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Pupal Color Polyphenism Regulated by Temperature and Photoperiod in the Asian Comma Butterfly, *Polygonia c-aureum* (Lepidoptera: Nymphalidae)

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Akira Yamanaka, Ayuki Tanaka, and Chisato Kitazawa (2012) Pupal color polyphenism regulated by temperature and photoperiod in the Asian comma butterfly, *Polygonia c-aureum* (Lepidoptera: Nymphalidae). *Zoological Studies* **51**(8): 1432-1437. To investigate the effects of environmental factors on pupal coloration of the Asian comma butterfly *Polygonia c-aureum* L., newly hatched larvae were reared and allowed to reach pupation under different temperature and photoperiodic conditions. Pupal colors which developed after pupal molting were classified into 5 types of dark brown, brown, yellowish-brown, light yellow, and brilliant yellow on the basis of the coloration of the ventral side of pupae. Under long-day conditions, developmental ratios of combined dark brown and brown types in male and female pupae were respectively 68% and 70% at 16°C, 42% and 7% at 23°C, and 0% and 0% at 32°C. Under short-day conditions, developmental ratios of combined dark brown and brown types in male and female pupae were respectively 43% and 29% at 20°C, and 29% and 7% at 32°C. Pupal color development in *P. c-aureum* was significantly affected by temperature and not by photoperiod, although short-day conditions induced pupae that were slightly darker than long-day pupae at 32°C. A higher temperature induced pupae of lighter types than did a lower temperature under the same photoperiodic conditions. Between the sexes, pupal coloration of males showed a tendency towards a slightly darker type than that of females at all tested photoperiodic and temperature conditions. However, there were no significant differences between male and female pupae. These results indicate that pupal color polyphenism in *P. c-aureum* is co-regulated by 2 factors, mainly temperature and to a small degree photoperiod, as environmental cues, suggesting that these 2 factors contribute to regulating phenotypic plasticity in life history traits of *P. c-aureum*. http://zoolstud.sinica.edu.tw/Journals/51.8/1432.pdf

Key words: *Polygonia c-aureum*, Nymphalidae, Pupal color polyphenism, Temperature, Photoperiod.

Some butterflies belonging to the Nymphalidae, as one of their phenotypically plastic morphological traits, exhibit pupal color polyphenism, the development of which is influenced by various environmental cues experienced during larval and/or pupal stages (Brecher 1922, Koch and Bückmann 1984, Yamanaka et al. 2009).

Pupal color polyphenism of the peacock butterfly *Inachis io* L. and the small tortoiseshell butterfly *Aglais urticae* L. is affected by the background color of the pupational site (Brecher 1922, Koch and Bückmann 1984), whereas that of the painted lady butterfly *Vanessa cardui* L. is affected by the temperature experienced during the larval and pupal stages irrespective of photoperiod (Brecher 1922, Yamanaka et al. 2009). Physiological studies indicated that pupal coloration of some nymphalid butterflies is controlled by secretion of a cerebral factor, such

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as a pupal melanization-reducing factor (PMRF) in *I. io*, a PMRF-like molecule in *A. urticae*, and a hormonal factor inducing whiteness of the pupal color in *V. cardui*, from the head-thoracic region when reaching the pharate pupal stage (Bückmann 1960, Bückmann and Maisch 1987, Koch et al. 1990, Yamanaka et al. 2009).

The Asian comma butterfly *Polygonia c-aureum* L., which is well known as a species that shows seasonal diphenism in wing coloration of summer/autumn morphs, also exhibits pupal color polyphenism from light to dark brown types. In *P. c-aureum*, Hiroyoshi (1992) revealed that the photoperiod experienced during larval stages was one of the environmental cues affecting pupal coloration of dark brown/brilliant-yellow types under moderate temperature (21 and 25°C) conditions, although it was suggested that other environmental cues may affect pupal color expression, and that darker pupae are formed under short-day (SD) conditions compared to those formed under long-day (LD) conditions.

Additionally, both the photoperiod and temperature experienced during larval stages are involved in the regulatory mechanism of seasonal morph determination (Endo 1984, Endo et al. 1992), whereas the photoperiod experienced during adult stages is involved in the maintenance of adult diapause- or non-diapause-type reproductive activity in *P. c-aureum* (Fujita et al. 2009). Therefore, those previous studies suggested that some of the developmental events in this species may also be regulated by correlations with photoperiod and/or temperature.

