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SOME ASPECTS CONCERNING MELANIN FORMATION IN LATE LARVAE OF THE OLEANDER HAWK-MOTH, *Deilephila nerii* (L.) (LEPIDOPTERA: SPHINGIDAE)

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Franklin Chang (1978) Some Aspects Concerning Melanin Formation in Late Larvae of the Oleander Hawk Moth, *Deilephila nerii* (L.). *Bull. Inst. Zool. Academia Sinica* **17**(2): 85-95. The oleander hawk moth, *Deilephila nerii*, characteristically melanizes 96 hours before larval-pupal ecdysis. The onset of melanization appears to be hormonally-controlled. Injection of large amounts (40-100 μg/larva) of β-ecdysone will block or partially interfere with melanization. Juvenile hormones I and III appear to have no effect except in combination with ecdysone. A series of ligation experiments show that the region responsible for the initiation of melanization is the thoracic region. Furthermore, melanization appears to be independent of any influence from the head, except for the timing of onset of melanization.

The oleander hawk moth, *Deilephila nerii* (L.), is a native of Africa and Europe, but can also be found throughout the Pacific area. The adult moth has cryptically green-colored wings and feeds on nectar (Fig. 1). The larval stage has typically five instars, feeding mainly on leaves of the oleander plant as well as a small number of unrelated plants, such as crepe gardenia.

During the last or fifth instar, the green-colored larva gradually develops a black coloration on the dorsum of all eight abdominal segments (Fig. 2). The switch from green to black appears to be hormonally controlled by blood-borne factors. In a series of ligation experiments, it has been determined that the anterior region of the larva is involved in the initiation of pigment formation (Fig. 3)[2]. Furthermore, the pigment has been identified by infrared analysis and autoradiography to be indole-melanin and to be located in the exocuticle[2].

This paper was written in an attempt to communicate some of the results of a series of experiments conducted in order to shed more light on the endocrine mechanisms involved in the conversion to melanin synthesis during the late instar of the oleander hawk moth. A more detailed report will appear later.

MATERIALS AND METHODS

Insects: Larvae (fourth and fifth instars) used in these experiments were collected in the field on Oahu island, Hawaii, U. S. A., and brought to the laboratory where the larvae were maintained on fresh oleander leaves in plastic sweater boxes at 23°C until used.

Injection experiments: Various dosage levels of juvenile hormone I and III (20 μg/μl acetone) (Calbiochem, La Jolla, California) or β-ecdysone (10 μg/μl in 10% ethanol) (ECO Chemical Intermediates, Cambridge, Massachusetts), were injected by use of a Hamilton microliter syringe through the horn located at the posterior end of the larva or by topical application along the intersegmental membrane. Immediately after the injection, the horn was quickly tied with thread to prevent loss of hemolymph.

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Ligation experiments: Ligation of larvae was performed with medium cotton thread in the case of body trunk ligatures and by thin cotton thread when neck-ligated.

RESULTS AND DISCUSSION

Approximately 22-28 hours before melanization is initiated in the dorsal region of the abdomen, there appears on the prothorax an intense black area (Fig. 4). If one ligates fifth instars around the level of the second or third abdominal segment before the appearance of this black "marker", then in all cases, melanization occurs only in the region anterior to the ligature. If a ligature is made shortly after the appearance of this "marker" (within 10 hr), then 80% of the ligated larvae will show melanization both anterior and posterior to the ligation. The interpretation of this result is that in the former case, ligation was made before the factor responsible for melanization was secreted (precritical period) and hence no posterior melanization was observed, and in the latter case, ligation was made after the factor was secreted (postcritical period). In the experiments, this "marker" was used to determine what larvae were in the precritical state.

There are certain behavioral and morphological changes which precede melanization called prodromal signs. In the tobacco hornworm, *Manduca sexta* (a close relative of the oleander hawk moth), these prodromal signs signify the approach of larval-pupal eclosion, taking the form of (1) cessation of feeding (2) the heart and dorsal aorta become more distinct (3) clearing of the gut contents (4) wandering behavior. A few hours later, apolysis (separation of the old from the new cuticle) and ecdysis (shedding of the old cuticle) occurs. The oleander hawk moth also exhibits these prodromal signs, but in addition, melanization occurs along with the initial prodromal signs. In *Manduca*, melanization does not normally occur. Fig. 5 shows the gradual temporal change of color from green to greyish-black to dark black, the entire process of melanization being completed in a span of 4 hours.

