Hormonal Control of the Orange Coloration of Diapause Pupae in the Swallowtail Butterfly, Papilio xuthus L. (Lepidoptera: Papilionidae)

Authors: Yamanaka, Akira, Imai, Hiroshi, Adachi, Miwa, Komatsu, Mitsunobu, Islam, A. T. M. Fayezul, et al.

Source: Zoological Science, 21(10) : 1049-1055

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.1049

ZOOLOGICAL SCIENCE **21**: 1049–1055 (2004)

Hormonal Control of the Orange Coloration of Diapause Pupae in the Swallowtail Butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae)

Akira Yamanaka[1*], Hiroshi Imai[1], Miwa Adachi[1], Mitsunobu Komatsu[1], A.T.M.Fayezul Islam[1,2], Ichiro Kodama[1],Chisato Kitazawa[3] and Katsuhiko Endo[1]

[1]*Department of Physics, Biology and Informatics, Faculty of Science, Yamaguchi University, Yamaguchi 753-8512, Japan*
[2]*Institute of Food and Radiation Biology, AERE, GPO Box-3787, Dhaka-1000, Bangladesh*
[3]*Department of Biology, Duke University, Durham, NC 27708, USA*

ABSTRACT—Diapause pupae of *Papilio xuthus* show color polymorphism, represented by diapause-green, orange, and brownish-orange types that are each associated with specific pupation sites. We investigated the role of the site of pupation on the induction of the development of orange types (or brownish-orange types), and the endocrine mechanism underlying the control of color polymorphism in short-day pupae. All short-day larvae of the wandering stage developed into orange or brownish-orange type pupae when they were placed in rough-surfaced containers after gut-purge. Utilizing a pharate pupal ligation between the thorax and abdomen, the endocrine mechanism underlying the control of color polymorphism was shown to involve a head-thorax factor (Orange-Pupa-Inducing Factor: OPIF) that induced orange types in short-day pupae. OPIF was bioassayed using the ligated abdomens of short-day pharate pupae. OPIF was extractable with 2% NaCl solution from 5th-instar larval ganglia complexes following the mesothoracic complex ($TG_{2,3}$-AG_{1-7}), but it could not be extracted with either acetone or 80% ethanol solution. OPIF may not exist in the brains of day-0 pupae or in brain-subesophageal ganglion and prothoracic ganglion complexes of 5th-instar larvae. The short-day pharate pupae responded to OPIF in a dose-dependent manner.

Key words: *Papilio xuthus*, swallowtail butterfly, pupal coloration, diapause pupa, orange pupa

INTRODUCTION

Pupae of three lepidopteran families, the Pieridae, Papilionidae and Nymphalidae, show color polymorphism that is dependent on background color (Poulton, 1887; Bückmann 1960, Smith, 1978). Physiological mechanisms underlying the control of pupal polymorphisms were shown to involve hormonal factors (Ohtaki, 1960; Hidaka, 1961; Bückmann and Maisch, 1987).

Recent investigations of the yellow/black pupal polymorphism exhibited by the peacock butterfly *Inachis io* (Nymphalidae) have shown that pupal melanization is controlled by a pupal melanization reducing factor (PMRF) that exists in the central nervous system of larvae and adults (Bückmann and Maisch, 1987; Koch *et al*., 1990; Starnecker, 1997; Starnecker and Bückmann, 1997). Additionally, PMRF is thought to stimulate the incorporation of lutein into the pupal integument of *I. io* (Starnecker, 1997), though details of the regulatory mechanism have not been elucidated.

The coloration of pupal bodies in certain papilionid species is determined by environmental cues surrounding the site of pupation, such as background color, texture, humidity, curvature, and photoperiod (Ohnishi and Hidaka, 1956; Hazel and West, 1996; Smith, 1978; Honda, 1981; Shimada, 1983). It has been reported that the development of brown pupae in *Papilio xuthus* is determined by a neurohormone, the so-called browning hormone, which is secreted from the prothoracic ganglion in the pharate pupal stage. Pharate pupae are thought to develop into the green pupae in the absence of the browning hormone (Hidaka, 1961; Awiti and Hidaka, 1982).

