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Effects of hormonal factors on pupal cuticle coloration in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae)

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Abstract

We investigated the effects of hormonal factors on pupal cuticle coloration in the swallowtail butterfly, *Papilio xuthus* L. Neither juvenile hormone I, juvenile hormone analog methoprene, nor 20–hydroxyecdysone cause melanizing-stimulation and -inhibition to the pupal cuticle of *P. xuthus* by injection experiments. Further, the summer-morph-producing hormone (SMPH)-active peptide of *Bombyx mori*, which is involved in the regulation of wing coloration for seasonal morphs of the Asian comma butterfly, *Polygonia c-aureum*, did not show the melanizing-stimulation activity to the pupal cuticle of *P. xuthus*. However, when an extract including pupal cuticle-melanizing-hormone (PCMH) was injected into ligatured abdomen of pharate pupa, the pupal cuticle exhibited brown color instead of green to have appeared if no injection was given. Thus, it was suggested that the PCMH can trigger only the first step of the pupal cuticle melanizing cascade in *P. xuthus*.

Introduction

Pupae of many species of swallowtail butterflies show color polymorphism in the pupal cuticle body, including green, brown and orange types. In *Papilio xuthus*, the two main pupal color types, green and brown, are determinated by environmental factors, *e. g.*, humidity, light, and the texture of pupational sites (Ishizaki and Kato, 1956; Hidaka, 1961), and the development of brown pupal color is regulated by a neurosecretory hormone called the browning hormone (Hidaka, 1961; Awiti and Hidaka, 1982).

Recently, we have reported that a hormone producing brown pupae (pupal cuticle-melanizing hormone: PCMH) was extracted from brain–suboesophageal ganglion–prothoracic ganglion (Br–SG–PG) complexes of *P. xuthus* pupae, and that the extracts of Br–SG complexes of silkmoths, *Bombyx mori*, also showed a PCMH activity (Yamanaka *et al.*, 1999). By contrast, several endocrinological studies on pupal coloration have revealed that the juvenile hormone (JH) is involved in the green color expression of pupae in cabbage white butterflies, *Pieris rapae crucivora* and *P. brassicae* (Hidaka and Ohtaki, 1963; Ressin, 1980). However, nothing is known about the effects of the JH and insect neuropeptides on the pupal cuticle coloration in swallowtail butterflies.

In the present study, we examined the effects on the pupal cuticle coloration of *P. xuthus* of various insect hormonal factors. The factors we tested were JH I, a JH analog methoprene, 20–hydroxyecdysone, and summer-morph-producing hormone (SMPH)-active peptide of *B. mori* adults, which shows an SMPH activity in the Asian comma butterfly, *Polygonia c-aureum*.

Materials and Methods

Insects

P. xuthus and *P. c-aureum* were collected from the town of Yamaguchi. Larvae of *P. xuthus* were reared on leaves of *Fagara ailanthoides* under a long day photoperiod (16 h light and 8 h dark; 16L:8D) at 23℃ using the methods described by Yamanaka *et al.* (1999). Pharate pupae and pupae of *P. xuthus*

were used for present experiments. Larvae of *P. c-aureum* were raised on leaves of *Humulus japonicus* under a long day photoperiod (16L:8D) at 25℃ or a short day photoperiod (8L:16D) at 20℃ (Endo *et al.*, 1988). Cocoons of *B. mori* of a commercial race (Kinshu × Showa) were obtained from a silk farm in Yamaguchi Prefecture. They were allowed to develop at room temperature.

PCMH and SMPH-active peptide

Three hundred Br–SG complexes were obtained from *P. xuthus* green pupae and *B. mori* adults by dissection in 0.9% NaCl. One hundred Br–SG complexes from *P. xuthus* and *B. mori* were grouped and stored at −85℃ until use, respectively. A batch of 300 Br–SG complexes of *P. xuthus*, and *B. mori* was homogenized in 1.5 ml of ice-cold acetone with a Teflon homogenizer and centrifuged for 15 min at 12,100 ×g at 4℃. The pellet was washed with 1.5 ml of 80% ethanol and centrifuged under the same conditions. The resulting pellet was extracted with 1.0 ml of 2% NaCl in a boiling water bath for 4 min, cooled rapidly on ice, and centrifuged under the same conditions. The resulting supernatant of *P. xuthus* was applied to a Sep–Pak cartridge C18 column (Waters) for desalting and eluted with 50% acetonitrile solution. The eluate was lyophilized and used as PCMH extract. On the other hand, to separate SMPH-active peptide from PCMH-active peptide, ammonium sulfate was added to the resulting supernatant of *B. mori* to 80% saturation. The precipitate from centrifugation (12,100×g, 30 min) was recovered and dissolved in 1.0 ml of 0.1 M ammonium acetate. The solution was applied to a gel filtration column (12×912 mm) of Sephadex G–50 equilibrated with 0.1 M ammonium acetate and was eluted with the same solution according to the procedure previously reported (Tanaka *et al.*, 1997). The SMPH-active fractions were pooled, lyophilized and used as the SMPH-active peptide fraction.

Injection samples

Five, and 10 μg of JH I, and 1.0, 5.0, and 10 μg of 20–hydroxyecdysone were dissolved in 10 μl of ethanol, respectively. Fifty Br–SG complexes equivalent of PCMH, and SMPH-active peptide, and 1.25 and 6.25 μg of methoprene were dissolved in 10 μl of distilled water, respectively.

