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Photoperiod- and Temperature-dependent Regulation of Pupal Beige/Black Polymorphism in the Small Copper Butterfly, *Lycaena phlaeas daimio* Seitz

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ABSTRACT—The small copper butterfly, *Lycaena phlaeas daimio*, has pupal beige/black polymorphism, the development of which is found to be controlled in an apparent association with the development of adult seasonal polymorphism (spring and summer morphs) by photoperiod and temperature in the larval stages. That is, the pupae of beige and black types developed under long-day and short-day conditions tend to develop into brown-winged and red-winged adults, respectively. In addition, a large proportion of long-day pharate pupae chilled at 4°C for 5 days were observed to develop into pupae whose head-thoracic complexes and abdomens were judged to be of the black and intermediate types, respectively. They developed into adults with redder wings as compared to those obtained from unchilled pupae.

The results indicate that the physiological mechanism underlying the photoperiodic control of the development of adult seasonal polymorphism may also play a significant role in the determination of pupal beige/black polymorphism in *L. phlaeas daimio*. Furthermore, cuticle melanization was found to be induced in the head-thoracic complexes of pupae by chilling of the pharate pupae. Melanization of pupal cuticle seems to occur in a close association with the development of reddish-winged adults.

Key words: *Lycaena phlaeas daimio*, pupal polymorphism, photoperiod, pharate pupae, chilling

INTRODUCTION

Many butterflies exhibit color polymorphisms in the pupal stage. Pupal color polymorphisms are mostly determined by environmental factors at the pupating sites around the end of wandering stage, such as smells, colors, humidity and surface characteristics (Hidaka and Ohtaki, 1963; Rassin, 1980; Maisch and Buckmann, 1987; Nijhout, 1994). The physiological mechanisms underlying the control of pupal color polymorphisms involve neuroendocrine (or endocrine) factors (Nijhout, 1994) which may vary according to butterfly families.

Pupae of the swallowtail butterfly, *Papilio xuthus* L., exhibit a green/brown polymorphism which is determined by surface characteristics at the pupating sites in addition to the effects of smells and humidity (Hidaka, 1961). The physiological mechanism underlying the control of pupal color polymorphism in *P. xuthus* has been shown to involve a neuroendocrine factor named a browning hormone (Hidaka, 1961). The browning hormone is believed to be synthesized in the brain and released from the prothoracic ganglion in the pharate pupal stage (Hidaka, 1961; Awiti and Hidaka, 1982). Furthermore, a neuropeptide stimulating melanization of the pupal cuticle (named pupal-cuticle-melanizing hormone: PCMH) has been shown to exist in *P. xuthus*. The PCMH was purified partially from brain, suboesophageal ganglion and prothoracic ganglion (Br-SG-PG) complexes of 5th-instar larvae (Yamanaka *et al*., 1999). In addition, in *P. xuthus* it has been demonstrated that there are pupae of orange types, which appear only in diapause pupae induced by short-day conditions in the larval stages (Ishizaki and Kato, 1956). The nymphalid butterfly, *Inachis io*, has light- and dark-brown pupae, development of which is regulated by a neuroendocrine factor inhibiting melanization of the pupal cuticles, the so-called pupal cuticle melanization reducing factor (PMRF). This factor was purified partially from the anterior parts (head-thorax complexes) of *I. io*

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pupae. Pupal green/gray polymorphism of the cabbage white butterfly, *Pieris brassicae*, has been proposed to be controlled by two neuroendocrine factors, named the melanization-inhibiting and melanization-stimulating hormones (Kayser-Wegmann, 1975). In addition, in *P. rapae*, pupal epidermal cells were shown to require the presence of juvenile hormone (JH) for the development of green pigmentation (Hidaka and Ohtaki, 1963).

The small copper butterfly, *L. phlaeas daimio* Seitz, exhibits seasonal polymorphism (spring and summer morphs), development of which is controlled by photoperiod and temperature during the larval stages (Sakai and Masaki, 1965; Endo *et al*., 1985) as has been reported in many other butterflies (Muller, 1955; Danilevskii, 1961; Beck, 1980; Saunders, 2002). The physiological mechanism underlying the photoperiodic control of seasonal polymorphism was shown to involve a cerebral factor, named the summer-morph-producing hormone (SMPH), which changes the wing color to be dark-brown. Besides, chilling of pharate pupae reddens the wing color by shifting the appearance of hemolymph ecdysteroids at the earlier pupal stage (Endo and Kamata, 1985).

