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Interactions of environmental factors influencing pupal coloration in swallowtail butterfly *Papilio xuthus*

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Abstract

The swallowtail butterfly *Papilio xuthus* Linné [Lepidoptera: Papilionidae] exhibits pupal protective color polyphenism. Interactions of various environmental factors on pupal coloration were analyzed in non-diapausing individuals. Under sufficient light (200 lux), most pupating larvae became green pupae when the surface of the pupation site was smooth, while they became brown when the surface was rough. Tactile signals are the positive environmental factors causing induction of the brown pupal coloration. In dark boxes, the induction of the brown pupal coloration was easily induced even on a smooth surface, suggesting that light suppresses induction of brown coloration. Different colors of pupation sites did not affect pupal coloration under sufficient light. Environmental factors received during a critical period both before girdling and after girdling affected pupal coloration. When tactile signals received from rough surfaces reach threshold levels during pupation, brown pupal coloration is determined. Larvae reared under a daily periodicity of natural light formed a girdle at midnight, subsequently, the prepupae received strong daylight the following day. Under natural light most larvae produced brown pupae on rough surfaces and green pupae on smooth surfaces.

Keywords: Butterfly; Pupal color polyphenism; Protective coloration; Tactile signal; Light intensity

1. Introduction

Many species of butterflies in the natural world exhibit pupal color polyphenism to enable them to mimic the colors of a variety of pupation sites as a survival mechanism against insectivorous birds (Dyck et al., 1998; Hazel, 1977; Hazel et al., 1998; Hidaka et al., 1959; West and Hazel, 1982). In the Papilionidae family, Ohnishi and Hidaka (1956) reported that the pupal color of *Papilio xuthus* and *Papilio protenor* was not determined by the background color of the pupation site. Hidaka (1961, 1975) presumed the involvement of a particular chemical stimulus (odor) emanating from green live food and non-food plants under dark conditions. When larvae of *Papilio xuthus* were reared under short-day photoperiodic conditions, the larvae formed frequently the orange- (reddish brown-) type of pupae that underwent a long pupal diapause (Ishizaki and Kato, 1956; Yamanaka et al., 2005). Formation of the orange-type of pupae depended on the relative humidity, temperature, and the photoperiod perceived during the larval growth period (Ishizaki and Kato, 1956). Ishizaki and Kato (1956) also reported that pupal coloration is affected by humidity and darkness during pupation in non-diapausing individuals. The determination of pupal coloration in the butterflies *Papilio polytes*, *Papilio demoleus* and *Papilio polyxenes* depends upon variations in environmental conditions, and brown pupae tend to be found on rough surfaces and green pupae on smooth ones (Smith, 1978). The texture of the pupation substrates, chemical stimulants, conditions of light intensity, relative humidity, temperature, photoperiod during the larval stage, and the diameter of pupal substrates are regarded as factors influencing the species of *Papilio*, but none of these appear to be the major determinant (Clarke and Sheppard, 1972; Honda, 1979, 1981). Honda (1979) described the way how leaves and known odors of live food plants exerted no obvious effect on the formation of green pupae on rough surfaces in light, although a weak effect due to live food plant extracts was observed at 100%

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relative humidity. Wiklund (1972) described the weak effect of various wavelengths of light on pupal coloration in *Papilio machaon* L. Differential effects of colors of pupation sites or color rays were described in some species of Papilionidae (Hazel and West, 1979; West and Hazel, 1985; Smith, 1978). In *Papilio polyxenes* and *Battus philenor*, the pupal coloration results from the joint action of genotype and environment and usually makes the pupae cryptic in their habitats (Hazel, 1977; Hazel and West, 1979). The idea that textural signals accumulate over some threshold level to induce brown pupae was first proposed by Hazel (1977) and in subsequent papers by Hazel and West (1982) and Smith, 1978. It was described that genetic evidence in the pupal color in swallowtails is a threshold trait (Hazel, 1977; Hazel and West, 1982; Sims, 1983).