To gain a better understanding of the diversity of phenotypic plasticity in pupal color among nymphalid butterflies, an investigation is needed of the developmental mechanism of pupal coloration in butterfly species showing seasonal polyphenism in wing coloration such as *P. c-aureum* because adults of 3 nymphalid butterflies, *I. io*, *A. urticae*, and *V. cardui*, do not exhibit seasonal polyphenism of the wing ground-coloration.

In this study, we focused on identifying environmental cues affecting pupal coloration and investigated the influence of different photoperiods and temperatures experienced during larval-pupal stages on pupal coloration as a 1st step toward a better understanding of the regulatory mechanism of pupal coloration in *P. c-aureum*.

MATERIALS AND METHODS

Insects

Adults of the Asian comma butterfly *Polygonia c-aureum* were collected from suburbs of Yamaguchi City, Japan. Female adults which were fed on a 10% sucrose solution at 25°C were allowed to lay eggs on leaves of the host plant *Humulus japonicus* at intervals of 4 d. Eggs removed from leaves were collected in a Petri dish and kept at room temperature. Newly hatched *P. c-aureum* larvae were transferred to transparent plastic containers (13 × 20 × 6 cm) and reared on fresh *H. japonicus* leaves, which were exchanged daily in the light period, under LD conditions (16 h of light: 8 h of dark) at 16, 23, and 32°C, and then mature larvae molted to pupae (hereafter referred to as LD16-, LD23-, and LD32-pupae). Additionally, newly hatched *P. c-aureum* larvae were also reared on fresh *H. japonicus* leaves under SD conditions (10 h of light: 14 h of dark) at 20 and 32°C, and then mature larvae molted to pupae (hereafter referred to as SD20- and SD32-pupae). Inland regions of Central Japan (Honshu), particularly basins in these areas including Yamaguchi City, are subject to large temperature variations. For example, monthly mean, highest, and lowest air temperatures taken from the Yamaguchi Meteorological Station, Yamaguchi prefecture, of the Japan Meteorological Agency were respectively 18.7, 29.5, and 6.7°C in May; 23.0, 32.3, and 14.2°C in June; 26.4, 35.3, and 19.2°C in July; 28.3, 36.6, and 21.4°C in Aug.; 22.7, 32.3, and 13.6°C in Sept.; and 19.4, 29.3, and 10.7°C in Oct. 2006. On the basis of climate data of Yamaguchi from 2002 to 2006 and a report published by Hidaka and Takahashi (1967), the temperatures used in this study were designed to investigate the development of pupal color and wing ground-color.

Pupal molting conditions

Mature larvae that had entered the wandering stage after gut purge moved to the upper side of the rearing containers, the upper sides of which were covered with white paper (Nepia, Tokyo, Japan), in order to find a suitable pupational site. Mature larvae attached to the white paper were transferred to new, empty rearing containers and maintained under the same pupational conditions, such as background color, texture, and a relative humidity of < 55%, and were allowed to pupate under each photoperiodic and temperature condition. Pupal coloration was classified by the grade of brown/yellow coloration of the ventral side of pupae 2 d after pupation at 20, 23, and 32°C, and 3 d after pupation at 16°C.

An average grade score for yellowness on the ventral side of pupae

An average grade score for yellowness (AGY) was obtained using the following formula:

AGY = [(0 × number of insects of grade 0) + (1 × number of insects of grade 1) + (2 × number of insects of grade 2) + (3 × number of insects of grade 3) + (4 × number of insects of grade 4)]/total number of insects.

Statistical analysis

The nonparametric Mann-Whitney *U*-test was used to determine differences between 2 groups, and the nonparametric Kruskal-Wallis *H* test was used to analyze differences among more than 2 groups, followed by the Mann-Whitney *U*-test to identify specific group differences when the Kruskal-Wallis test showed significance ($p < 0.05$). Significance was set at $p < 0.05$ in all statistical analyses.

RESULTS

Classification of pupal coloration in *Polygonia c-aureum*

To provide an index of pupal coloration in *P. c-aureum*, pupae that developed under LD conditions at 23°C were used to classify pupal coloration in this species.

