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Photoperiodic Regulation of Reproductive Activity in Summer- and Autumn-Morph Butterflies of *Polygonia c-aureum* L.

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Keijiro Fujita, Moeko Inoue, Masao Watanabe, Abu Taher Md. Fayezul Islam, Reza Md. Shahjahan, Katsuhiko Endo, and Akira Yamanaka (2009) Photoperiodic regulation of reproductive activity in summer- and autumn-morph butterflies of *Polygonia c-aureum* L. *Zoological Studies* **48**(3): 291-297. The Asian comma butterfly *Polygonia c-aureum* has seasonal morphs of summer- and autumn-types, the development of which is determined by photoperiod and temperature during larval stages in close relation to the determination of adult reproductive diapause. In this paper, we investigated how the period of reproductive diapause changes in response to photoperiodic conditions available after adult emergence by determining ovarian development in female butterflies of each morph-type. Females of autumn-morph types did not produce eggs for about 80 d under short-day conditions at 20°C. When they were transferred to long-day conditions at 20°C after emergence, however, these females began to accumulate yolk within 8 d, and in ovaries about 15 d later, the female possessed more than 3 eggs in each ovariole, totaling > 24 eggs. When females of the summer-morph type were transferred to short-day conditions at 20°C after emergence, their ovarian maturation was delayed and took about twice as long compared to that of females continuously exposed to long-day conditions. The maintenance of adult diapause- or non-diapause-type reproductive activity thus respectively requires short- or long-day conditions, suggesting that *P. c-aureum* is also responsive to photoperiod during its adult stages. http://zoolstud.sinica.edu.tw/Journals/48.3/291.pdf

Key words: Egg production, Nymphalid butterfly, Photoperiod, Reproductive diapause, Temperature.

Many species of bivoltine and multivoltine insects undergo diapause during various developmental phases such as egg, larval, pupal, and adult stages, which is determined by photoperiod and temperature conditions during the embryonic or post-embryonic stages (Danks 1987). Adult diapause is generally characterized by the cessation of egg development in the adult stage, and is referred to as reproductive diapause (Nijhout 1994).

In terms of the regulatory mechanism of reproductive diapause, it is well-known that adult females of the Colorado potato beetle *Leptinotarsa decemlineata* enter reproductive diapause in the absence of juvenile hormones (JHs) under short-day (SD) conditions because their corpora allata (CA) remain inactive after emergence. They terminate or reenter reproductive diapause in response to exposure to long-day (LD) or SD conditions in the adult stage (De Wilde 1954, De Wilde and de Boer 1961, Sláma 1964). Additionally, adult brains of this species were

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shown to suppress CA secretion of JHs during diapause through nerves connecting the brain and CA (nervi corporis cardiaci and allati), thus causing the secretion of a factor activating CA to secrete JHs when they are subjected to LD conditions (De Wilde and de Boer 1969).

Similarly, several researchers revealed that *Danaus plexippus*, *Hypolimnas bolina*, and *Polygonia c-aureum* also enter reproductive diapause in the adult stage when larvae are reared under an SD photoperiod and relatively low-temperature conditions (Endo 1972, Barker and Herman 1976, Pieloor and Seymour 2001). Others have shown that the development of ovarian maturation or the termination of reproductive diapause in *D. plexippus* and *P. c-aureum* is controlled by the secretion of JHs which is essential for vitellogenesis, i.e., yolk protein uptake by oocytes (Endo 1972, Barker and Herman 1973, Herman 1981, Muranaka et al. 2002).

Kataoka et al. (1989) reported that allatotropin (AT) is a factor that activates the CA, which is a neuropeptide hormone consisting of 13 amino acids that stimulates JH secretion by CA, and was initially identified from adult brains of the tobacco hornworm *Manduca sexta*, a lepidopteran insect. Later, a 20 kD peptide obtained from larval brains of *Galleria mellonella* was also demonstrated to activate larval CA of this species *in vitro* (Bogus and Scheller 1994). However, until now there has been no physiological information regarding the factor activating CA, which is a necessary factor in females for them to develop ovarian maturation regardless of the presence of adult reproductive diapause, and we have no information on whether reproductive diapause (or non-diapause reproduction) in lepidopterans once it is determined in their larval stages can be re-regulated by photoperiodic and temperature conditions after adult emergence.