Recently, Hiraga (2005) found a new type of sensory mechanism for the control of pupal coloration in *Graphium sarpedon nipponum* Fruhstorfer [Lepidoptera: Papilionidae]. Pupal color is determined by the illuminant difference between the dorsal direction of incident light and the ventral direction of light in *G. sarpedon nipponum*. In addition, Hiraga (2005) reported that tactile signals received successively from rough surfaces at the pupation site cause the induction of the brown pupal coloration under conditions where there is sufficient light in *Papilio xuthus*. Based on these results, a 'tactile signal accumulation hypothesis' was proposed for the control of pupal coloration in non-diapause pupation of *Papilio xuthus*.

In the present work, the author analyzed the effects of the color of the pupation site, tactile signals, light intensity, living food plant leaves, 100% humidity and 5% carbon dioxide gas on the pupal coloration in non-diapause pupation of *Papilio xuthus*. The results strongly supported the 'tactile signal accumulation hypothesis', although light, living plant leaves, and 100% humidity might significantly increase the threshold levels of the tactile signals that are required for the induction of brown pupal coloration. Pupation experiments with larvae reared under a daily periodicity of natural light indicated that the presence of a rough texture at the pupation site is important for the induction of brown pupal coloration in the field.

2. Materials and methods

2.1. Insects

The eggs and larvae of *Papilio xuthus* were gathered from leaves of the food plants *Poncirus trifoliata*, *Zanthoxylum piperitum*, and the orange tree *Citrus* species. Both eggs and larvae were reared in plastic cages (15 × 25 × H15 cm) and fed on leaves from food plants under continuous lighting at 200-lux intensity (white fluorescent lamps) to ensure the production of non-diapausing pupae. The eggs and larvae were gathered in Kyoto city.

2.2. Pupation apparatus

When a larva in the final insta finished its final evacuation (gut purge) in preparation for pupation and was wandering in search of a pupation site, the larva was placed on the following apparatus to investigate the pupal coloration mechanism. To analyze the effect of the color and surface roughness of the pupation site, a paper board (5 × 7 cm) was fixed to a wire (0.7-mm diameter, 14-cm length) and the wire was then placed in a bottle. The entire apparatus was then covered with a transparent polyethylene bag (Fig. 1A). The pupation boards were made of two sheets that were fixed together on their reverse sides with a wire inserted between them. A range of boards were made using a combination of glossy photopaper (EPSON PM photopaper), filter paper (Whatman no. 17 CRH), abrasive cloth AA-100 (black aluminum oxide particles, Sankyo Rikagaku Co. Ltd., Saitama, Japan), or abrasive paper G-40 (brown sand particles, Sankyo Rikagaku Co. Ltd.). Microscopic images of. AA-100 and G-40 by microscopy were analyzed by MacSCOPE version 2.5 (Mitani Corp., Maruoka, Japan) run on a personal computer to measure the particle size and particle number per 100 mm^2 (Fig. 2). The sheets of photopaper of black, white, green or brown were used to test the effects of color. They were colored using an EPSON ink-jet printer (PM-950C) connected to a computer (Apple Macintosh PowerBook G4; Adobe Illustrator 9.0): white (CMYK color index: C0, M0, Y0, K0), black (C0, M0, Y0, K100), green (C50, M0, Y100, K0), and brown (C0, M60, Y100, K20). All larvae pupated on the boards, rather than on the wire of the apparatus.

To investigate the effects of living plant leaves (*Poncirus trifoliata*) and 100% humidity on pupal coloration, pupation boards were made of two sheets (10 × 30 cm) of photopaper, filter paper, AA-100 or G-40, which were fastened together on their reverse sides using staples. The boards were folded to form a folding screen (Fig. 1B) and placed in a transparent plastic container (16 cm × 12 cm × H12 cm), which was sealed tightly with a plastic cap. In some cases, leaves of the living food plant *Poncirus trifoliata* (total; approximately 200 cm^2 of leaves) or absorbent cotton that was soaked in water on a small plate were placed in the container together with the pupation board (Fig. 1B).

