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Studies on the Insect Metamorphosis
VI. Effect of Low Temperature on the Morphogenesis of *Luehdorfia*-Pupae[1)]

By

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It has been verified by our previous experiments that the pupal diapause in *Luehdorfia* is caused by the dormant state of the brain and that it is simply interrupted by the introduction of active brains either from the 5th instar of this species or from the mature larvae of *Bombyx mori* (1951, 1955). In the natural life-cycle of this butterfly, the temperature-decrease is surmised to be one of the factors that break this dormant state of the brain. In his excellent experiments with *Cecropia*-silkworm, Williams (1946) found that the pupal diapause of this moth is also conditioned by the inactive state of the brain, which is activated by subjecting the pupae to low temperatures. According to Koidsumi and Shibata (1953), low temperature in October in Formosa is also necessary for breaking the pupal diapause of the tropical moth, *Eriogyna pyretorum.* A wealth of literatures are available for showing from the ecological point of view that exposure to adequate low temperature is one of the surest ways of bringing the insect diapause to an end.

The present paper is concerned with the similar effect of low temperature on the emergence of butterflies from *Luehdorfia*-pupae.

Materials and Methods

Luehdorfia-eggs were collected from the fields, and larvae hatched from them were reared in the laboratory. As experimental materials, the pupae that had passed more than 2 weeks after pupation were used. They were chilled for

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different days according to the experiment either in the refrigerator or ice-chest or both of them, and kept at a room temperature of around 22°C all the while upto the day enough to discern the imaginal differentiation. Precise procedure will be given in each section.

Experimental Results

In 1950, as a preliminary experiment, 5 groups containing 30~40 diapausing pupae each were chilled in ice-chest (6°~12°C) from the middle of July for 25, 35, 45, 55, and 65 days respectively. After cooling, all pupae were held at a room temperature for about 45 days, and then dissected in order to ascertain how far the imaginal development proceeded. Dissection revealed that the cooling treatment yielded in every group the precocious metamorphosis. However, in that year some of pupae among the non-treated controls were found to start the imaginal development in late October, when pupae of 55- and 65-day groups were dissected. Therefore, the data of these groups became unreliable.

Taking the fact into account, the experiments were repeated again in 1951 i. e., 7 groups comprising 40 diapausing pupae each were kept at 6°C from the middle of June for 7, 14, 21, 28, 35, 42 and 64 days respectively. Being afraid of the ill-effect to expose the pupae suddenly from high temperature of the laboratory to low temperature just mentioned, we put them in the ice-chest (12°±3°C) for the first 5 days, and then moved them to the refrigerator (6°C) for the remaining period of experiments. After cooling, they were placed in a room at 25°C for about 45 days, and then dissected in order to decide their differentiating state. It was found from this experiment that cooling for 14 days was sufficient to break the dormant state of the brain in some specimens, and the effect of cooling was more conspicuous in number when the pupae were chilled for more than 35 days.

In addition, implantation and extirpation of the chilled brain were carried out in that year. Five brains from the chilled pupae just taken out from the refrigerator were implanted into each non-treated pupa through a little cut made at the top of the head. The wound was coated with melted paraffin. The donors of brain served as the material of the extirpation experiments. Beyond expectation, only a few recipients started the imaginal differentiation, while many of donors could realize it in spite of lack of brain.

In 1954, the following series of experiments were undertaken: first, continuous treatment of low temperature was carried out, i. e., for the first 5 days the pupae were chilled in ice-chest at 12°±3°C, and then kept in refrigerator at 7°±2°C for the rest of experimental period (Series A). Secondly, after 5 days chilling in ice-chest, pupae were moved every other day from ice-chest to refrigerator or vice versa (Series B). Thirdly, after 5 days chilling in ice-chest, they were

chilled alternately every third day in ice-chest and refrigerator (Series C). Non-treated pupae kept in a room at 22°C served as control. After cooling all pupae were held in the same room keeping the control specimens. The results are shown in Table 1.

Table 1. Exposure to low temperatures.

Period of cooling	Series of experiment	No. of Specimens	Date of termination of cooling	Date of dissection	Result of dissection		Percentage of adult formation
					non-differentiation	adult formation	
15 days	Series A	50	10/VII	27/VIII	34	16	32
	B	30	10/VII	26/VIII	25	5	17
	C	30	10/VII	27/VIII	12	18	60
20 days	Series A	50	15/VII	3/IX	34	16	32
	B	30	15/VII	3/IX	18	12	40
	C	29	15/VII	3/IX	7	22	76
25 days	Series A	50	20/VII	3/IX	28	22	44
	B	30	20/VII	3/IX	10	20	67
	C	30	20/VII	3/IX	3	27	90
30 days	Series A	50	25/VII	8/IX	27	23	46
	B	27	25/VII	8/IX	6	21	78
	C	29	25/VII	8/IX	2	27	93
Control	non-treated	50	—	8/IX	35	15	30