LD23-pupae 2 d after pupation were divided into 5 grades with respect to the brownness/yellowness of the ventral side of the pupal body: grade 0, almost dark brown; grade 1, brown (pupae of grades 0 and 1 were referred to as brown-type pupae); grade 2, an intermediate yellowish-brown color between grades 1 and 3 (pupae of grade 2 were referred to as intermediate-type pupae); grade 3, ocher or light yellow; and grade 4, brilliant-yellow (pupae of grades 3 and 4 were referred to as yellow-type pupae) (Fig. 1, Table 1). These observations indicated that pupae of *P. c-aureum* exhibited a variety of pupal colorations, and these varieties of color were clearly classified into 5 grades according to the coloration of the ventral side of pupae.

Influences of temperature and photoperiod

As shown in table 1, pupal coloration of male

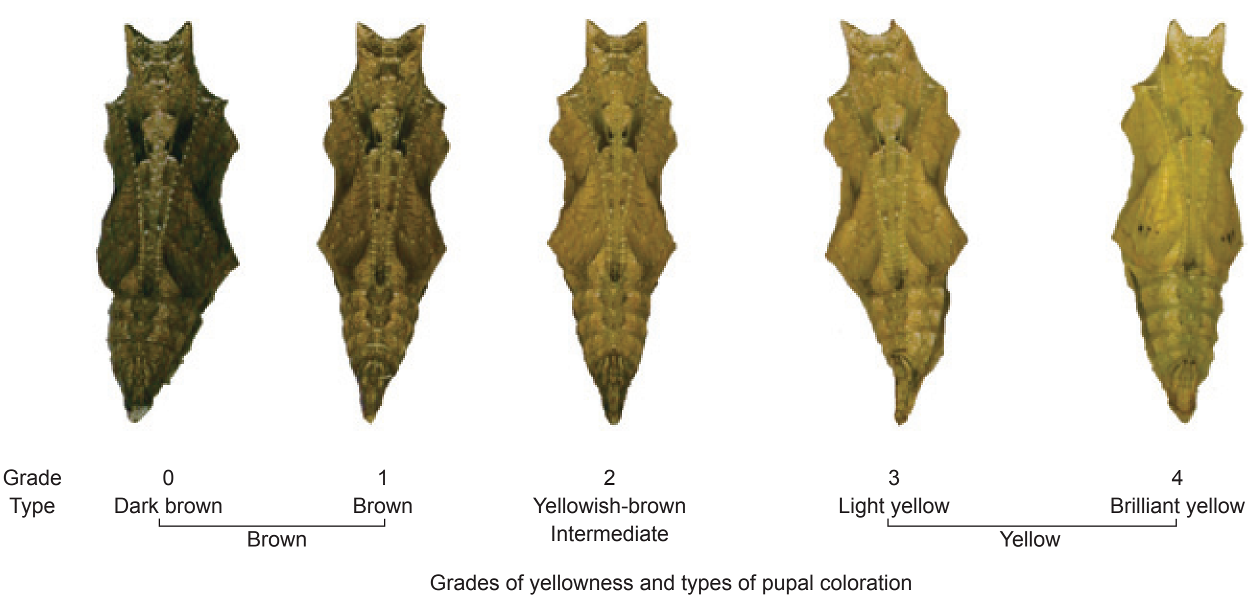

Fig. 1. Classification of the degree of yellowness coloration in pupae of *Polygonia c-aureum*. All larvae reared under long-day conditions at 23°C developed into pupae, and they were then graded and classified into 5 types according to pupal coloration. Grade 0 represents a brown type, whereas grade 4 represents a brilliant-yellow type. Intermediates were classified as either grades 1, 2, or 3 depending on the extent of yellowness on the ventral side of a pupa.

and female pupae, that appeared after pupation, changed in response to rearing conditions when newly hatched larvae were reared under different photoperiodic and temperature conditions during larval and pupal stages.