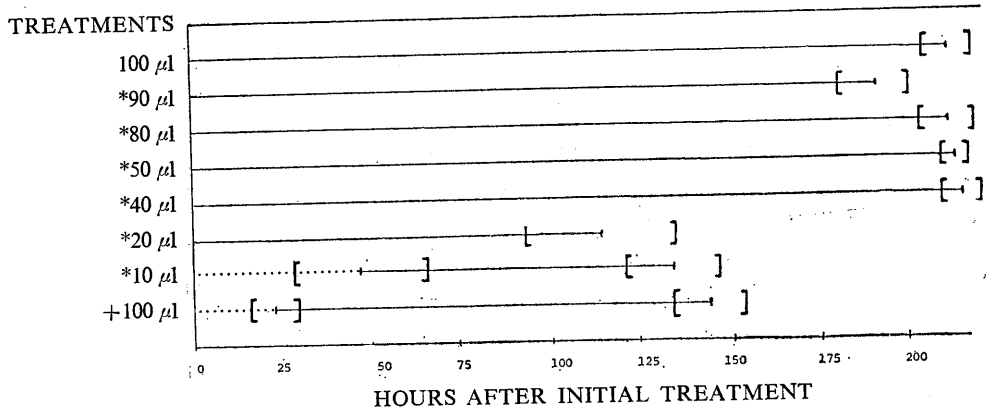
Previous work with *Manduca sexta* by Truman and Riddiford[5] and Bollenbacher *et al.*[1] have shown that two pulses of ecdysone (the molting hormone) occurs in late fifth instars. The first pulse is associated with the initial prodromal signs, and the second with apolysis and ecdysis. There is evidence that in *Deilephila*, there may also be two pulses of ecdysone during the last instar (to be published in another paper elsewhere). This is based on observations that during the process of molting, the larval body contracts and there is retraction of the prolegs (Fig. 6). These events are highly characteristic and are separate from the initial prodromal signs. If larvae are ligated around the abdomen 10 hours after the appearance of the "marker", then the region posterior to the ligature will melanize. However, the prolegs never retract. Injection of the abdomen with β-ecdysone will cause the prolegs to retract and begin apolysis. An interpretation of this result is that the ligation may have prevented the second pulse of ecdysone from reaching the abdomen and that the injected dose substituted for this second pulse. If the above is correct, then melanization, which is temporally very closely associated with the initial prodromal signs, may be blocked or interfered with by injecting large amounts (10 to 100 μg) of ecdysone into the larva at appropriate times so that this first distinct peak is never seen.

With this in mind, various amounts of ecdysone were injected into precritical fifth instars and then observed for melanization over the next several hours. As can be seen from Table 1, 40 μg and above of β-ecdysone is fully effective in interfering with or blocking melanization. Fig. 7 shows a fifth instar treated with 80 μg β-ecdysone. Note in this case there was a complete block of pigment formation in the abdominal dorsum. Fig. 8. shows partial interference with melanization. Juvenile hormones I and III separately administered by topical application are without effect except in combination with β-ecdysone (Tables 2, 3, and 4a, b).

The following series of ligation experiments have supported, along with the above results, the view that ecdysone may be responsible for

TABLE 1

Effect of β-ecdysone injected into late precritical and postcritical larvae of the oleander hawk moth, *Deilephila nerii*, in respect to the onset of melanization and subsequent pupation[a,b]



* Died after number of hours indicated.

\+ Postcritically injected.

[a] Single doses of β-ecdysone (10 µg/µl in 10% ethanol) were injected through the horn of late instars (10 larvae injected/dosage level). Time of injection is 0 hr.