To investigate the effect of 5% carbon dioxide gas, a plastic container (16 cm × 12 cm × H12 cm) covered with a nylon net in which a folding screen of photopaper was inserted was placed in a carbon dioxide incubator (ESPEC, BNA-121D) with 5% carbon dioxide gas at 28 °C (in darkness).

Room temperature was maintained at 24–30 °C using an air conditioner. White fluorescent lamps were used for lighting (continuous lighting, 200 lux) in the room during the pupation period. Light intensity was measured using an illumination meter LX-1108 (Sato Shouji Inc., Yokohama). For those experiments that were conducted in complete darkness, the pupation apparatus described above (Fig. 1A

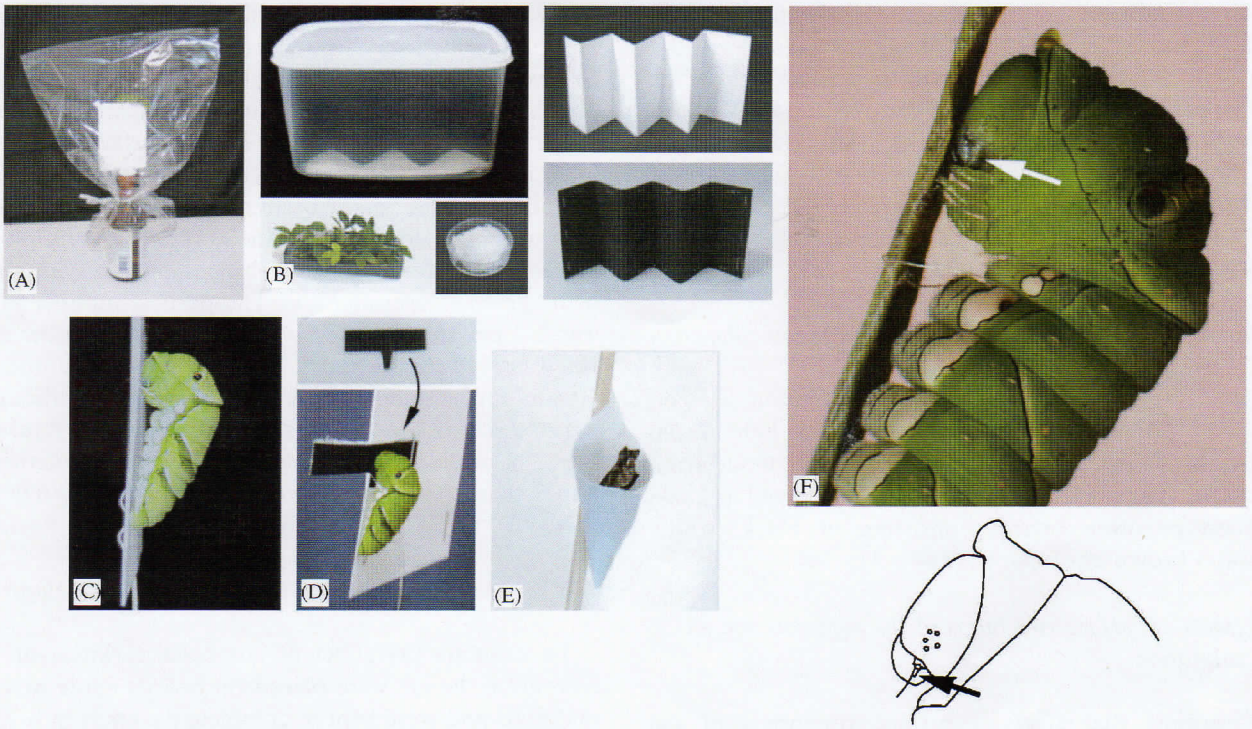

Fig. 1. Apparatus used to examine effects of various environmental factors on pupal coloration in *Papilio xuthus*. (A) Apparatus with a board. (B) Apparatus used to analyze effects of living plant leaves or 100% humidity. A container closed tightly with a cap, folding screens of photopaper and AA-100, leaves of living food plant, and a plate of absorbent cotton with water. (C) Prepupa releasing abdominal prolegs from the pupation boards. Note that the front of the head touches the pupation board. (D) Changing materials of the pupation site using a T-shaped sheet. (E) Changing materials of the pupation site using a conical bag. (F) Prepupa releasing abdominal prolegs from the pupation material. Arrow shows an antenna that does not touch the pupation site.