* Corresponding author: Tel. +81-83-933-5720;
FAX. +81-83-933-5720.
E-mail: yamanaka@yamaguchi-u.ac.jp

In order to analyze the function and structure of hormonal factor(s) involved in pupal color polymorphism of *P. xuthus*, we have been focusing on the biochemical aspects of hormonal factor(s) producing brown-type pupae. In our previous studies, we reported a neuropeptide (Pupal-Cuticle-Melanizing Hormone: PCMH, or PCMH-active factor) involved in the production of brown-type pupae that exists in the brain (Br), subesophageal ganglion (SG) and prothoracic ganglion (PG) complex of *P. xuthus* pupa (or in the Br-SG complex of *Bombyx mori* adult), and that may trigger the first step of pupal cuticle melanization in *P. xuthus* (Yamanaka *et al*., 1999, 2000).

Pupal diapause in *P. xuthus* is regulated by photoperiodic conditions. Larvae reared from the egg stage under long-day or short-day conditions developed into non-diapause or diapause pupae, respectively (Ishizaki and Kato, 1956; Ishizaki, 1958; Hidaka and Hirai, 1970). Additionally, diapause pupae of *P. xuthus* exhibit color polymorphism, as represented by diapause-green, orange, and brownish-orange types (Ishizaki and Kato, 1956; Ishizaki, 1958). About half of the specimens reared under darkness, high humidity and low temperature at the young larval period developed into orange-type pupae, which invariably enter diapause (Ishizaki and Kato, 1956). Field observations during late autumn revealed that pupae pupating on a rough surface, such as a dead twig or wooden wall, tended to develop into orange and brownish-orange types, while those that pupated on the fresh twig of a food plant tended to develop into green types (Ishizaki, 1958). However, it is still not clear how underlying physiological mechanisms control and influence the coloration of diapause pupae in *P. xuthus*, and there is little information concerning the relationship and difference between the brown type of non-diapause pupae and the orange type (or brownish-orange types) of diapause pupae.

The present study aims to determine the environmental conditions inducing the development of orange types (or brownish-orange types) in diapause pupae of *P. xuthus*. A further goal of this research is to establish methods for the extraction and assay of a factor from the nerve cord of 5th-instar short-day larvae that induces orange-type pupae (hereafter designated as the Orange-Pupa-Inducing Factor: OPIF).

MATERIALS AND METHODS

Insects

Adults of *P. xuthus* L. were collected near towns within the Yamaguchi prefecture. Female adults fed on an 8–10% sucrose solution at 25°C were allowed to oviposit on leaves of *Fagara ailanthoides* at intervals of four days. Larvae were reared on leaves of *F. ailanthoides* under either long-day (LD) conditions (alternating 16 hr light and 8 hr dark periods, 16L:8D) at 23°C, or short-day (SD) conditions (8L:16D) at 20°C. Under LD conditions, larvae developed into non-diapause pupae (LD-pupae), whereas under SD conditions they developed into diapause pupae (SD-pupae).

Expression of pupal colors of SD-pupae

Fifth-instar larvae of *P. xuthus* entered the wandering stage after gut-purge. Larvae of the wandering stage were selected from the stock cultures under SD conditions, and placed in transparent plastic containers at 25°C. After pupation, SD-pupae were classified into three types with respect to body color: diapause-green, orange, and brownish-orange (Fig. 1A). Additionally, the assay and classification of OPIF activity involved short-day pharate pupae being ligated between the thorax and abdomen with a cotton thread. The ligated abdomens of pupae belonging to the orange type were classified into one of four grades with respect to the degree of orange