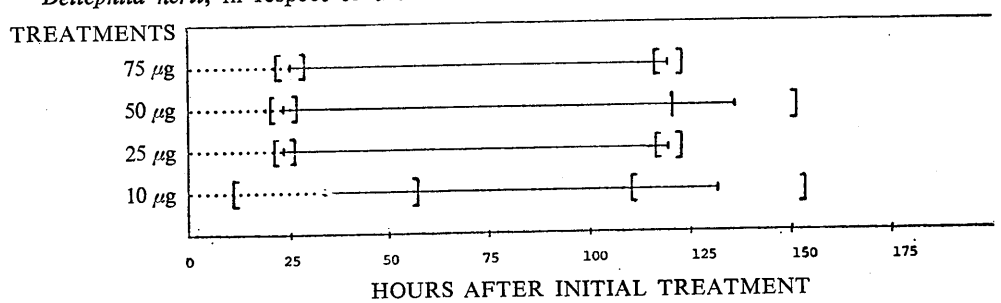
[b] Time to melanization and subsequent pupation were compared using one-way ANOVA statistics (P=0.05). S. D. in brackets.

——— No melanization observed from 0 time.

.....—— Time to larval-pupal eclosion from 0 time. Dotted line is time to melanization from 0 time.

TABLE 2

Effect of topically applied JH I on late precritical larvae of the oleander hawk moth, *Deilephila nerii*, in respect to the onset of melanization and subsequent pupation[a,b]



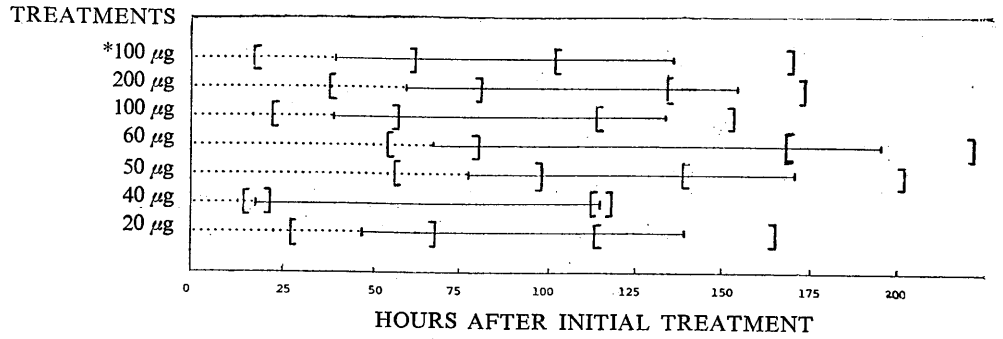
[a] Single doses of JH I (20 µg/µl in acetone) were topically applied to the intersegmental membranes of late instars (10 larvae/dosage level). Time of injection is 0 hr.

[b] Time to melanization and subsequent pupation were compared with the solvent controls using one-way ANOVA statistics (P=0.05). S. D. in brackets.

.....—— Time to larval-pupal eclosion from 0 time. Dotted line is time to melanization from 0 time.

TABLE 3

Effect of topically applied JH III on late precritical larvae of the oleander hawk moth in respect to the onset of melanization and subsequent pupation[a,b]



* Postcritically injected.

[a] Single doses of JH III (20 μg/μl in acetone) were topically applied to the intersegmental membranes of late instars (10 larvae/dosage level). Time of injection is 0 hr.

[b] Time to melanization and subsequent pupation were compared with the solvent controls using one-way ANOVA statistics (P=0.05). S. D. in brackets.

·····—— Time to larval-pupal eclosion from 0 time. Dotted line is time to melanization from 0 time.

