures may upset the interac-

tion of these two opposing reactions through their different temperature coefficients. If it be true, such a circumstance will bring about an anomality of diapause development. The reduced efficiency of a discontinuous cold exposure upon the termination of diapause, as compared with a continuous one, may be concordant with this incident.

It has already been pointed out that the final phase of the diapause development in the cabbage moth pupae can be completed at either low or high temperatures (MASAKI, 1956). Owing partly to the heterogeniety of the present material, and partly to an inadequacy of the procedure, the experiments have failed to follow the changing thermal requirement of the diapausing pupae in quantitative terms. The diapausing pupae of the cabbage moth, which are kept at a high temperature, require a very long period of time before initiating adult development. Whereas in the present samples, a considerable number of pupae resumed development at high temperatures during six months of observation. This fact may indicate that they had already passed through an initial period of diapause development, in which a low range of temperature is more or less indispensable, under the field conditions. It may also be possible that the "intensity" of the diapause condition varies year by year, as has been reported in the grasshopper, *Melanoplus bivittatus* (CHURCH and SALT, 1952). One or both of these complicating factor might have caused some but no substantial discrepancies of the results between the present and previous experiments. In the present experiments, however, the absence of accelerated response of the pupae by a high temperature of 30°C. was clearly indicated at an early stage of cold storage. Inability of the diapausing pupae to initiate metamorphosis at such a high temperature was similarly indicated in the data obtained by OTUKA and SANTA (1956). As the length of cold storage was prolonged, the normal response which is encountered in most morphogenetic processes was restored. These changes in thermal response were rather gradual, when considered in terms of the sample as a whole. However, the spread of variation in time which was required to initiate metamorphosis was very large, and the frequency distirbution of emerging moths apparently did not represent a homogeneous sample. Naturally, in a number of cases, it contained both diapause-free and diapause-persisting pupae. These two types of pupae have different attributes with regard to the responsiveness to temperature, and the quantitative continuity between the two types could hardly be pursued. Such situations were

an unavoidable obstacle in analysing the experimental results, making almost impossible the management of the results by statistical methods.

In the cecropia moth, WILLIAMS (1956) has found two responses of the pupae in diapause; by an application of high temperature, one preceding response is retarded, and the other subsequent response is accelerated. This is just comparable to the finding in the cabbage moth pupae that the first phase of the diapause requires a paticular range of low temperature and the final phase can be progressed in a much wider range, covering optimal temperatures for morphogenesis. The primary aim of the present investigation was to determine the temperature coefficient of this latter process in the cabbage moth pupae, though the attempt was unsuccessful due to some reasons state above. The substantial mechanism of diapause may, of course, be a biochemical or biophysical event, and efforts should be made by using such methods of approach. An analysis of the thermal relations of diapause development chiefly concerns the ecological aspects of the phenomenon, but at the same time it may also offer a clue for solving the problem by much more critical way.

V. Summary

1. The effect of intermittent chilling, daily alternation of temperatures, and incubation temperatures after various periods of chilling—all upon the completion of the diapause in the cabbage moth pupae have been analysed.

2. The heterogeneity of the material which had been collected in the field caused some discrepancies among the results. But in general the action of low temperature needed to continue over a certain length of time to overcome diapause condition simultaneously in many pupae.

3. Daily exposure to cold for 8 hours exerted only a little effect even if it was lasted for as long as 135 days. Daily chilling of 16 hours was more effective than the former, but in neither of these cases, a prompt and simultaneous development of the pupae has not been observed. The effect of daily cold exposure seemed not to be accumulated in the pupae.

4. At an early stage of cold storage, a temperature as high as 30°C. exerted a retarding effect upon the initiation of adult formation,

as compared with lower temperatures. In later stages, such an adverse effect of high temperature became disappeared, and finally a normal temperature-velocity relationship was regained by the pupae as they were free of diapause and initiating metamorphosis into the adults.

VI. Acknowledgement

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