The table indicates that the continuous cooling for 15 days is effective in 32 percent for breaking the diapause of *Luehdorfia*, and longer cooling improves generally the effectiveness to a certain degree. But, when pupae are exposed alternately every other day to 12°±3°C and 7°±2°C in ice-chest and refrigerator, percentage of the imaginal differentiation increases fairly higher than when the pupae are exposed continuously to 7°±2°C, excepting 15-day group The increase of this percentage is more marked when the pupae are treated alternately every third day in ice-chest and in refrigerator. For instance, in 25-day group, when the pupae were subjected to continuous cooling, 44 percent of them emerged, while when the pupae were chilled alternately every other day, 67 percent of emergence was obtained, and when the pupae were cooled alternately every third day, they developed into butterflies in as high as 90 percent. In every series, the longer the treatment, the higher the frequency of emergence, so far as the present experiments are concerned.

That the brain hormone is secreted from the brain some days after cooling, in *Luehdorfia* like in *Cecropia*, is doubtful in the light of transplantation and extirpation of brain in 1951. In order to ascertain this point the following experiments were carried out.

The duration of continuous cooling was 15, 20, 25 and 30 days each. At

the time when the cooling was finished, about two-thirds of the specimens in each group were dissected to see their differentiating state, and one-third of them was kept at 22°C for 45～55 days to be dissected at the close of experiments. The results are shown in Table 2.

Table 2. Exposure to low temperatures

Period of cooling	No. of specimens	Date of dissection	Days after cooling to dissection	Result of dissection					Percentage of differentiation
				non-diff.	differentiation				
					A	B	C	D·E	
15 days	92	10/VII	0	78	14	—	—	—	15
	50	27/VIII	48	34	—	—	—	16	32
20 days	93	15/VII	0	64	29	—	—	—	31
	50	3/IX	50	34	—	1	—	15	32
25 days	90	20/VII	0	64	26	—	—	—	29
	50	3/IX	45	28	—	2	—	20	44
30 days	101	25/VII	0	57	40	—	4	—	44
	50	8/IX	45	27	—	—	—	23	46

As is shown in this table, some of the pupae dissected at the time the chilling was finished, indicated the inception of imaginal differentiation in 15, 31, 29 and 44 percent in respective groups, but they remained all in A grade of differentiation of our classification, i. e., the pupal brain was apparently larger and fat bodies were somewhat dissociable. In the case that the treated pupae were kept at 22°C for aforesaid periods, imaginal differentiation advanced to grade D or E, i. e., nearly to perfect adult. However, the percentages were shown not so much increased during the culturing period, except for those in 15- and 25-day groups. In the case of 15-day group we cannot reject a possibility that the brain hormone was secreted continuously after cooling was over. In 25-day group, however, the increase of percentage may be ascribed to the miss-classifying of pupae just after cooling, because the pupae that were doubtful in their differentiation were classed in the non-differentiating group. At any rate, in other groups, it is highly probable that brain hormone was released in the course of cooling.

To afford data favourable to this conclusion from the different aspect, following experiments were performed. The pupae were chilled as above for respective days and after cooling, brain alone was removed, and 3 brains thus removed were implanted into each non-treated intact pupa. Both of these donors and recipients were kept at 22°C for 41～51 days. The data are arranged in Tables 3 and 4.

Table 3 indicates the percentage of the adult-formation in the case of extirpation of the brain. The pupae that had already differentiated to grade A on extirpation of brain could advance to the adult form in as high as 81～100 percent of pupae. On the other hand, pupae that had shown no clear differenti-

Table 3. Extirpation of brain after cooling.

Period of cooling	No. of specimens	Differentiating state at the time of extirpation of brain	Result of dissection			Percentage of adult form
			non-differentiat.		adult form	
			lived	dead		
10 days	84	non-differentiation 68	39	28	1	1
		differentiation in grade A 16	0	3	13	81
15 days	90	non-differentiation 76	8	68	0	0
		differentiation in grade A 14	0	2	12	86
20 days	93	non-differentiation 66	36	27	3	5
		differentiation in grade A 27	0	5	22	81
25 days	90	non-differentiation 64	30	28	6	9
		differentiation in grade A 26	0	0	26	100
30 days	96	non-differentiation 57	28	21	8	14
		differentiation in grade A 39	0	7	32	82

ation on extirpation of brain developed to the adult form at most in 14 percent.

From these facts it seems difficult to avoid the conclusion that the brain hormone is secreted, as a rule, from the brain until the cooling is finished.

Table 4. Implantation of 3 chilled brains into each pupa.