TABLE 4a

Effect of sequential topical application of JH I to late precritical larvae of the oleander hawk moth in respect to the onset of melanization and subsequent pupation[a,b]

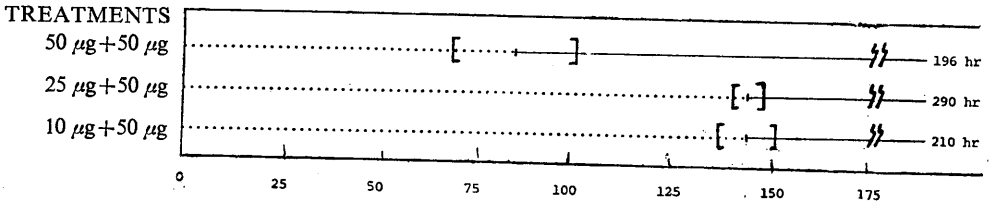
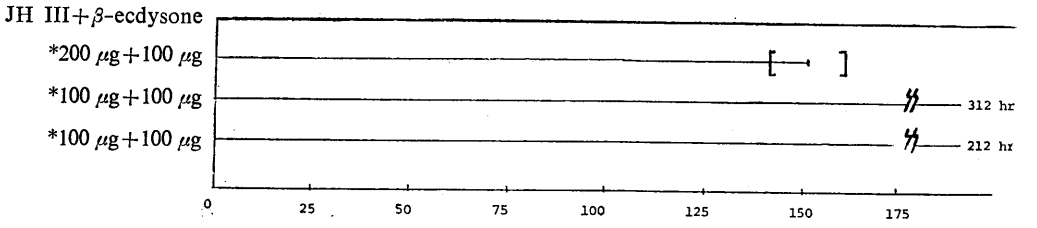


TABLE 4b

Effect of simultaneous injection of β-ecdysone and topical application of JH III on precritical larvae of the oleander hawk moth in respect to the onset of melanization and subsequent pupation[c,d]



* Died after number of hours indicated.

[a] Topical application of JH I 24 hr apart. Time of application is 0 hr.

[b, c] Time to melanization and subsequent pupation were compared with the solvent controls using one-way ANOVA statistics (P=0.05). S. D. in brackets.

[d] Time of administration is 0 hr.

——— No melanization observed from 0 time.

·····—— Time to larval-pupal eclosion from 0 time. Dotted line is time to melanization from 0 time.

TABLE 5

Effect of ligation made at specific levels around the body of precritical late larvae of the oleander hawk moth in respect to the onset of melanization

LEVEL OF LIGATURE	OBSERVATION
(1) Neck	Melanization posterior to ligature within 96-102 hr; head capsule remains greenish-yellow in color.
(2) Prothorax and mesothorax	Head capsule develops black color; melanization posterior to ligature.
(3) Metathorax and first abdominal segment	Absence of melanization posterior to ligature; head capsule develops black color.
(4) Double ligation: (a) Neck (b) Metathorax and first abdominal segment	Absence of melanization anterior to neck ligature (head capsule) and posterior to second ligature (abdomen).

the induction of melanization. When precritical larvae are neck-ligated, the abdominal region posterior to the ligature eventually melanizes while the head remains green in color (Fig. 9). However, when a ligation is placed around the third thoracic and first abdominal segment, the abdominal region (posterior to the abdominal ligature) does not melanize while the head capsule, nevertheless, melanizes, as it normally does. A double ligation around the neck and another around the third thoracic and first abdominal segment results in absence of melanization anterior to the former ligature and posterior to the latter. Table 5 summarizes these and other results. From the above information, it appears that the thoracic region is involved in the initiation of melanization.

It is interesting to note that in spite of the neck ligation, the thoracic region still has the capability of initiating melanization, i. e., in the absence of any influence from the head. However, the timing of the onset of melanization is delayed, taking, on the average, an additional 3 days to melanize in comparison with unligated controls. Fain and Riddiford[3,4] have shown that in neck-ligated and precritical (in respect to the brain) fourth instar *Manduca*, the prothoracic gland still has the potential to secrete low amounts of ecdysone (sufficient enough for pupation) in spite of the absence of prothoracotropic hormone from the brain. It appears that *Deilephila* may also have this capability and that prothoracic gland activation by the brain is unnecessary.