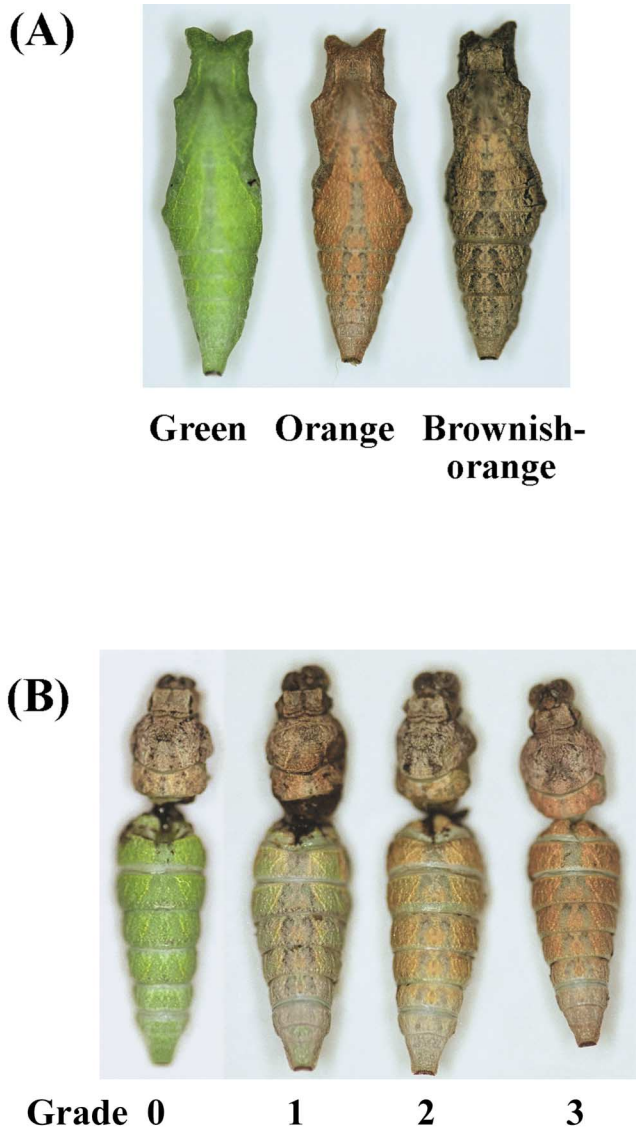

Fig. 1. **(A)** Classification of the pupal body colors of SD-pupae in *P. xuthus*, showing the color types of diapause-green (left), orange (middle) and brownish-orange (right). **(B)** Classification of the orange coloration of the ligated abdomen in *P. xuthus*. Grade 3 represents an orange type, whereas grade 0 represents a diapause-green type. Intermediates were classified as either grade 1 or 2, depending on the degree of orange coloration. Grades 3, 2, and 1 resulted after injection of an extract containing 50, 30, and 10 TG_2-AG_{1-7} complex equivalents in a 2% NaCl solution derived from day-0 last instar SD-larvae of *P. xuthus*, respectively. A coloration corresponding to grade 0 resulted after injection with H_2O.

coloration (grades 0 to 3). Grade 0 represented the diapause-green type, whereas grade 3 represented the orange type. Grades 1 and 2 were classified according to the degree of orange coloration of the cuticle (Fig. 1B).

Staging of pharate pupae

Pharate pupal stage (stage P1–6) was determined by observing the development of antennal buds and eye pigment visible through the head capsule, as described by Hidaka (1961).

Dissections of pupal brains and larval nerve cord systems

Three-hundred brains were obtained from day-0 LD-pupae and day-0 SD-pupae of *P. xuthus* by dissection in 0.9% NaCl solution, transferred into ice-cold acetone, and stored at –85°C until use. Seven-hundred and sixty parts of nerves, representing brain (Br), subesophageal ganglion (SG) and prothoracic ganglion (TG_1) complex, mesothoracic and metathoracic ganglia ($TG_{2,3}$), and all abdominal ganglia ($AG_{1–7}$) complexes, were obtained from day-0 last (5th) instar short-day larvae (SD-larvae) by dissection in 0.9% NaCl solution, and then transferred immediately into ice-cold acetone and stored at –85°C until use.

Preparation of crude extracts from pupal brains and larval nerve cord systems

A batch of 100 brains of day-0 LD-pupae and day-0 SD-pupae, as well as 100 Br-SG-TG_1 and $TG_{2,3}$-$AG_{1–7}$ complexes of day-0 last instar SD-larvae, were homogenized in 1.5 ml of ice-cold acetone with a Teflon homogenizer and centrifuged for 10 min at 12,100×*g* at 4°C. Each resulting supernatant (hereafter referred to as the acetone extract) was lyophilized and stored at –85°C until use. Each pellet was re-extracted with 1.5 ml of 80% ethanol, centrifuged for 10 min at 12,100×*g* at 4°C, and then the resulting supernatant (hereafter referred to as the 80% ethanol extract) was lyophilized and stored at –85°C until use. Finally, each pellet was extracted with 1.5 ml of 2% NaCl solution at 95°C for 5 min, cooled rapidly on ice and centrifuged for 10 min at 12,100×*g* at 4°C. After centrifugation, the resulting supernatant was applied to a Sep-Pak C18 cartridge column (Waters) for desalting. After washing with 10 ml of distilled water, the column was eluted with 50% acetonitrile solution. The eluate (hereafter referred to as the 2% NaCl extract) was lyophilized and stored at –85°C until use.