The Asian comma butterfly *P. c-aureum* has seasonal (summer and autumn) morphs, the development of which is determined by photoperiod and temperature during the larval stages (Fukuda and Endo 1966, Hidaka and Aida 1963, Hidaka and Takahashi 1967, Endo et al. 1992). It is conceivable that brains of *P. c-aureum* LD pupae are essential to secretion of summer-morph-producing hormone (SMPH), prothoracicotropic hormone (PTTH), and an AT-like factor as neuroendocrine factors for the development of summer-morphs, i.e., reproductively active adults, whereas brains of SD-pupae secrete PTTH but neither the SMPH nor AT-like factor during the development of autumn-morphs, which is associated with reproductive diapause in the adult stage (Fukuda and Endo 1966, Endo 1970 1972 1984, Endo et al. 1992, Muranaka et al. 2002). However, we have no evidence to determine whether the secretion of the AT-like factor, which should be essential for reproductive activity, is controlled by photoperiod and temperature.

In this study, we investigated whether the maintenance of reproductive diapause or reproductive activity of the non-diapause type is re-regulated by photoperiod and temperature after adult emergence in summer- and autumn-morph females of *P. c-aureum*. Additionally, we investigated the length of LD exposure required for the termination of reproductive diapause in autumn-morph females of *P. c-aureum*.

MATERIALS AND METHODS

Insects

Female adults of *P. c-aureum* were collected from the suburbs of Yamaguchi City, Japan. Mated females which fed on a 10% sucrose solution for 4 d at 25°C were allowed to lay eggs on leaves of the larval food plant *Humulus japonicus* at intervals of 4 d. Larvae after hatching at 25°C were subjected to either LD conditions (alternating 14 h light and 10 h dark periods; 14L-10D), or SD conditions (10L-14D) at 20°C. Larvae were fed fresh leaves of *H. japonicus*, which were exchanged every day during the light period (Endo 1970). Larvae reared under LD conditions at 20°C developed into adults of the summer-morph type, the females of which produced many eggs 4 d after adult emergence at 25°C. Under SD conditions at 20°C, larvae developed into adults of the autumn-morph type, and these females entered reproductive diapause and did not accumulate blue-green yolk in their oocytes far as long as 15 d after emergence at 25°C (Endo 1970). The newly emerged females obtained were subjected to the following experiments. They were fed a 10% sucrose solution every day using a plastic syringe with a needle. Ages of the females were counted as the time since emergence in days.

Photoperiodic and temperature conditions

Adult females of the summer- or autumn-morph type were divided into 2 groups within 12 h

after emergence and kept under either LD or SD conditions at 20°C. Other adult females of the autumn-morph type were divided into 2 groups as described above and kept under SD conditions at either 25 or 30°C. They were fed a 10% sucrose solution every day. Five to 20 females were selected from each experimental group at predetermined ages of 0-109 d and immediately dissected in a 0.9% NaCl solution to observe the state of ovarian development.

Exposure to LD conditions

Adult females of the autumn-morph type obtained under SD conditions at 20°C were subjected to a 10 d LD exposure within 12 h after emergence at 20°C. After LD exposure, they were re-transferred to SD conditions at 20°C. Ten females were selected from the experimental group at predetermined ages and immediately subjected to dissection to observe the state of ovarian development.

Criteria for classifying the state of ovarian development

After dissection, adult females were classified into 5 grades on a scale according to the state of ovarian development observed: adult females which had accumulated no yolk in their oocytes (grade 0), those which had accumulated a small amount of yolk in their oocytes (grade 1), those which had fewer than 8 eggs in their ovaries (grade 2), those which had 9-16 eggs in their ovaries (grade 3), and those which had > 17 eggs in their ovaries (grade 4). The average grade of ovarian development (AGOD) together with the standard deviation was then calculated for each experimental group.