Bioassay for melanizing-stimulation and -inhibition activites

To assess the melanizing-stimulation activities on pupal cuticle, larvae of *P. xuthus* in the wandering stage were placed in cardboard boxes with a rough inner surface (brown pupa producing condition) and ligatured between the thorax and abdomen at the stage P2 of Hidaka (1961) that was judged by the morphology of pupal antennal buds and the tone of eye pigment observable through the head capsule. Ten μl each at a desired concentration of JH I, methoprene, 20–hydroxyecdysone, SMPH-active peptide, or PCMH was injected into each ligatured abdomen when the development of anterior part reached stage P4. On the other hand, the bioassay for the melanizing inhibition activities was carried out with the

Table 1 Melanizing-stimulation effects of JH I, methoprene, 20–hydroxyecdysone, SMPH-active peptide and PCMH on the pupal cuticle coloration of *Papilio xuthus*.

Hormonal factors	Concentration (μg/insect)	N	Grades of cuticular melanization			
			0	1	2	3
JH I	5.0	10	10	0	0	0
	10.0	5	5	0	0	0
Methoprene	1.25	10	10	0	0	0
	6.25	5	5	0	0	0
20–hydroxyecdysone	1.0	10	10	0	0	0
	5.0	5	5	0	0	0
	10.0	5	5	0	0	0
SMPH-active peptide	50 Br–SG eq.	4	4	0	0	0
PCMH	50 Br–SG eq.	4	0	0	0	4
Distilled water		10	10	0	0	0

non-ligatured abdomen of pharate pupae under the brown pupa-producing condition. Ten μl of JH I, methoprene, or 20-hydroxyecdysone at desired concentration was injected into each non-ligatured abdomen when the development of anterior part reached stage P4. Controls were injected only with 10 μl of distilled water. The wound by injection was sealed with paraffin wax. After pupation, the larval cuticle was removed in a 0.9% NaCl solution using fine forceps. Non-ligatured abdomens were classified into one of grades 0–4, and ligatured abdomens were classified into one of grades 0–3 as described by Yamanaka *et al.* (1999), where grade 4 represents the most intense and grade 1 the least intense melanization. Grade 0 corresponds to no melanization (green color).

Bioassay of SMPH-activity

SMPH activity in each fraction of Sephadex G-50 column chromatography was assayed by the *Polygonia* pupal assay (Endo *et al.*, 1988). Each fraction was dissolved in distilled water (10 Br-SG equivalents/10 μl) and injected into each abdomen of short day *Polygonia* Day 0 female pupae. The grades of summer morphs in the female butterflies were classified into one of grades 0–4, where grade 4 and 3 were regarded as summer morphs and grade 0 and 1 were regarded as autumn morphs. Grade 2 was intermediate morph.

Chemicals

JH I and 20-hydroxyecdysone were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Methoprene was obtained from Otsuka Chemical Co. (Osaka, Japan). Sephadex G-50 was purchased from Pharmacia Biotech Co. (Uppsala, Sweden). All chemicals were of analytical grades.

Results and Discussion

To test whether JH I, methoprene, 20-hydroxyecdysone, and SMPH-active peptide solely act on the melanizing-stimulation to pupal cuticle, we performed injection experiments using ligatured abdomens of pharate pupae, which were to produce brown pupae of *P. xuthus*. Table 1 indicates that these hormonal factors did not cause the melanizing-stimulation to pupal cuticle of ligatured abdomens. Similar results have been reported in the peacock butterfly, *Inachis io* (Lepidoptera: Nymphalidae), from a black background that injections of JH I, II, III, ecdysone and 20-hydroxyecdysone give no effect on pupal melanization (Maisch and Bückmann, 1987). On the contrary, the PCMH of *P. xuthus* green pupae showed high melanization activity to the pupal cuticle. Taken together we conclude that JH I, methoprene, 20-hydroxyecdysone, and SMPH-active peptide are not involved in the melanizing-stimulation effect on pupal cuticle coloration of *P. xuthus*, and that PCMH can trigger only the first step of the pupal cuticle melanizing cascade of *P. xuthus*.

Furthermore, to investigate their melanizing-inhibition effects on pupal cuticle, JH I, methoprene, and 20-hydroxyecdysone were injected into the non-ligatured abdomens of pharate pupae, which were to produce brown pupae. As shown in Table 2, neither JH I, methoprene nor 20-hydroxyecdysone inhibited the melanization of pupal cuticle. JH is known to play a role in the green coloration of *P. rapae crucivora* and *P. brassicae* pupae although its pathway has yet been poorly understood (Hidaka and Ohtaki, 1963; Ressin, 1980). The present result provides evidence that JH I, methoprene, and 20-hydroxyecdysone do not act

Table 2 Melanizing-inhibition effects of JH I, methoprene, and 20-hydroxyecdysone on pupal cuticle coloration of *Papilio xuthus*.

Hormonal factors	Concentration (μg/insect)	N	Grades of cuticular melanization				
			0	1	2	3	4
JH I	5.0	10	0	0	0	0	10
	10.0	5	0	0	0	0	5
Methoprene	1.25	10	0	0	0	0	10
	6.25	5	0	0	0	0	5
20-hydroxyecdysone	1.0	10	0	0	0	0	10
Distilled water		10	0	0	0	0	10

on the green coloration in *P. xuthus*.

To understand the pupal cuticle coloration system of *P. xuthus* in detail, further studies on purification of the PCMH, and characterization of morphological and physiological differences between brown and green pupae are now in progress.

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