In the present study, we attempted to clarify whether pupal beige/black polymorphism is determined in a significant association with the development of adult (seasonal) morphs in *L. phlaeas daimio*.

MATERIAL AND METHODS

Animal

The small copper butterfly, *Lycaena phlaeas daimio*, collected near the town of Yamaguchi was used. Female butterflies were fed on 7–10% sugar solution for four days at room temperature and allowed to lay eggs on leaves of the larval food plant, *Rumex acetosa*. The eggs were divided into two groups. One group was exposed to long-day conditions, consisting of alternating 14-hr light and 10-hr dark periods (14L-10D), at 18°C, 24°C and 28°C and the other group was exposed to short-day conditions (10L-14D) at 18°C, 24°C and 28°C. Each photoperiod-controlled box (62×39×110 cm^3) was equipped with a 20-W white fluorescent tube controlled by a 24-hr time-switch. In the light period, the light intensity was 400–500 lux. The larval containers were transparent plastic (13×20×6 cm^3).

Classification of body colors in pupae

Pharate pupae were selected from each stock culture everyday in the morning, kept in transparent plastic containers, and allowed to pupate at 25°C. Two days after pupation, they were classified into three types, beige, intermediate and black (Fig. 1), depending on their body color.

Digitalization of body blackness in pupae

Photographs of the dorsal figures of the pupae were taken with a digital camera (COOLPIX 995, Nikon, Tokyo, Japan) two days after pupation. The photographs were processed by increasing the contrast and decreasing the pixels. Hard copies were made for counting the numbers of black, dark-gray, light-gray and white squares in 56- and 80-square areas of the head-thoraxes and abdomens, respectively (Fig. 2). Then, the average grade of pupal head-thoracic blackness (t-AGPB) and of abdominal blackness (a-AGPB) were determined by assigning scores of 3, 2, 1 and 0 for black, dark-gray, light-gray and white squares, respectively.

Fig. 1. Pupal polymorphism in *Lycaena phlaeas daimio*. Pupae of black (right), intermediate (middle) and beige types (left) obtained under long-day conditions at 28°C.

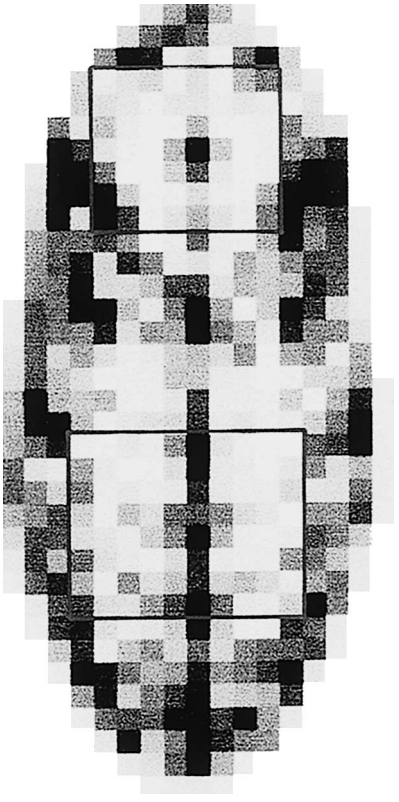

Fig. 2. A processed photograph of an intermediate pupa for obtaining the score of AGPB (average grade of pupal blackness). Thick lines enclose the head-thoraic and abdominal areas of the pupa where the numbers of black, dark-gray, light-gray and white squares were counted.

Classification of wing patterns in male adults

Male butterflies emerging from each pupal group were killed, made into specimens and the numbers of red scales distributed on the central line of the 4th (CLIV) and the 7th cells (CLVII) of the anterior wings were counted.

Comparisons were made by an F-test based on these indices.