RESULTS

Effects of photoperiod in the adult stage on ovarian development in females of the autumn-morph type

To examine whether the duration and termination of reproductive diapause are regulated by photoperiodic conditions in adult-stage females of *P. c-aureum*, females of the autumn-morph type, grown under SD conditions at 20°C during the larval stage, were subjected to either SD or LD conditions at 20°C. Five to 20 females in each experimental group were dissected at predetermined ages of 0-109 d.

As shown in figure 1, females accumulated no blue-green yolk in their oocytes until 54 d, except at 40 d when kept under SD conditions at 20°C. The proportion of females accumulating yolk or having eggs gradually increased thereafter, and the AGOD had increased to 2.2 ± 1.8 at 83 d. At 109 d, the majority of females possessed more than 17 eggs (or more than 2 eggs in each ovariole) with an AGOD of 3.5 ± 0.7. On the other hand, when females were transferred to LD conditions after emergence, their ovarian development was rapid, and three of 20 females had accumulated a small amount of yolk at 8 d and the AGOD was 0.2 ± 0.4, while all females at 32 d possessed > 17 eggs in their ovaries and the AGOD had reached 3.6 ± 0.7 (Fig. 1).

These results indicate that the maintenance and termination of adult reproductive diapause in female *P. c-aureum* are determined by SD and LD photoperiodic conditions, respectively, although the former persisted only ca. 60 d even under SD conditions at 20°C.

Effects of temperature conditions on the duration of adult reproductive diapause in females of the autumn-morph type

To investigate whether the duration of

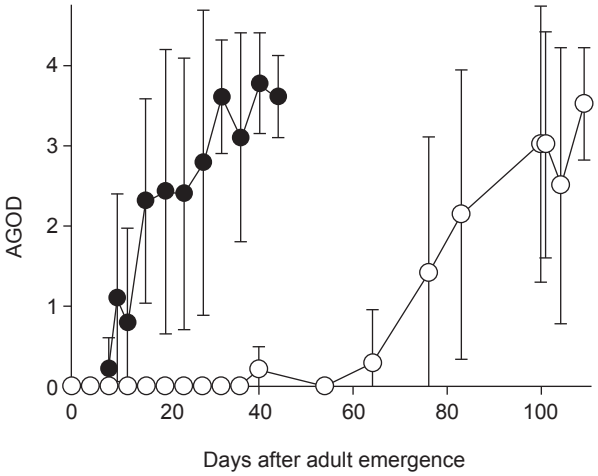

Fig. 1. Effects of long-day (LD) and short-day (SD) exposures on egg production in females of the autumn-morph type of *Polygonia c-aureum*. Solid and open circles show the average grade of ovarian development (AGOD) at 20°C under LD and SD conditions, respectively, and bars indicate standard deviations. Five to 20 females were examined in each experimental group.

reproductive diapause is affected by temperature conditions after emergence, females of the autumn-morph type obtained under SD conditions at 20°C were divided into 2 groups and subjected to SD conditions at different temperatures of 25 or 30°C. Ten to 20 females at respective ages were dissected to observe the state of ovarian development. Reproductive diapause was defined as an AGOD of < 2 in the present paper.

When females were kept at 25°C, their ovarian development was fairly slow (Fig. 2). One of 10 females had accumulated a small amount of yolk at 20 d, and the AGOD was 0.1 ± 0.3, which reached 2.8 ± 1.5 at 70 d, and all 10 females possessed > 16 eggs at 80 d. The duration of reproductive diapause observed at 25°C was ca. 60 d, which was 20 d shorter than that observed at 20°C (Figs. 1, 2). When the temperature was raised to 30°C, the duration was far shorter, ca. 22 d (Fig. 2).

These results indicate that females of the autumn-morph type maintain a temperature-dependent reproductive diapause under SD condition, the period of which is shortened if the temperature increases.