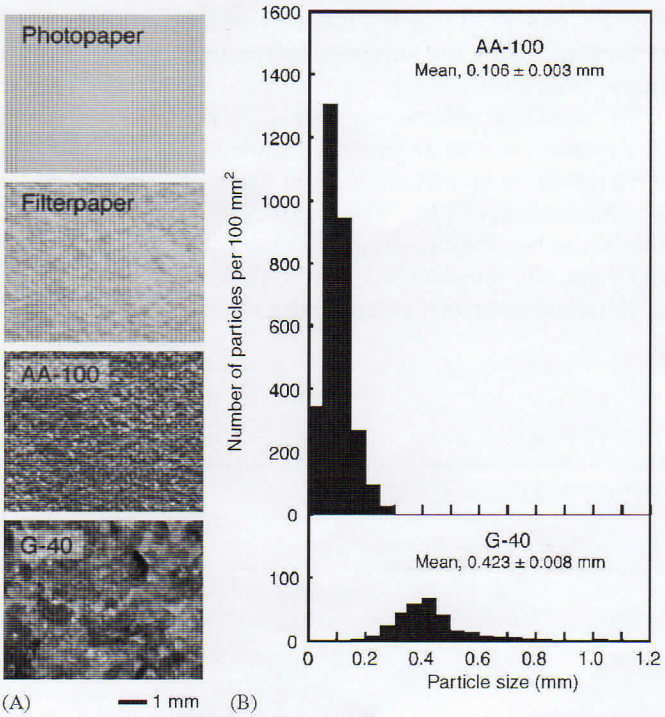

Fig. 2. (A) Microscopic images of photopaper, filter paper (Whatman no. 17 CHR), abrasive cloth AA-100, and abrasive paper G-40. Scale bar represents 1 mm. (B) Particle sizes and the number of particles of AA-100 and G-40. The number of particles per 100 mm^2 was 3014 in AA-100 and 371 in G-40.

or B) was kept within large dark plastic boxes (60 cm × 40 cm × H40 cm) that were tightly covered with black drapes.

All of the experiments were performed between May and September of 2005 in Kyoto city. All of the pupae used in this work emerged as adult butterflies approximately 10 days after pupation (no hibernation of the pupae). The final pupal color was judged 24 h after larva–pupal ecdysis. Pupae were classified into green, brown, and intermediate (beige or pale brown) types. The orange-type (or red-type) pupae (Ishizaki and Kato, 1956; Yamanaka et al., 2005) that undergo a long pupal diapause did not appear in this work.

2.3. Changing materials at the pupation site during pupation

To investigate the critical period that is required to determine the pupal color, the materials used at the pupation site were changed with other materials during the pupation period as follows. When a larva had formed a silken girdle on an AA-100 board, the larva was released from the board by cutting the girdle with scissors and was then transferred to a conical bag made from white photopaper (Fig. 1E). In other experiments, when the larva had formed a girdle on the AA-100 board, a T-shaped sheet of black photopaper was inserted between the head–thorax section and the pupation board and was fixed

to the board with staples. In a reverse experiment, when the larva had released its abdominal prolegs from the pupation board (which was made from either black or white photopaper) after formation of the girdle, a T-shaped sheet of AA-100 (black) was inserted between the head–thorax part and the pupation board and fixed in place (Fig. 1D).