Developmental ratios of brown, intermediate, and yellow types of male LD16-, LD23- and LD32-pupae were 68%, 32%, and 0%; 31%, 42%, and 27%; and 0%, 56%, and 44%, whereas those of female LD16-, LD23-, and LD32-pupae were 70%, 23%, and 7%; 9%, 56%, and 35%; and 0%, 25%, and 75%, respectively. AYG values of male LD16-, LD23-, and LD32-pupae were 1.00, 1.81, and 2.56, whereas those of female LD16-, LD23-, and LD32-pupae were 1.16, 2.30, and 2.90, respectively (Table 1).

On the other hand, developmental ratios of brown, intermediate, and yellow types of male SD20- and SD32-pupae were 43%, 43%, and 14%; and 21%, 31%, and 48%, whereas those of female SD20- and SD32-pupae were 29%, 47%, and 24%; and 7%, 27%, and 66%, respectively. AGY values of male SD20- and SD32-pupae were 1.63 and 2.24, whereas those of female SD20- and SD32-pupae were 1.88 and 2.63, respectively (Table 1).

Regarding the influence of temperature under LD conditions, results of the Kruskal-Wallis *H* test on this data indicated that there were significant differences among the 16, 23, and 32°C groups (males: $H = 22.394$, $p < 0.001$; females: $H = 60.106$, $p < 0.001$; both sexes: $H = 82.072$, $p < 0.001$). Between-group comparisons with the Mann-Whitney *U*-test indicated that there were significant differences in frequencies of pupal color grades between 16 and 23°C in males ($U = 434.5$, $Z = 3.490$, $p < 0.001$), females ($U = 211.5$, $Z = 4.896$, $p < 0.001$), and both sexes ($U = 1826$, $Z = 5.625$, $p < 0.001$), and between 23 and 32°C in males ($U = 356$, $Z = 2.624$, $p = 0.0087$), females ($U = 460$, $Z = 3.195$, $p = 0.0014$), and both sexes ($U = 2523$, $Z = 5.299$, $p < 0.001$) under LD conditions. Under SD conditions, the Mann-Whitney *U*-test indicated that there were significant differences in frequencies of pupal color grades between 20 and 32°C in males ($U = 316$, $Z = 2.709$, $p = 0.0068$), females ($U = 180$, $Z = 3.144$, $p = 0.0017$), and both sexes ($U = 961$, $Z = 4.708$, $p < 0.001$).

Concerning the influence of photoperiod, the Mann-Whitney *U*-test indicated that there were no significant differences in frequencies of pupal color grades between LD32 and SD32 in both sexes ($U = 1692$, $Z = 1.788$, $p = 0.0737$), males ($U = 231$, $Z = 0.705$, $p = 0.4808$), or females ($U = 679$, $Z = 1.528$, $p = 0.1266$), but the AGY value for LD conditions was slightly higher than that for SD conditions.

Additionally, the Mann-Whitney *U*-test

Table 1. Influences of photoperiod and temperature on pupal coloration of *Polygonia c-aureum* during larval and pupal stages

Rearing conditions	Number of pupae classified by grade of yellowness of pupal coloration							
	Sex	*n*	0	1	2	3	4	AGY
LD16	M	25	8	9	8	0	0	1.00
	F	56	12	27	13	4	0	1.16
	M+F	81	20	36	21	4	0	1.11
LD23	M	64	9	11	27	17	0	1.81
	F	23	0	2	13	7	1	2.30
	M+F	87	9	13	40	24	1	1.94
LD32	M	18	0	0	10	6	2	2.56
	F	40	0	0	10	24	6	2.90
	M+F	58	0	0	20	30	8	2.79
SD20	M	35	3	12	15	5	0	1.63
	F	17	1	4	8	4	0	1.88
	M+F	52	4	16	23	9	0	1.71
SD32	M	29	2	4	9	13	1	2.24
	F	41	0	3	11	25	2	2.63
	M+F	70	2	7	20	38	3	2.47

LD and SD represent long- (16 h of light: 8 h of dark) and short-day (10 h of light: 14 h of dark) photoperiodic conditions, respectively. Each number immediately to the right of the LD/SD designation indicates the temperature (°C) during the larval-pupal stages. *n*, number of insects; AGY, average grade score for yellowness on the ventral side of pupae; M, male, F, female.

indicated that there were no significant differences in frequencies of pupal color grades between males and females when considering all tested conditions (LD16: $U = 641.5$, $Z = 0.638$, $p = 0.5237$; LD23: $U = 555$, $Z = 1.860$, $p = 0.6292$; LD32: $U = 256$, $Z = 1.932$, $p = 0.0533$; SD20: $U = 247.5$, $Z = 1.040$, $p = 0.2982$; SD32: $U = 467$, $Z = 1.683$, $p = 0.0923$), but the AGY value for males was somewhat lower than that for females.