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關於夾竹桃鷹天蛾 (Oleander Hawk Moth, *Deilephila nerii* (L.)) 幼蟲末期黑色素形成的幾個要點

張 永 吉

夾竹桃鷹天蛾有一特性，就是在幼蟲蛻皮變爲蛹的 96 小時以前，開始有黑色素形成。而這個機制的啓動，是由激素來控制的。注射大量的（每隻幼蟲注射 40～100 μg）β-蛻皮素會抑制或部份干擾黑色素的形成。青春激素及除非與蛻皮素混合使用，否則就不產生影響。一連串的結紮實驗顯示黑色素之形成是由胸部起始的。此外，黑色素之形成並不受頭部的影響。頭部只是控制它何時啓動形成黑色素的機制。

Fig. 1. An adult female oleander hawk moth.

Fig. 2. Two fifth instars of the oleander hawk moth on an oleander leaf. The green-colored larva or early fifth instar is contrasted with a melanized larva or late fifth instar.

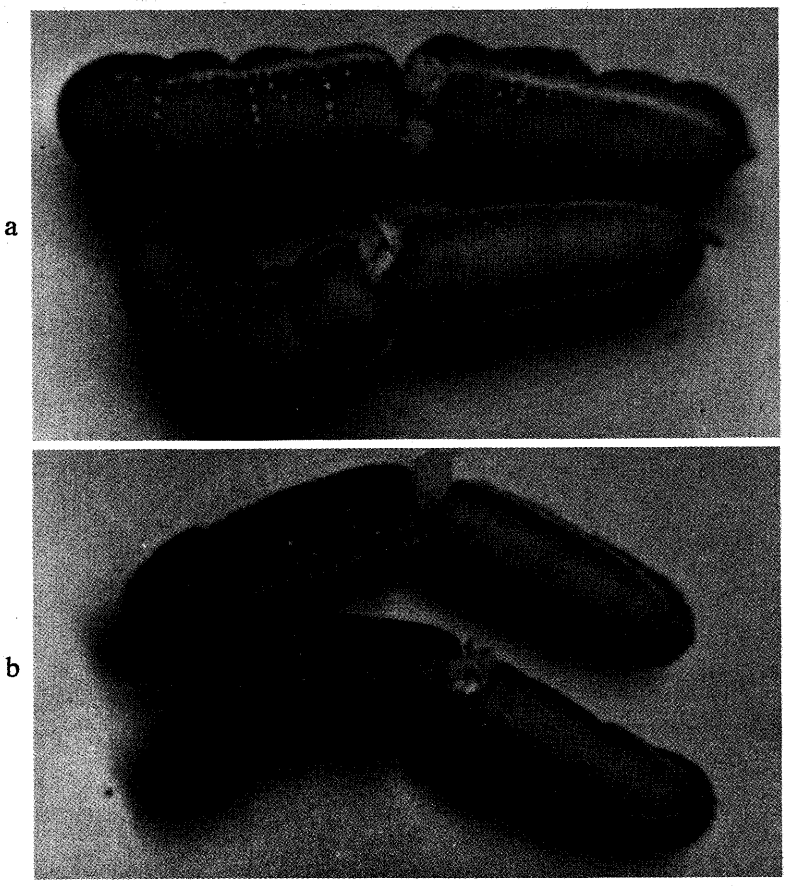

Fig. 3. a: Two precritical fifth instars of the oleander hawk moth ligated around the abdominal region.
b: Same two larvae as above except 24 hours later. Note the absence of melanization posterior to the ligation.

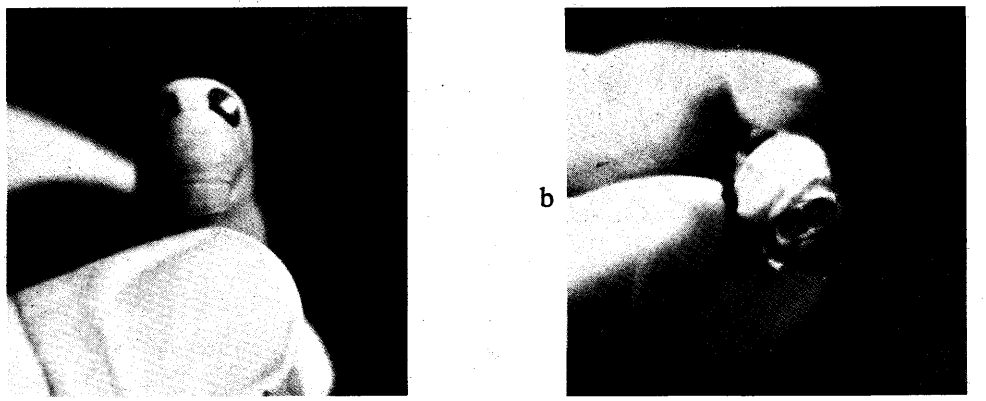

Fig. 4. a: Precritical fifth instar of the oleander hawk moth at this time bearing no black "marker" on the prothorax.
b: Same larva except in the postcritical state. Note black "markers" are prominent on the prothorax.