Bioassay of OPIF activity

SD-larvae of the wandering stage were placed in rough-surfaced transparent plastic containers, the insides of which were covered with white kitchen towel paper (Nepia, Japan) (referred to as rough-surfaced paper containers). Larvae at stage P2 were ligated between the thorax and abdomen with a cotton thread, as described by Hidaka (1961).

Ten μl of samples containing the equivalent of 50 brains, 50 Br-SG-TG_1 complexes, and 50 (30, 10, or 5) $TG_{2,3}$-$AG_{1–7}$ complexes were injected into each ligated abdomen when the region of the pharate pupa anterior to the ligature reached stage P4. The injection was made from the dorso-lateral intersegmental region between the 6th and 7th abdominal segments, and the wound was sealed with paraffin wax. After pupation, the larval cuticle was removed in 0.9% NaCl solution using fine forceps, and pupae were classified into one of the four grades. An average orange-coloration degree (AOD) was obtained from the responses of four ligated abdomens. When pupae classified as belonging to the orange type were not further classified into one of the four grades representing the degree of orange coloration, pupae were classified into the three body color types of diapause-green, orange, and brownish-orange.

RESULTS

Degree of orange coloration of pupae and its correlation with pupation site

Table 1 shows that the degree of development of orange coloration in pupae varies with differences in pupation site. SD-larvae developed into diapause-green (12.4%), orange (59.9%), and brownish-orange type pupae (22.7%) in containers of smooth-surfaced transparent plastic under constant light conditions at 25°C. A large proportion (95.3%) of larvae developed into orange type pupae in rough-surfaced paper containers under the same conditions, while less than 5% of pupae placed in the same containers developed into the brownish-orange type. Furthermore, the majority of SD-larvae in cardboard boxes under constant dark conditions at 25°C developed into pupae of the brownish-orange type, with the proportion of diapause-green, orange, and brownish-orange types being 0%, 3.1%, and 96.9%, respectively.

The results indicate that a relatively large proportion of SD-larvae, even when kept in smooth-surfaced containers of transparent plastic, developed into orange type or brownish-orange type pupae under conditions of constant light at 25°C, and no SD-larvae developed into the diapause-green type pupae when kept in rough-surfaced paper containers or cardboard boxes.

Stage-dependent changes of the effect of pharate pupal ligation on body coloration

Pharate pupae destined to develop into orange type and brownish-orange type pupae (O-pharate pupae) were ligated between the thorax and abdomen with cotton thread at six different stages (P1–6). As shown in Table 2, all of the

Table 1. Color types of pupated SD-pupae observed under different pupation conditions.

Pupation sites	Number of insects	Color type of pupal body (%)		
		Diapause-green	Orange	Brownish-orange
Constant light conditions at 25°C				
Transparent plastic container	137	17(12.4%)	82(59.9%)	38(22.7%)
Rough-surfaced paper container*[1]	278	0(0.0%)	273(95.3%)	5(4.7%)
Constant dark conditions at 25°C				
Cardboard box	96	0(0.0%)	3(3.1%)	93(96.9%)

*[1] Rough-surfaced paper container represents a transparent plastic box, the inner surfaces of which were covered with white kitchen towel paper, as descried in Materials and Methods.

Table 2. Pupal color types of ligated head-thoracic and abdominal parts at different life-cycle stages of O-pharate pupae.

	Ligated stages of O-pharate pupae					
Color type of ligated part	P1	P2	P3	P4	P5	P6
Head-thoracic part						
Diapause-green	0	0	0	0	0	0
Orange	5	5	5	5	5	5
Brownish-orange	0	0	0	0	0	0
Abdominal part						
Diapause-green	5	5	5	5	2	0
Orange	0	0	0	0	3	5
Brownish-orange	0	0	0	0	0	0

Five O-pharate pupae were ligated between the thorax and abdomen with a cotton thread at each life-cycle stage. The stage assigned to each O-pharate pupa (stage P1–6) was determined by observing the development of antennal buds and eye pigment visible through the head capsule, as described by Hidaka (1961).

head-thorax parts of O-pharate pupae developed into the orange type, regardless of the stage of the ligated pharate pupa, but the color of the abdomen varied depending on the stage at which the ligature was applied. That is, all pupal abdomens were classified as diapause-green types when they were ligated prior to stage P4. The proportion of orange type abdomens exceeded 50% and approached 100% when pupae were ligated at stages P5 and P6, respectively.