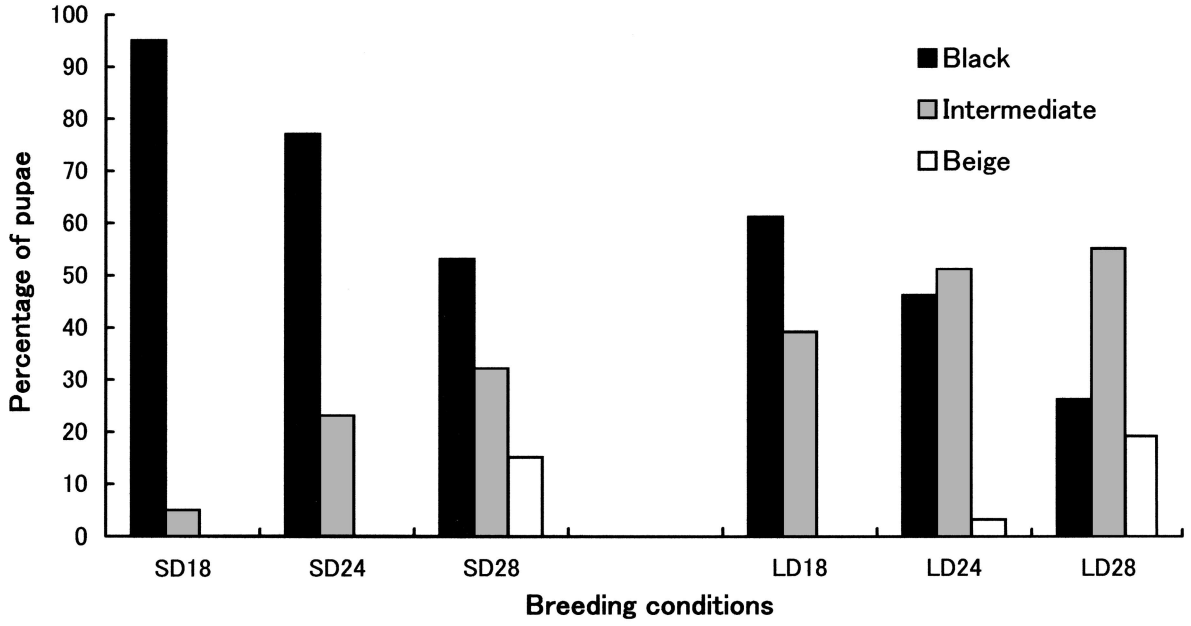

Fig. 3. Proportions of black, intermediate and beige pupae raised up from the egg stage under long-day and short-day conditions at 18°C (n=256 and 256), 24°C (n=290 and 300) and 28°C (n=300 and 300).

RESULTS

Effects of photoperiod and temperature on pupal body color

To determine whether the body colors of *L. phlaeas* pupae are regulated by photoperiod and temperature during the larval stages, groups (n=250–300) of *L. phlaeas* larvae were reared under long-day and short-day conditions at 18°C, 24°C and 28°C. Pupae were classified into beige, intermediate and black types two days after pupation (Fig. 1).

When raised from the egg stage under short-day conditions at 18°C, the majority (approx. 95%) of pupae were classified as black types (Fig. 3). The proportion of black types decreased as the temperature rose, reaching about 50% at 28°C. At 28°C about 15% of pupae were classified as beige and 30% of pupae were classified as intermediate.

Under long-day conditions, the proportion of black types was at its maximum (about 60%) at 18°C (Fig. 3). The proportion of black types decreased when the temperature was raised, standing at about 25% at 28°C. At 28°C about 20% of pupae were classified as beige and about 55% of pupae were classified as intermediate.

The results indicate that, in *L. phlaeas daimio*, photoperiod and temperature in the larval stages may play a significant role in the determination of the pupal body color.

Developmental association of black pupae and red-winged adults

To ascertain whether red-winged adults develop in a certain correlation with the development of black pupae in *L. phlaeas daimio*, pupae obtained under short-day conditions at 18°C (n=95), 24°C (n=162) and 28°C (n=78) were classified into beige, intermediate and black types. Male adults were made into specimens, in order to count the numbers of red scales in CLIV and CLVII.