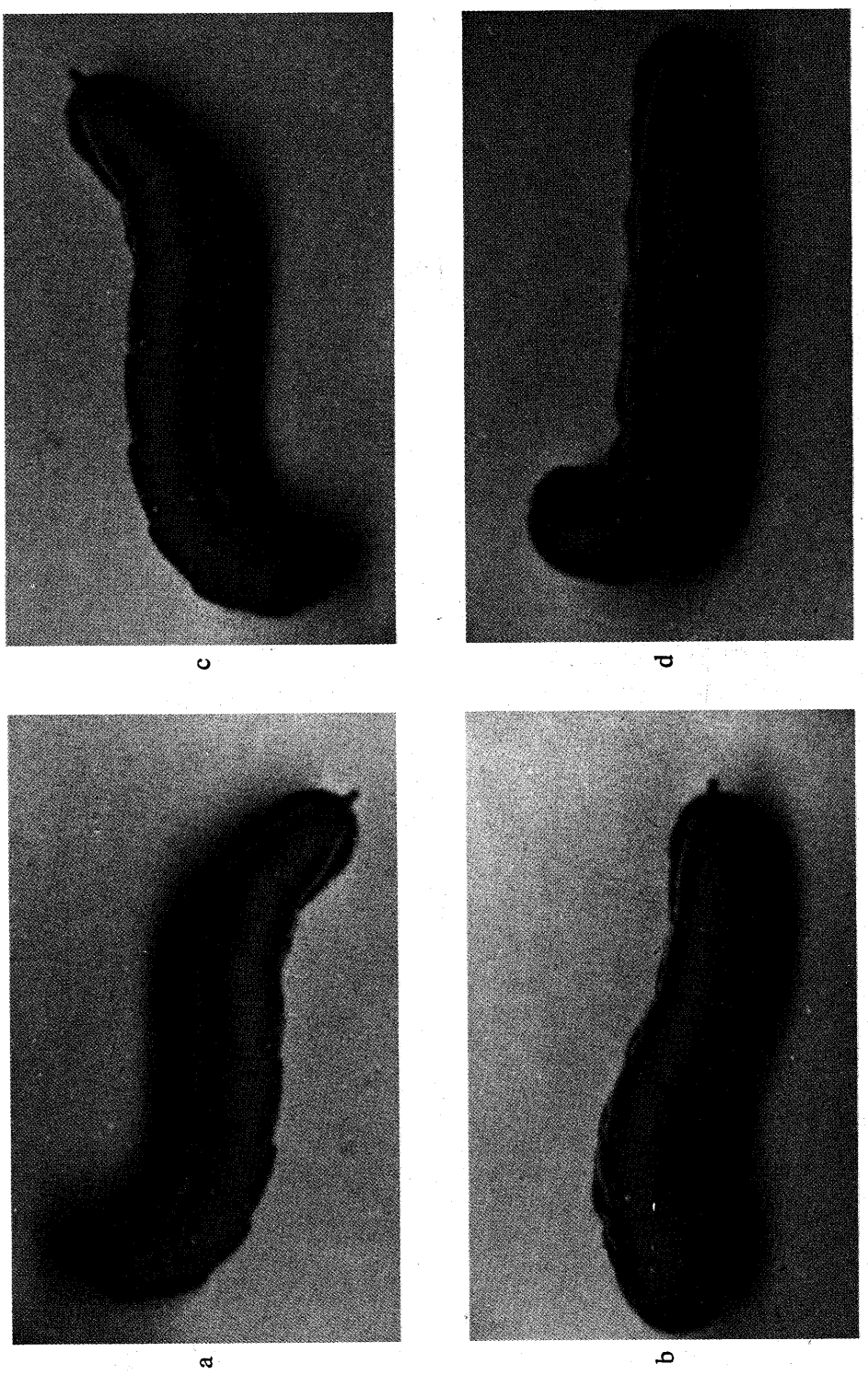

Fig. 5 a–h. Temporal sequence of the gradual melanization of the dorsum of a fifth-instar oleander hawk moth. Melanization is complete within 4 hours after its initiation.