The results indicate that orange type coloration in SD-pupae of *P. xuthus* may be induced by a humoral factor(s) originating from the head-thoracic glands or from the abdominal ones which is under the control of brain. The humoral factor(s) seems to be secreted in the O-pharate pupa at around the P5 stage.

Effect of brain extracts of LD- and SD-pupae on pupal abdominal coloration

We investigated whether a humoral factor(s) exists in the brains of LD- and SD-pupae that induces orange type coloration in SD-pupae, a so-called orange-pupa-inducing factor (OPIF). As shown in Table 3, pupal abdomens injected with the acetone, 80% ethanol or 2% NaCl fraction derived from the brain extracts were classified as diapause-green types, except for the abdomens of some pupae that displayed a spotted melanin cuticle. Such cuticles were possessed by pupae that had received the 2% NaCl extract.

These results indicate that OPIF activity is not present in the brain of day-0 LD and day-0 SD-pupae, and that pupal-cuticle-melanizing hormone (PCMH) activity may exist in the brain of LD and SD-pupae.

Effect of extracts of Br-SG-TG_1 and $TG_{2,3}$-$AG_{1–7}$ complexes of SD-larvae

We assayed whether OPIF activity exists in the central nerve system of day-0 last instar SD-larvae. As shown in Table 4, abdomens that had received the acetone, 80% ethanol, or 2% NaCl extracts of 50 Br-SG-TG_1 complexes were classified as diapause-green types, with the exception of some abdomens that displayed a spotted melanin cuticle. These cuticles were found on pupae that had received the 80% ethanol or 2% NaCl extracts. The abdomens of pupae that had received the 2% NaCl extracts of 50 $TG_{2,3}$-$AG_{1–7}$ complexes were classified as belonging to the orange type, but those that had received the acetone or 80% ethanol extracts of $TG_{2,3}$-$AG_{1–7}$ complexes were classified as having a diapause-green type body color.

No OPIF activity was detected in abdominally ligated O-pharate pupae injected with extracts prepared from Br-SG-TG_1 complexes of SD-larvae. However, slight PCMH activity

Table 3. Pupal color types of ligated abdomens relative to different extracts of brain from LD and SD-pupae.

Source of extract and eluate fractions	Number of insects	Pupal color type of ligated abdomen			
		Diapause-green	Orange	Brownish-orange	Brown*
Brains of LD-pupae					
Acetone	6	6	0	0	0
80% ethanol	6	6	0	0	0
2% NaCl	6	4	0	0	2
Distilled water	6	6	0	0	0
Brains of SD-pupae					
Acetone	6	6	0	0	0
80% ethanol	6	6	0	0	0
2% NaCl	6	5	0	0	1
Distilled water	6	6	0	0	0

A ten μl sample representing an equivalent of 50 brains was injected into a ligated abdomen when the region of the O-pharate pupa anterior to the ligature reached the P4 stage. Asterisk (*) indicates a brown color type, and reflects a grade 1 level of cuticle melanization based on the classification of ligated pupal cuticular melanization in non-diapause pupae of *P. xuthus*, as described by Yamanaka *et al.* (1999).

Table 4. Pupal color types of ligated abdomens with respect to different extracts of Br-SG-TG_1 and $TG_{2,3}$-$AG_{1–7}$ complexes derived from SD-larvae.

Source of Extracts and eluate fractions	Number of insects	Pupal color types of ligated abdomen			
		Diapause-green	Orange	Brownish-orange	Brown*
Br-SG-TG_1 complexes					
Acetone	8	8	0	0	0
80% ethanol	8	7	0	0	1
2% NaCl	8	5	0	0	3
Distilled water	8	8	0	0	0
$TG_{2,3}$-$AG_{1–7}$ complexes					
Acetone	8	8	0	0	0
80% ethanol	8	8	0	0	0
2% NaCl	8	0	8	0	0
Distilled water	8	8	0	0	0