Effects of the length of LD exposure on termination of adult reproductive diapause in females of the autumn-morph type

To investigate the length of LD exposure required for terminating reproductive diapause in females of the autumn-morph type, adult females obtained under SD conditions at 20°C were subjected to LD conditions for 10 d at 20°C within 12 h after emergence. After 10 d of LD exposure, females were returned to SD conditions at 20°C. Ten adult females at predetermined ages were dissected to observe the state of ovarian development.

Figure 3 shows the changes in ovarian development of females subjected to the above LD exposure for 10 d. There was no accumulation of yolk in oocytes at 0, 4, and 8 d, when the AGOD was 0. An accumulation of a small amount of yolk was observed at 14 d (4 d after re-transfer to SD conditions) with an AGOD of 1.3 ± 0.8. Females showed a gradual accumulation of yolk in oocytes, and the AGOD had reached 2.3 ± 1.9 at 18 d. At 34 d, all females possessed > 16 eggs, and the AGOD had reached 4 ± 0. Females of the autumn-morph type that were exposed to LD conditions

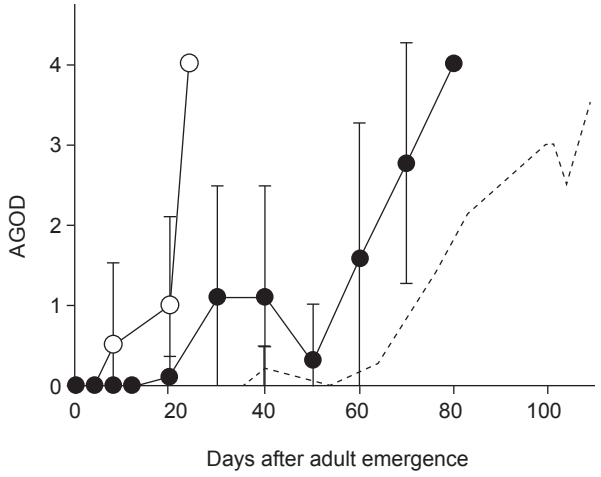

Fig. 2. Effects of temperature on egg production in females of the autumn-morph type of *Polygonia c-aureum* maintained under short-day (SD) conditions (10 h of light and 14 h of dark; 10L-14D). Solid and open circles show fluctuations in the average grade of ovarian development (AGOD) scores at 25 and at 30°C, respectively, with bars showing standard deviations. The broken line shows the profiles of AGOD scores under SD conditions at 20°C (cf. Fig. 1). Ten to 20 females were used for each experimental group.

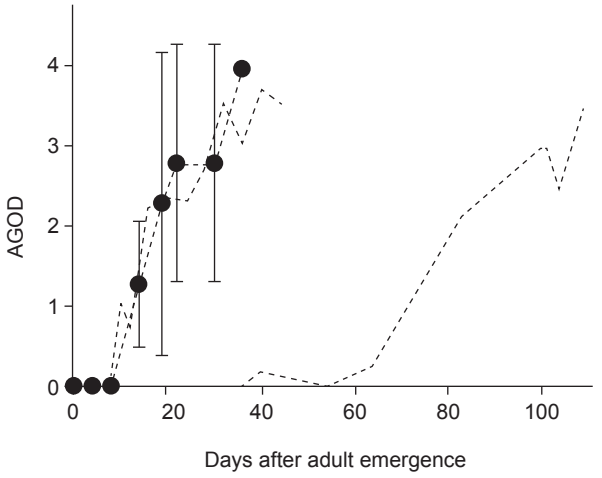

Fig. 3. Effects of a limited long-day (LD) exposure on egg production in females of the autumn-morph type of *Polygonia c-aureum*. Solid circles show fluctuations in average grade of ovarian development (AGOD) scores of treated females. Females were exposed to LD conditions (14 h of light and 10 h of dark; 14L-10D) for 10 d after emergence, and then transferred to short-day (SD) conditions (10L-14D) at 20°C. Bars show standard deviations. Broken lines on the left and right show profiles of AGOD scores in females of the autumn-morph type continuously maintained at 20°C under LD and SD conditions, respectively (cf. Fig. 1). Ten females were used for each experimental group.

for 10 d at 20°C after emergence showed almost the same fluctuating patterns with those kept continuously under LD conditions at 20°C after emergence (Fig. 3). In females exposed to a shorter LD period for 8 d after emergence, ovarian development was fairly arrested, and the AGOD was 1.5 at 30 d (data not shown).