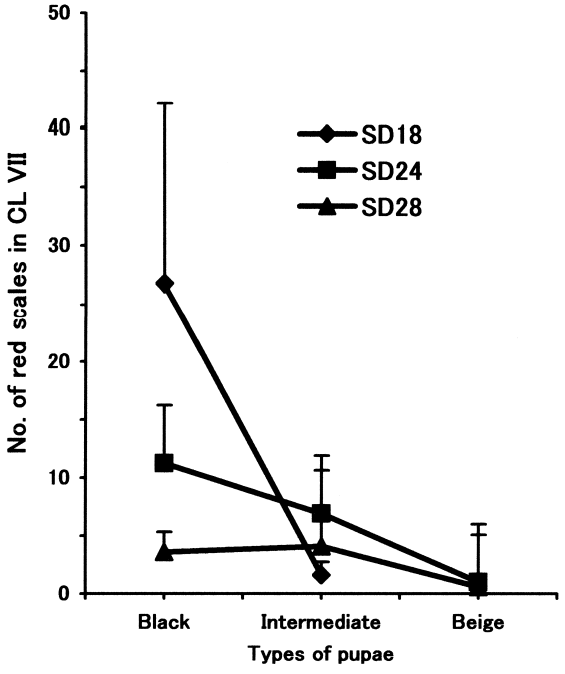

Fig. 4. Numbers of red scales distributed in CLVII of anterior wings of male adults raised up from the egg stage under short-day conditions at 18°C (n=95), 24°C (n=162) and 28°C (n=78). "Black", "Intermediate" and "Beige" show the color types of pupae from which the male adults were obtained. Vertical bars show the standard deviations.

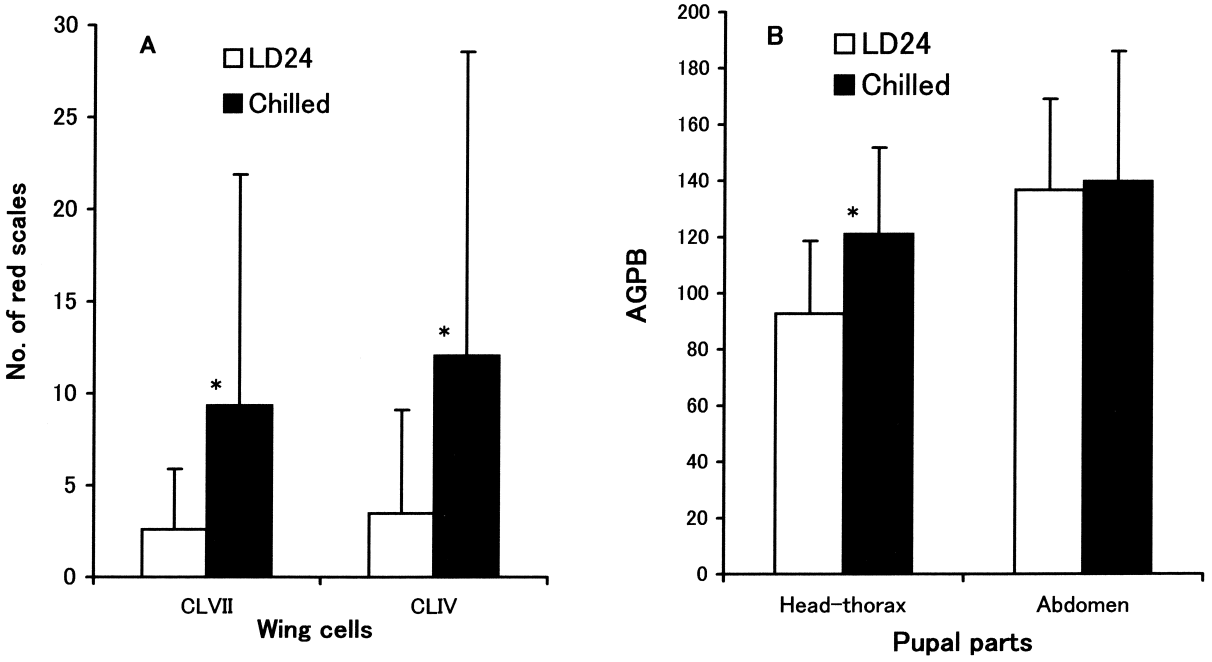

Fig. 5. Effects of chilling of long-day (24°C) pharate pupae on the number of red scales in CLVII of male adults (A), and pupal color (B). A: Open and solid columns show the numbers of red scales in CLVII of male adults emerged from chilled and unchilled pupae (n=75 and 162), respectively. B: Open and solid columns show the head-thoracic and abdomimal scores of AGPB in chilled and unchilled pupae (n=329 and 649), respectively. P<0.01, significantly different from control (LD24) analyzed by F-test. Vertical bars show the standard deviations.

The numbers of red scales distributed in CLIV and CLVII were found to vary depending on the color types of the pupae from which the adults had emerged (Fig. 4). Male adults emerged from black pupae had significantly larger numbers of red scales in the CLIV and CLVII as compared to those emerged from intermediate and beige pupae (P<0.01, F-test). But the relationship between pupal body color and adult wing color is not apparent in males reared under short-day conditions at 28°C (P>0.5, F-test).

The results indicate that, in *L. phlaeas daimio*, the wing color of adults seems to be determined in association with the body color of pupae; that is, black and beige pupae tend to develop into red-winged and brown-winged adults, respectively.