3. Results

3.1. Effect of color of the pupation site on pupal coloration

To study the effects of color of the pupation sites on pupal coloration, larvae were tested in a light room (200 lux) on various colored pupation boards made from photopaper (Fig. 1A). Most larvae (90%) emerged as green pupae on pupation boards consisting of black, white, green, and brown photopaper (Table 1).

3.2. Effects of surface roughness of the pupation site on pupal coloration

To examine the effect of surface roughness of the pupation site on pupal coloration, smooth plastic, photopaper, filter paper, abrasive cloth AA-100, and abrasive paper G-40 were used as materials for the pupation board, which was tested in a light room (200 lux). As shown in Table 2 Experiment 1, most pupating larvae (89%) became green in color on smooth surfaces of photopaper and on the plastic boards. In contrast, most pupating larvae became brown in color on filter paper and on AA-100 boards, which both had rough surfaces (Experiments 2 and 3). These results indicated that rough surfaces caused the brown pupal coloration. Thus, tactile signals received from the pupation site might be important for determining the brown pupal coloration. On the abrasive paper G-40, 43% of the larvae tested became brown pupae, with the rest being green (Experiment 4). The particle number of G-40 was 371 per 100 mm^2, whereas, the particle number of AA-100 was 3014 per 100 mm^2. Thus, the particle number in G-40 was much smaller than that of AA-100, although the particle size of G-40 (mean size 0.423 ± 0.008 mm, errors are 95% confidence intervals of Student's t estimation) was larger than that of AA-100 (mean size 0.106 ± 0.003 mm) (Fig. 2B).

Table 1
Effect of colors of the pupation site in a lighted room

Pupation site		Number of pupae		Total number
Material	Color	Green	Brown	
Photopaper	Black	13	0	13
	White	12	4	16
	Green	5	0	5
	Brown	5	0	5
	Sum	35 (90%)	4 (10%)	39

3.3. Effects of illumination intensity on pupal coloration

To examine the effect of complete darkness on pupal coloration, larvae were placed on boards made of various materials and were kept in complete darkness in boxes. As shown in Table 3, 77% of the larvae became brown pupae on smooth surfaces of the plastic and photopaper boards when kept in the dark (Table 3 Experiment 1), in contrast to the case where there was sufficient light (Table 2 Experiment 1). The number of brown pupae increased significantly by darkness for all of the different materials that were used to form the pupation sites (cf. Tables 2 and 3; Fig. 3A and B). Thus, the intensity of ambient light is an important factor for suppressing the induction of brown pupal coloration.

To examine effects on pupal coloration when the illumination was at an intensity lower than 200 lux, larvae were tested on boards made from black photopaper under an illumination of 1 lux. The majority (9 out of 10; 90%) of the larvae became brown pupae (Fig. 3C), suggesting that the 1-lux illumination was not sufficient to suppress the appearance of brown pupae under these conditions.

Table 2
Pupal color of *Papilio xuthus* under various conditions in a lighted room

Pupation site	Addition	Sample size	Brown pupae	2 × 2 comparisons (p-value)**						
				2	3	4	5	6	7	8
1. Smooth[a]	None	18	2 (11%)	++	++	−	+	−	++	−
2. Filter paper	None	15	12 (80%)		++	+	++	+	−	+
3. AA-100	None	27	27 (100%)			++	++	++	++	++
4. G-40	None	54	23 (43%)				++	−	++	−
5. Smooth[a]	Leaves	33	0 (0%)					++	++	+
6. Filter paper	Leaves	16	5 (31%)						++	−
7. AA-100	Leaves	27	21 (81%)							++
8. G-40	Leaves	7	1 (14%)							

[a]Sum of pupae on smooth surface materials; plastic board and photopaper.
**p-value by χ^2 test for independence. ++, $p<0.01$; +, $0.01<p<0.05$, −, not significant ($p>0.05$).