A ten μl sample representing an equivalent of 50 Br-SG-TG_1 and 50 $TG_{2,3}$-$AG_{1–7}$ complexes was injected into a ligated abdomen when the region of the O-pharate pupa anterior to the ligature reached the P4 stage. Asterisk (*) indicates a brown color type, and reflects a grade 1 level of cuticle melanization based on the classification of ligated pupal cuticular melanization in non-diapause pupae of *P. xuthus*, as described by Yamanaka *et al*. (1999).

was detected in pupae that had received the 80% ethanol or 2% NaCl extracts of Br-SG-TG_1 complexes derived from SD-larvae. On the other hand, OPIF activity was only found in pupae that had received the 2% NaCl extracts of $TG_{2,3}$-$AG_{1–7}$ complexes of SD-larvae, with no OPIF activity being detected in the acetone or 80% ethanol treatments.

The results indicate that OPIF activity is present in $TG_{2,3}$-$AG_{1–7}$ complexes of day-0 last instar SD-larvae, but is absent in Br-SG-TG_1 complexes. OPIF activity is detected when using an extract derived from 2% NaCl solution, but is not detected when the extract is derived from acetone or 80% ethanol solution.

Dose-dependent changes of the response of abdomens of O-pharate pupae to the crude OPIF-active extract of $TG_{2,3}$-$AG_{1–7}$ complexes

Finally, we examined whether the response of the abdomens of O-pharate pupae is dependent on the dose of the OPIF-active extract from $TG_{2,3}$-$AG_{1–7}$ complexes. The 2% NaCl extracts representing 5, 10, 30, and 50 $TG_{2,3}$-$AG_{1–7}$ complex equivalents were injected into the ligated abdomens of O-pharate pupae. After pupation, the abdomen of each pupa was classified into one of four color grades (grade 0–3).

As shown in Table 5, half of the O-pharate pupae receiving the 2% NaCl extract of 5 $TG_{2,3}$-$AG_{1–7}$ complex equivalents developed abdomens of an intermediate orange type. The degree of orange coloration of the abdomen of an O-pharate pupa increased with every increment in dose of injected 2% NaCl extract, and the coloration reached the maximum grade of 3.0 when the 2% NaCl extract representing 50 $TG_{2,3}$-$AG_{1–7}$ complex equivalents was injected into the ligated abdomens of O-pharate pupae.

The results indicate that the degree of orange coloration of the pupal epidermis resulting from the experimental manipulation of ligated abdomens of O-pharate pupae shows a dose-dependent response to crude OPIF-active extracts of $TG_{2,3}$-$AG_{1–7}$ complexes.

Table 5. Dose response and the effect of crude OPIF-active extracts from day-0 last instar SD-larvae on the development of orange coloration.

Dose: Number of $TG_{2,3}$-$AG_{1–7}$ complex equivalents	Number of insects	Degree of orange coloration of ligated abdomen				AOD
		0	1	2	3	
0 (H_2O)	8	8	0	0	0	0
5	8	4	4	0	0	0.5
10	8	0	3	5	0	1.625
30	8	0	1	3	4	2.375
50	8	0	0	0	8	3

A ten μl sample containing the desired $TG_{2,3}$-$AG_{1–7}$ complex equivalents was injected into a ligated abdomen when the region of the O-pharate pupa anterior to the ligature reached the P4 stage. An average orange-coloration degree (AOD) was obtained from the responses of four ligated abdomens.

DISCUSSION

All SD-larvae placed in a cardboard box developed into orange (3.1%) and brownish-orange (96.9%) type pupae (Table 1). This result is supported by the finding of Ishizaki (1958), who found that all mature larvae collected from the field in autumn developed into orange-1 (20.5%) and orange-2 (79.5%) type pupae when placed in cardboard boxes. Thus, it appears that the texture of the cardboard during pupation is an important factor in producing the brownish-orange type of SD-pupae and the brown type of LD-pupae (Ohnishi and Hidaka, 1956), but not orange-type pupae. We found that approximately 95% of SD-larvae placed in containers of rough-surfaced paper under constant light conditions at 25°C developed into orange-type pupae, and less than 5% developed into brownish-orange types (Table 1). This may be explained by the existence of two factors, such as a pupal-cuticle-melanizing hormone (PCMH) and orange-pupa-inducing factor (OPIF), which have different thresholds for texture or brightness. On the other hand, it is known that over 90% of LD-larvae kept in smooth-surfaced containers containing food plants developed into green-type pupae (Ohnishi and Hidaka, 1956; Yamanaka *et al*., 1999), while it has been reported that 67.5% of mature larvae collected from the field in autumn developed into diapause-green type pupae when placed in glass cups containing food plants (Ishizaki, 1958). Our data show that only 12.4% of SD-larvae kept in smooth-surfaced containers without a food plant developed into the diapause-green type (Table 1). Therefore, one environmental factor that may play a role in inducing pupae of the diapause-green type in *P. xuthus* could be the smell of a food plant, but the nature of such a mechanism is not clearly known at present. These results suggest that the environmental factors inducing green types of SD-pupae may differ from those affecting LD-pupae.