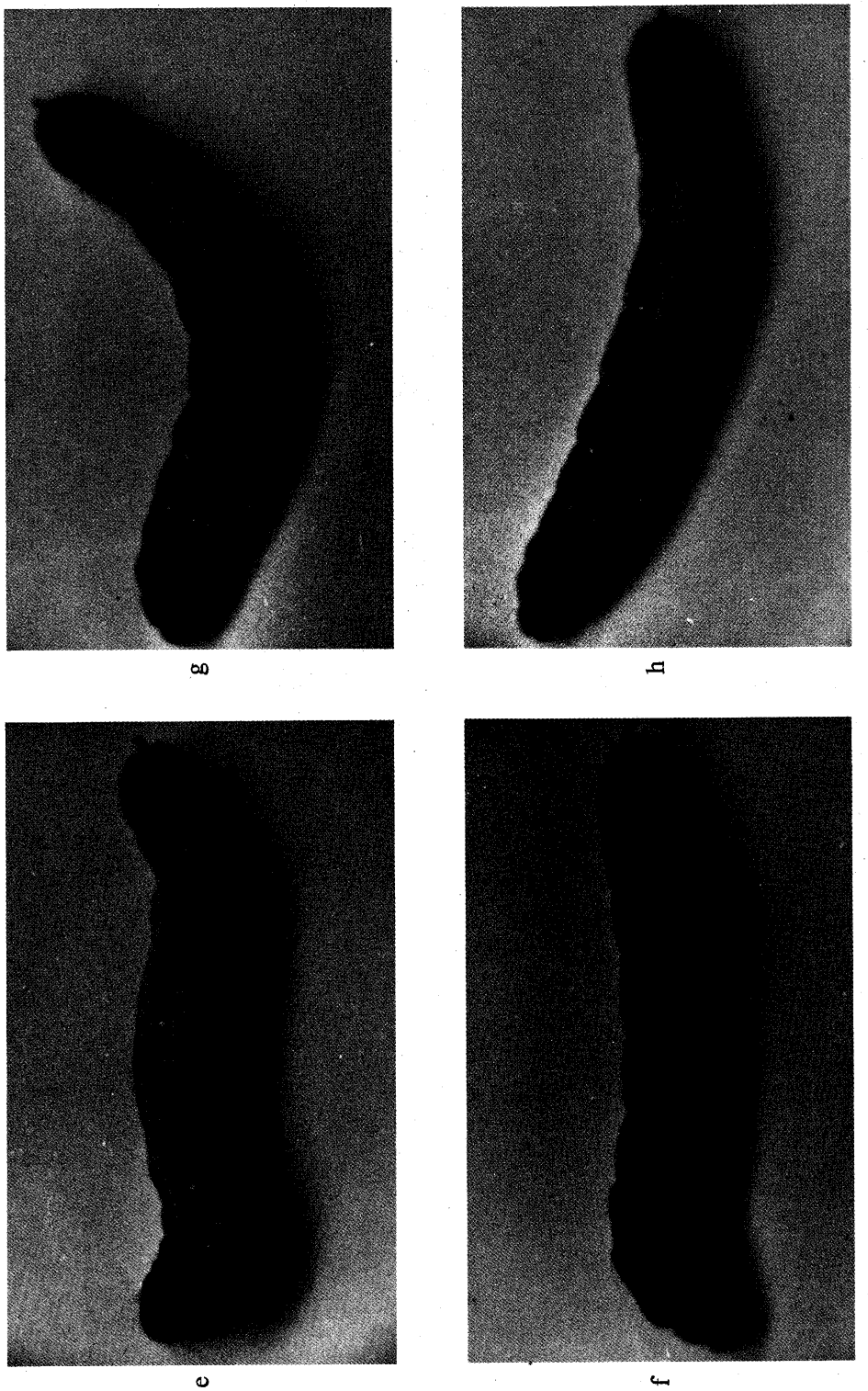

Fig. 5 a-h. (Continued)