DISCUSSION

Pupal color polyphenism in butterflies is known to be influenced by various environmental cues and is controlled by neurosecretory hormones in some species (Nijhout 1994 2010). The Asian comma butterfly *Polygonia c-aureum* also shows pupal color polyphenism. A previous study revealed that photoperiod is one of the environmental cues affecting pupal coloration in *P. c-aureum*, in which prolongation of day-length resulted in a slight increase of the ratio of yellow-type pupae under the same rearing temperature (Hiroyoshi 1992).

In order to identify other environmental cues affecting pupal coloration in this species, we investigated variations in pupal coloration of *P. c-aureum* and classified the coloration into 5 grades from dark brown to brilliant yellow according to the coloration on the ventral side of pupae (Fig. 1). Based on this classification, our results indicated that ratios of yellow- and brown-type pupae significantly increased with high and low temperatures under the same photoperiodic conditions, respectively, and more pupae of darker types were produced under SD conditions than LD conditions at the same temperature (Table 1). Therefore, the pupal color of *P. c-aureum* is co-regulated by temperature and photoperiodic conditions as environmental cues.

In a similar study conducted with the small copper butterfly *Lycaena phlaeas daimio* Seitz (Lycaenidae), which exhibits seasonal polyphenism in wing coloration (Endo et al. 1985), it was revealed that both the photoperiod and temperature experienced during larval-pupal stages play significant roles as environmental cues in determining pupal beige/black polyphenism, and that there was a clear inverse correlation between black pigmentation of a pupal body and dorsal wings, suggesting that a tradeoff exists between black pigmentation of the pupal and wing colors (Usui et al. 2004).

In *P. c-aureum*, however, we observed that all LD16-, LD23-, LD32-, and SD32-pupae developed into adults of summer-morph types with a yellow color, whereas only SD20-pupae developed into adults of autumn-morph types with a brown color (data not shown) as previously described by Hidaka and Takahashi (1967). Therefore, there was no direct correlation between pupal and wing coloration in *P*. *c-aureum*, indicating that the regulatory mechanisms of pupal colors and seasonal morphs are controlled independently of each other by a correlation of both photoperiod and temperature as environmental cues. According to physiological studies, a cerebral factor affecting pupal coloration is located in the entire central nervous system of larvae of some nymphalid species such as *I. io* and *V. cardui*, which produce pupae of light-color types (Starnecker et al. 1994, Yamanaka at al. 2009). However, this factor has not yet been identified in *P. c-aureum*, whereas a summer morph-producing hormone regulating seasonal diphenism of wing color was identified in the pupal brain (Endo et al. 1988, Masaki et al. 1988). Based on our results, more yellow pupae of *P. c-aureum* can be produced under LD conditions at a high temperature (e.g., LD32), and its use in further studies may lead to the establishment of a bioassay method to detect the activity of a cerebral factor affecting pupal coloration.

From ecological aspects, larvae of *P. c-aureum* usually molt into pupae within a bell-shaped leaf near the ground on a host plant. Hence, pupae of this species may be influenced by thermal adaptation, producing pupae of darker-color types in autumn/spring and lighter pupae in summer, in response to temperature and photoperiod rather than camouflage as in pupae of *I. io*, which hang on the underside of a leaf or twig and adapt to the background color. The findings in this and previous studies indicate that developmental events of morphological traits during the life cycle of the nymphalid butterfly species *P. c-aureum* are highly responsive to temperature and/or photoperiod.

In conclusion, the pupal color of *P. c-aureum* was co-regulated by 2 environmental cues: temperature and photoperiod. The results of this study contribute to our knowledge of the diversity of phenotypic plasticity of pupal color in nymphalid butterflies. However, further study is needed to elucidate the endocrinological mechanism underlying the control of pupal color polyphenism in *P. c-aureum*.

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