By experimenting with O-pharate pupae kept in rough-surfaced paper containers, we further investigated whether the orange coloration of pupae changes by employing a technique involving a ligation between the thorax and abdomen at six different life-cycle stages of O-pharate pupae. As shown in Table 2, all ligated abdomens of O-pharate pupae developed into the diapause-green type (or orange type) when the ligature was applied before the P4 stage, or later than the P5 stage. Interestingly, the timing of this humoral factor(s)'s release was similar to that of browning hormone's or PCMH's release in LD-pharate pupae (Hidaka, 1961; Yamanaka *et al*., 1999). However, orange-type pupae are a specific color type of diapause pupae, and there is no report as yet of LD-larvae developing into orange-type pupae in *P. xuthus*. Thus, these results suggest that humoral factor(s) originating from the pupal head-thoracic and abdominal ganglions may play a significant role in the determination of orange coloration of SD-pupae under the control of brain.

To investigate whether there is a humoral factor(s) inducing orange coloration (orange-pupa-inducing factor: OPIF) in *P. xuthus*, OPIF activity was assayed utilizing a bioassay system and the ligated abdomens of O-pharate pupae. As shown to Table 3, OPIF-activity was not detected in brain extracts of day-0 LD- and SD-pupae. However, slight PCMH activity was detected in 2% NaCl extracts prepared from the brains of LD- and SD-pupae, as well as in 2% NaCl extracts prepared from Br-SG-PG complexes of LD-pupae (Yamanaka *et al*., 1999). However, OPIF activity was detected in 2% NaCl extracts of $TG_{2,3}$-$AG_{1–7}$ complexes of day-0 last instar SD-larvae, but not in extracts consisting of Br-SG-TG_1 complexes (Table 4). These results show that $TG_{2,3}$-$AG_{1–7}$ complexes and Br-SG-TG_1 complexes of *P. xuthus* contain OPIF and PCMH, respectively, and furthermore, that the crude administration of OPIF revealed that the OPIF activity produced an intensity of orange on the abdomens of ligated O-pharate pupae in a dose-dependent manner. The crude extracts of $TG_{2,3}$-$AG_{1–7}$ complexes required for the development of orange types were estimated to be in the vicinity of 30 complex equivalents (Table 5).

The results revealed the presence of a neuroendocrine factor controlling pupal color polymorphisms in diapause pupae of a Papilionidae species. Our experiments indicated that the factor was extracted with 2% NaCl, but not with acetone or 80% ethanol solution.

It has recently been shown that pharate pupae of *P. polyxenes* destined to develop into pupae of a green type developed into pupae of a brown type when injected with crude PMRF extracts derived from *I. io* (Starnecker and Hazel, 1999). The PMRF of *I. io* is a neuropeptide that reduces black-type pigmentation in pupae of *I. io* (Starnecker and Hazel, 1999). Based on our present results, the timing of secretion and location of OPIF is considered similar to that demonstrated for PMRF. However, we have no evidence indicating that OPIF in *P. xuthus* changes pupal coloration in other butterfly species.

We are now trying to purify OPIF from $TG_{2,3}$-$AG_{1–7}$ complexes of 5th-instar SD-larvae in *P. xuthus*. Further investigation of the physiological roles played by OPIF is needed to clarify the regulatory mechanism of pupal coloration and the differences in regulation between SD and LD insects. These studies are aimed at clarifying the mechanisms underlying the endocrine control of pupal color polymorphism in *P. xuthus*.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Young Scientists (B) (No. 12740462) from the Japan Society for the Promotion of Science (JSPA) to A. Y., a Grant-in-Aid for Scientific Research (C) (No. 12640662) from The Ministry of Education, Culture, Sports, Science and Technology of Japan to K. E., and in part by a grant of The Naito Foundation to A. Y.