Effect of pharate pupal chilling on pupal body color

To investigate whether pharate pupal exposure to 4°C changes pupal body color as has been observed in the wing color of adults, larvae reared under long-day conditions at 24°C and pharate pupae were chilled at 4°C for 5 days (n=75). Two days after pupation at 25°C, they were classified into beige, intermediate and black types. Male adults were made into specimens, in order to count the numbers of red scales in CLIV and CLVII.

The numbers of red scales in both CLIV and CLVII apparently increased in pupae chilled at 4°C for 5 days at the pharate pupal stage (Fig. 5A). t-AGPB scores showing the degree of blackness in head-thoracic complexes apparently increased (P<0.01, F-test), but a-AGPB scores showing the degree of blackness in abdomens did not increase (P>0.5, F-test), by the pharate pupal exposure to 4°C (Fig. 5B). This observation is supported by another piece of evidence namely that t-black pupae, the head-thoraxes and abdomens of which were judged to be of the black and intermediate types respectively, appeared with the chilling of pharate pupae. The proportion of t-black pupae reached 25.7% in pupae developed from chilled pharate pupae (Fig. 6). In addition, 34.3%, 39.2% and 0.6% of pupae were classified into black, intermediate and beige types respectively.

The results indicate that, in *L. phlaeas daimio*, the chilling of pharate pupae may induce the melanization of pupal head-thorax cuticles, which occurs in an apparent correlation with the reddish shift in the wing color of male adults.

Fig. 6. A side view of t-black pupae (middle), the head-thorax and abdomen of which were judged to be of the black and intermediate types, respectively. The left and right pupae show black and intermediate types, respectively.

DISCUSSON

The small copper butterfly, *L. phlaeas daimio*, displays pupal color polymorphism, i. e. beige, intermediate and black types, the development of which was found to be determined by photoperiod and temperature during the larval stages in a close association with the development of adult seasonal polymorphism. Both pupal and adult polymorphisms, once determined by photoperiod and temperature during the larval stages, were found to be shifted toward short-day types by exposing pharate pupae at 4°C for 5 days.

We did not have any evidence to clarify whether environmental factors in the surroundings of pupating sites, such as smell, color, humidity and surface characteristics, play a significant role in the control of pupal color polymorphism in *L. phlaeas daimio*, as has been demonstrated in several other butterflies (Ishizaki and Kato, 1956; Hidaka, 1961; Hidaka and Ohtaki, 1963; Maisch and Buckmann, 1987; Yamanaka *et al*., 1999). The majority (approx. 95%) of larvae reared under short-day conditions at a relatively low temperature (18°C) developed into black pupae in the larval rearing containers. Under long-day conditions at 18°C, about 60% of pupae were judged to be of the black type. The proportions of black pupae decreased in both long-day and short-day insect groups as the temperature was raised. Furthermore, the exposure of pharate pupae to 4°C for 5 days was found to increase significantly t-AGPB scores showing the degree blackness of head-thoracic complexes including the pupal wings, but did not increase a-AGPB scores showing the degree of blackness of abdomens, in long-day *L. phlaeas* pupae.

Based on a line of our evidence about the determination of seasonal polymorphism in *L. phlaeas daimio*, the development of adult (seasonal) morphs has been shown to be regulated by SMPH, secretion of which is controlled by photoperiod and temperature in the larval stages (Endo and Kamata, 1985). Wing patterns, once determined by SMPH, shifted toward reddish types (spring morphs) by the secretion of ecdysteroids for adult development at earlier pupal stages. The early secretion of ecdysteroids was shown to occur in pupae raised up from the egg stage under short-day conditions at relatively low temperatures, or in those subjected to 4°C for 10 days in the pharate pupal stage (Endo and Kamata, 1985).

Rearing under short-day conditions at low temperatures during the larval stages, as well as chilling in the pharate pupal stage, have already been shown to shift wing patterns toward reddish types (spring or typical spring morphs) in *L. phlaeas daimio* (Endo and Kamata, 1985). Both environmental factors are judged to increase the proportion of black types and t-AGPB scores in pupae.

We do not have any evidence about a physiological mechanism underlying the control of pupal beige/black polymorphism. But, photoperiod and temperature in the larval stages and a low temprature in the pharate pupal (or early pupal) stage may play significant adaptive roles in the control of the correlational development of pupal and adult polymorphisms in *L. phlaeas daimio*.

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