These results indicate that exposure of *P. c-aureum* to LD conditions for 10 d at 20°C seemed to be sufficient to terminate reproductive diapause in females of the autumn-morph type.

Effects of an SD photoperiod on reproductive activity in females of the summer-morph type

Females of the summer-morph type were reproductively active soon after emergence. To investigate whether the reproductive activity of the summer-morph (or non-diapause) type is affected by an SD photoperiod in the adult stage, females obtained under LD condition at 20°C were divided into 2 groups within 12 h after emergence and subjected to either SD or LD conditions at 20°C. Ten females for each treatment were dissected every day to observe the state of ovarian development.

As shown in figure 4, all females showed an accumulation of a small amount of yolk at 1 d irrespective of the photoperiodic conditions; the AGOD was 1.3 ± 0.4 in the SD group and 1.0 ± 0.0 in the LD group. The majority of females under LD conditions had produced the 1st eggs in each ovariole within 3 d, and the AGOD was 1.4 ± 0.6. Each ovariole of these females possessed 3 eggs at 6 d, and the AGOD was 3.8 ± 0.4. However, 1/3 of the females kept under SD conditions required 10 d to produce 3 eggs in each of their ovarioles, when their AGOD had reached 3.2 ± 1.0.

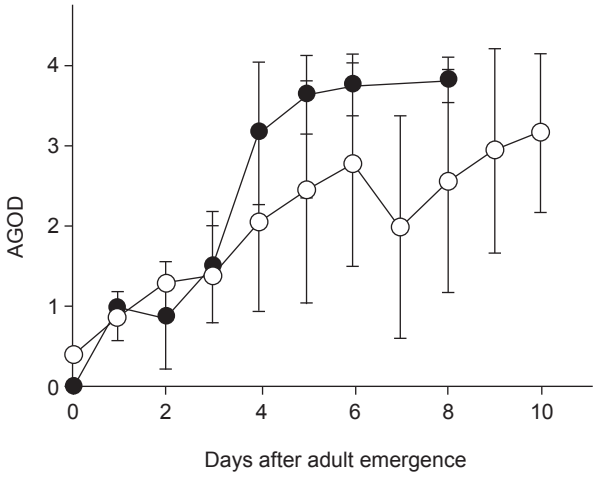

Fig. 4. Effects of long- (LD) and short-day (SD) photoperiods on egg production in females of the summer-morph type of *Polygonia c-aureum*. Solid and open circles show fluctuations in average grade of ovarian development (AGOD) scores of females of the summer-morph type maintained under LD (14 h of light and 10 h of dark; 14L-10D) and SD (10L-14D) conditions, respectively, at 20°C. Bars are standard deviations. Ten females were used for each experimental group.

These results indicate that the SD photoperiod caused a delay of 2nd and 3rd egg production in females of the summer-morph type at 20°C, and that LD conditions are required to maintain reproductive activity in the non-diapause type, suggesting that adult females of the summer-morph type are also responsive to photoperiodic conditions.