Table 3
Pupal color of *Papilio xuthus* under various conditions in a dark box

Pupation site	Addition	Sample size	Brown pupae	2 × 2 comparisons (*p*-value)						
				2	3	4	5	6	7	8
1. Smooth[a]	None	30	23 (77%)	+	+	–	++	–	–	–
2. Filter paper	None	11	11 (100%)		++	++	++	++	+	++
3. AA-100	None	17	16 (94%)			+	++	–	+	++
4. G-40	None	10	9 (90%)				++	–	–	+
5. Smooth[a]	Leaves	17	4 (25%)					–	+	++
6. Filter paper	Leaves	15	7 (47%)						–	++
7. AA-100	Leaves	10	8 (80%)							–
8. G-40	Leaves	14	13 (93%)							

Notation as in Table 2.

To examine whether illumination stronger than 200 lux is able to suppress the brown pupal coloration induced by the strong tactile signals produced by the AA-100 boards, larvae were tested on boards of AA-100 under 1000-lux illumination from white fluorescent lamps placed on both sides of the boards. All of the 13 larvae that were tested became brown pupae under this illumination regime (Fig. 3D). Thus, the formation of brown pupae that was induced by the tactile signals from AA-100 was not suppressed by even 1000-lux illumination, indicating that the strong tactile signals were dominant over the suppression function of even 1000-lux illumination, and the brown pupal coloration was still evident.

3.4. Effects of living food plant leaves on the pupal coloration

The effects of the presence of living leaves of the food plant *Poncirus trifoliata* were examined by using a plastic container covered tightly with a cap in a light room or in dark boxes, as described in Materials and Methods section (Fig. 1B). In the light room, the number of brown pupae was significantly reduced on the photopaper and filter paper by the presence of leaves (Table 2 and Fig. 3A). However, no significant reduction in the number of brown pupae due to the presence of leaves was observed on AA-100 boards under light. Within the dark boxes, a significant reduction in brown pupae due to the presence of leaves was observed on plastic, photopaper and filter paper boards, but not on AA-100 and G-40 (Table 3 and Fig. 3B). Thus, the presence of living plant leaves and light suppressed the induction of brown pupal coloration (Fig. 3A and B).

3.5. Effects of 100% humidity and 5% carbon dioxide gas on pupal coloration

As described above, the presence of living plant leaves decreased the number of brown pupae under some conditions. Living plant leaves might diffuse moisture, carbon dioxide and some other volatile compounds in dark conditions. When larvae were placed on a black photopaper apparatus and kept in the presence of 100% humidity in a large, completely dark box, only 43% of the pupae were brown under conditions of 100% humidity, suggesting that 100% humidity partially suppressed the formation of brown pupae (Table 4).

3.6. Time course of pupation events

Fig. 4A shows the time course of events observed during the pupation of 36 larvae, which were reared under continuous lighting (200 lux) from white fluorescent lamps during the larval period. After final evacuation, the larvae walked rapidly for 1–4 h to search for a suitable pupation site on the board and then remained stationary for 4–5 h. Subsequently, the larva formed a pad and a band string (silken girdle), which wound around the body and became a prepupa. After 2–4 h from the girdle formation, the prepupa released its abdominal prolegs from the pupation board. After approximately 20 h from girdle formation, the prepupa ecdysed (larva–pupal ecdysis). In the case of the brown pupal type, black mottled patterns were observed through the larval cuticle in the dorsal part of the prepupa at 2–3 h before the larva–pupal ecdysis, suggesting that induction of the black pigment had been determined more than 2–3 h prior to the ecdysis. The brown color expanded from the dorsal region to the rest of the body for several hours after the ecdysis. The larvae that had been reared under continuous lighting formed the girdle at random times of the day (Fig. 4A and C).

On the other hand, when larvae had been reared under natural light with a daily periodicity during the larval period, they carried out their final evacuation in early nighttime after sunset, formed girdles at around midnight, and then pupated the next night (Fig. 4B and D). The girdling of all larvae around midnight is likely due to a circadian rhythm caused by daily periodicity of natural light during the larval period. Note that all prepupae receive strong natural light through the daytime.