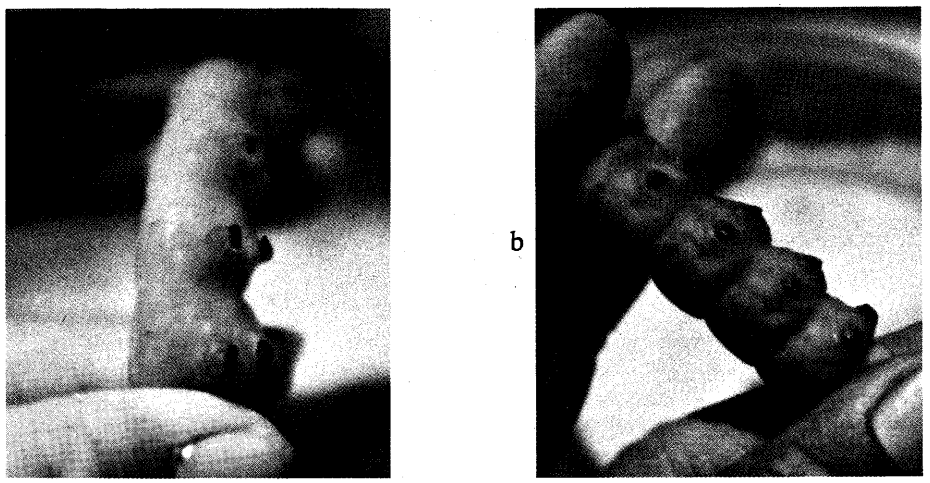

Fig. 6. a: Fifth instar of the oleander hawk moth one hour after melanization is complete. Note the presence of fleshy and unretracted prolegs.
b: Same larva 36 hours later. Note body trunk has contracted and prolegs are retracted. At this time, apolysis has taken place.

Fig. 7. Fifth instar of the oleander hawk moth injected with 80 μg β-ecdysone thirty hours earlier. Note absence of pigmentation in the dorsum.

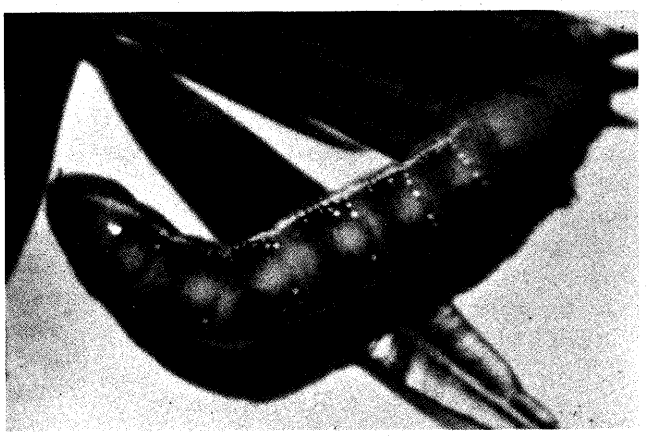

Fig. 8. Fifth instar of the oleander hawk injected with 60 μg β-ecdysone thirty-six hours earlier. Note mosaic-like pattern on the dorsum.

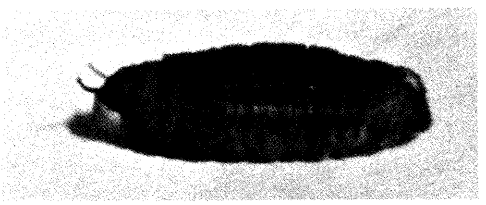

Fig. 9. A fifth instar of the oleander hawk neck-ligated eleven days earlier while in the precritical state. Note presence of melanization on the dorsum and body contraction. The head, however, remains green in color.