Period of cooling of donor	No. of specimens	Date of termination of cooling & implantation	Date of dissection	Result of dissection						Percentage of differentiation
				non-diff.		differentiation				
				Lived	dead	A	B	C	D·E	
10 days	30	5/VII	19/VIII	0	28	1	—	—	1	7
15 days	28	10/VII	27/VIII	2	20	—	—	—	6*	21
20 days	31	15/VII	4/IX	5	25	—	—	—	1*	3
25 days	30	20/VII	4/IX	8	19	—	1	—	2*	10
30 days	30	25/VII	4/IX	11	18	—	—	—	1	3

* dead

This table indicates the result of implantation of brains. As mortality is high beyond expectation due to unknown factors and moreover, as we cannot tell when death occurred, validity of this table may be decreased. Nevertheless, it will be allowed to say that the percentage of imaginal differentiation is much less than the theoretical value,* if a possibility of secretion from the implanted brain is admitted. In other words, the chilled brain would release its hormone before removal from cooling and the secretion would be finished or at least not

* 68% in the case of 15-days' cooling, 70% in the case of 20-days', 82% in the case of 25-days', 84% in the case of 30-days',

so active as is effective after implanting.

Next, pupae were chilled after extirpation of their brains From the day brains were extirpated, 5 groups containing 20 brainless pupae each were exposed to continuous cooling at 7°±2°C for 10, 15, 20, 25 and 30 days respectively and then moved to a room at 22°C. Then they were all dissected after 45~50 days. The results are given as Table 5.

Table 5. Cooling after extirpation of brain.

Period of cooling	No. of specimens.	Date of termination of cooling	Date of dissection	Result of dissection		Percentage of differentiation
				non-differentiation	differentiation	
10 days	20	5/VII	19/VIII	20	0	0
15 days	20	10/VII	27/VIII	20	0	0
20 days	20	15/VII	3/IX	20	0	0
25 days	20	20/VII	3/IX	20	0	0
30 days	21	25/VII	8/IX	21	0	0

As seen in the table, none of the pupae deprived of their brains showed any sign of imaginal development. It is clear from this fact that the brainless pupae cannot differentiate towards adults, even if they are exposed to low temperature.

Discussion

It has been revealed that low temperature is one of the factors terminating the pupal diapause of *Luehdorfia*. In addition, alternative exposure of pupae to 12°±3°C and 7°±2°C is more effective for breaking the diapause than continuous cooling at 7°±2°C, and further the exposure alternative every third day is better than that every other day. How do these differences arise?

In this connection we remind of our observations on neurosecretory cells in the brain of *Philosamia* subjected to low temperature of 3°C. Although *Philosamia* is non-diapausing species, by exposing the pupae to such low temperature, secretion products increase extraordinarily in the neurosecretory cells. These products are released from the cells promptly after pupae were moved to room temperatures. We assume that in this case the secretion of secretory material from the cell alone will be checked by low temperature, although its production occurs at low temperature. If it is available for *Luehdorfia*, the neurosecretory cells of *Luehdhorfia* is considered to generate actively the neurosecretory material at around 7°C, but this temperature is too cold, if not entirely, to permit its secretion from the cell to occur in due course. However, not only its production but its secretion from the cell would occur at 12°C. This would be the reason why alternative ex-

posure to 7°C and 12°C is more effective than the continuous cooling of 7°C. And the exposure alternating between the two temperatures at intervals of 2 days may keep the pupae in step with both phenomena of production and secretion of neurosecretory material better than that at intervals of one day. Further histological investigation of the neurosecretory cells in *Luehdorfia* itself is required to authorize the assumption.

Chilling the brain at −3°~−1°C is also effective, but it is not suitable because of high mortality of pupae (unpublished data). The temperatures a little higher than 12°C will be a'so effective, for some of the diapausing pupae develop occasionally in the laboratory in October. Koidsumi and Shibata (1953) demonstrated that in *Eriogyna pyretorum* a little decrease of temperature in October in Formosa is enough to cause the inception of emergence of this moth.

As stated in our previous papers, the brain hormone is not directly involved in the imaginal differentiation, but it stimulates the prothoracic gland so as to secrete a different hormone which is directly responsible for this differentiation. When the latter hormone is accumulated in the body fluid above a certain amount, the imaginal rudiments can react upon it, regardless of their previous cooling. The prothoracic gland is also always capable for reacting upon the brain hormone even in the course of cooling. But the imaginal rudiments do not necessarily undergo the development at low temperature. Morphogenesis of these rudiments occurs in general when the pupae are returned to the warmth.

Summary

1) *Luehdorfia japonica* has a long pupal diapause due to the dormant state of the brain. The diapause is completed by virtue of the reactivation with their exposure to low temperature.

2) The effect of low temperature is more evident when the pupae are chilled alternately at between 7°C and 12°C than when chilled continuously at 7°C. And the chilling alternative every third day is more effective than that every other day.

3) The pupae started the imaginal differentiation immediately after chilling, can develop into adult form in more than 81 percent, even if the brain is removed, while the diapausing pupae that have received 3 such chilled brains cannot resume the development. Therefore, the brain hormone is assumed to be secreted in the course of cooling at low temperatures.

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