REFERENCES

Awiti LRS, Hidaka T (1982) Neuroendocrine mechanism involved in

pupal colour dimorphism in swallowtail *Papilio xuthus* L.. Insect Sci Appl 3: 181–191

Bückmann D (1960) Die determination der puppenfarbung bei *Vanessa urticae* L.. Naturwissenschaften 47: 610–611

Bückmann D, Maisch A (1987) Extraction and partial purification of the pupal melanization reducing factor (PMRF) from *Inachis io* (Lepidoptera). Insect Biochem 17: 841–844

Hazel WN, West DA (1996) Pupation site preference and environmentally cued pupal colour dimorphism in the swallowtail butterfly *Papilio polyxenes* Fabr. (Lepidoptera: Papilionidae). Biol J Linn Soc 57: 81–87

Hidaka T (1961) Recherches sur le mécanisme endocrine de l'adaptation chromatique morphologique chez les nymphes de *Papilio xuthus* L.. J Fac Sci Univ Tokyo Sec IV Zool 9: 223–261

Hidaka T, Hirai Y (1970) Effect of non-24-hour photoperiod and light interruption of the dark phase on diapause determination in *Papilio xuthus* L.. Proc Jpn Acad 46: 541–545

Honda K (1981) Environmental factors affecting the pupal coloration in *Papilio protenor demetrius* Cr. (Lepidoptera: Papilionidae) II. Effect of physical stimuli. Appl Entomol Zool 16: 467–471

Ishizaki H (1958) Correlation between colour variation and diapause in the pupa of swallowtail, *Papilio xuthus*. Physiol Ecol Jpn 8: 32–35

Ishizaki H, Kato M (1956) Environmental factors affecting the formation of orange pupae in *Papilio xuthus*. Mem Coll Sci Univ Kyoto Ser B 23: 11–18

Koch PB, Starnecker G, Bückmann D (1990) Interspecific effects of the pupal melanization reducing factor on pupal coloration in different lepidopteran families. J Insect Physiol 36: 159–164

Ohnishi E, Hidaka T (1956) Effect of environmental factors on the determination of pupal types in some swallowtail, *Papilio xuthus* L. and *P. protenor demetrius* Cr.. Zool Mag 65: 185–187 (in Japanese with English summary)

Ohtaki T (1960) Humoral control of pupal coloration in the cabbage white butterfly, *Pieris rapae curucivora*. Annot Zool Japon 33: 97–103

Poulton EB (1887) An enquiry into the cause and extent of a special colour-relation between certain exposed lepidopterous pupae and the surfaces which immediately surround them. Phil Trans R Soc Lond B 178: 311–441

Smith AG (1978) Environmental factors influencing pupal colour determination in Lepidoptera I. Experiments with *Papilio polytes*, *Papilio demoleus* and *Papilio polyxenes*. Proc R Soc Lond B 200: 295–329

Shimada K (1983) Photoperiodic induction of diapause in normal and allatectomized precocious pupae of *Papilio machaon*. J Insect Physiol 29: 801–806

Starnecker G, Koch PB, Matsumoto S, Mitsui T, Bückmann D (1994) Localization of the pupal melanization reducing factor of *Inachis io* (L.) and comparison with melanization and reddish coloration hormone. Z Naturforsch 49c: 476–482

Starnecker G (1997) Hormonal control of lutein incorporation into pupal cuticle of the butterfly *Inachis io* and the pupal melanization reducing factor. Physiol Entomol 22: 65–72

Starnecker G, Bückmann D (1997) Temporal occurrence of the pupal melanization reducing factor during development of the butterfly, *Inachis io*. Physiol Entomol 22: 73–78

Starnecker G, Hazel W (1999) Convergent evolution of neuroendocrine control of phenotypic plasticity in pupal colour in butterflies. Proc R Soc Lond B 266: 2409–2412

Yamanaka A, Endo K, Nishida H, Kawamura N, Hatase Y, Kong W, Kataoka H, Suzuki A (1999) Extraction and partial characterizations of pupal-cuticle melanizing-hormone (PCMH) in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae). Zool Sci 16: 261–268

Yamanaka A, Yoshitomi A, Endo K (2000) Effects of hormonal factors on pupal cuticle coloration in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae). Proc Arthropod Embryol Soc Jpn 35: 13–16

(Received February 26, 2004 / Accepted July 26, 2004)