DISCUSSION

The results clearly show that in *P. c-aureum*, photoperiod and temperature conditions to which the adult stage are exposed affect the maintenance and termination of reproductive diapause in females of the autumn-morph type, and also the maintenance and duration of reproductive activity of non-diapause females of the summer-morph type. Females of the autumn-morph type that were subjected to LD conditions produced 8 (i.e., 1 egg in each of the 8 ovarioles) and 16 eggs (i.e., 2 eggs in each of the 8 ovarioles) within 10 and 38-40 d after emergence, respectively (Fig. 1). Furthermore, reproductive diapause in females of the autumn-morph type, which was maintained for 60-80 d under SD conditions at 20°C, was terminated at 20-24 d when the rearing temperature was raised to 30°C under the same photoperiod (Fig. 2). Therefore, adult females of the autumn-morph type require SD conditions at moderate temperatures to maintain reproductive diapause, suggesting that they may maintain some responsiveness to photoperiodic conditions in the adult stage. It appears that the duration (or termination) of reproductive diapause in autumn-morph females of *P. c-aureum* is determined by environmental photoperiodic conditions. Such phenomena have also been recorded for several species of coleopteran and hemipteran insects (Numata 1992, Saunders 2002).

Our results also indicate that the minimum length of LD exposure required to terminate adult reproductive diapause at 20°C was 10 d in adult females of the autumn-morph type (Fig. 3). This

observation is similar to our previous finding of the length of LD exposure required during larval stages to abort adult reproductive diapause at 20°C (Endo et al. 1992). Hence, breaking the SD photoperiod information which has accumulated in brains of SD insects might require exposure to LD conditions for at least 10 d in *P. c-aureum* in both the larval and adult stages.

In this study, we found that adult females of the summer-morph type, which are programmed to be reproductively active with LD conditions during the larval and pupal stages, showed slight responsiveness to SD conditions experienced after emergence, and this involved a deletion of production of the 3rd and 4th eggs of their ovarioles (Fig. 4).

Our previous studies showed that (i) adult females of the summer-morph type begin egg production at around adult emergence, (ii) egg production can be prevented by severing the nervi corporis allati (NCA) at the 5th instar larval stage, (iii) a cerebral factor terminating reproductive diapause in females of the autumn-morph type exists in female brains of both types, and (iv) the cerebral factor is extractable from both adult brains using a 2% NaCl solution, suggesting that this factor may be an AT-like factor (Endo 1972, Muranaka et al. 2002). Therefore, brains of adult females of the summer-morph (non-diapause) type kept under LD conditions are supposed to send an AT-like factor through the NCA in the days following adult emergence to maintain the activity of the CA of secreting JHs for ovarian maturation, as has been shown in other lepidopterans (Ramaswamy et al. 1997). Furthermore, brains of the summer-morph type may have regulatory mechanisms that delay egg production under SD conditions in the days following emergence, which may cause a decrease (or the cessation) of the secretion of the AT-like factor through the NCA in order to suppress JH secretion. Herein, we could provide no information about the physiological mechanism underlying the photoperiodic control of secretion (or production) of the AT-like factor in *P. c-aureum*. But, the results of the present study suggest that the photoperiodic regulatory mechanism of CA activity in adult females of *P. c-aureum* may play a significant role in readjusting the timing and period of reproductive activity to environmental conditions, and females may utilize photoperiodic responsiveness to save energy as a countermeasure against environmental extremes during the adult stages of each morph type.

Interestingly, recent studies of 2 tropical nymphalid butterflies, *Euploea core* and *E. sylvester* (Canzano et al. 2003), revealed that food resources, as well as rainfall, increased temperature, and photoperiod, are also used as direct cues for terminating adult reproductive diapause. Additionally, the egg size of offspring is controlled by oviposition temperature in females of *Bicyclus anynana*, which exhibit 2 seasonal morphs comprising dry and wet forms, although they do not enter adult reproductive diapause (Fischer et al. 2003a b).

In order to understand the life-history plasticity of nymphalid butterfly species, it will be necessary to accumulate detailed species-specific information under various environmental conditions.

In summary, adult female butterflies of *P. c-aureum* maintain some responsiveness to photoperiodic conditions in the days following adult emergence. Hence, *P. c-aureum* begins to count the numbers of LD and SD days from the first larval-larval ecdysis (Endo et al. 1972) and accumulates photoperiodic information throughout the larval, pupal, and adult stages. Additionally, we provide evidence that *P. c-aureum* can regulate the maintenance and termination of reproductive diapause based on photoperiodic conditions in the adult stage.

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