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ORIGINAL ARTICLE

Quantitative response to photoperiod and weak coupling between seasonal morphs and diapause regulation in the Asian comma butterfly, *Polygonia c-aureum* (Lepidoptera: Nymphalidae)

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Key words. Lepidoptera, Nymphalidae, *Polygonia c-aureum*, diapause induction, photoperiodism, ovary, reproduction, seasonal form

Abstract. Reproduction and wing patterns (shape and colouration) in *Polygonia c-aureum* L. (Lepidoptera: Nymphalidae) are regulated by both photoperiod and temperature experienced during the immature stages, which result in the development of summer or autumn forms. The critical day length for this seasonal change in form was 13.5L : 10.5D at 21°C and 13L : 11D at 25°C. We investigated the connection between seasonal form and female reproduction. Under a 15L : 9D photoperiod at 21°C, reproductively active summer form butterflies are produced, whereas under an 8L : 16D photoperiod at 21°C autumn form butterflies with a strong tendency to enter diapause were produced. On the other hand, under the critical day lengths at 21 or 25°C, autumn form butterflies developed with a weak tendency to enter diapause. When the adult butterflies were transferred from a critical or a short photoperiod to a long photoperiod shortly after emergence, the former were more likely to terminate diapause than the latter. If individuals are reared throughout their entire life cycle under a short photoperiod at 21°C, all the adults have a strong tendency to enter diapause. These results reveal the quantitative effects of photoperiod on diapause in this butterfly and strongly indicate that the determination of the autumn form and induction and maintenance of diapause are not rigidly coupled, at least under laboratory conditions.

INTRODUCTION

Seasonal polyphenism is recorded in many insects (Tauber et al., 1986). Various patterns exist in the relationship between seasonal forms or change in body colouration and diapause depending on the species. For example, as the planthopper *Stirellus bicolor* Van Duzee does not enter diapause, its polyphenism is not related to diapause (Whitcomb et al., 1972; Tauber et al., 1986). In the dung beetle, *Gymnopleurus humanus* Macleay, larvae overwinter and adult beetles show a temperature-dependent colour polymorphism (Davis et al., 2008). In the rice skipper *Parnara guttata guttata* (Bremer & Grey) and the horned beetle *Allomyrina dichotoma* (L.), it is the larvae that enter diapause and larval diapause is not tightly coupled with differences in the imaginal seasonal forms (Ishii & Hidaka, 1979; Nakasuji & Kimura, 1984; Plaistow et al., 2005). Similarly, the connection between pupal seasonal polyphenism and pupal or adult diapause is not rigid or incomplete in the black swallowtail butterfly *Papilio polyxenes* Fabricius (Hazel & West, 1983; Sims, 2007), the comma butterfly *Polygonia c-album* (L.) (Voigt, 1991), and the leafminer *Lyonetia prunifoliella malinella* (Matsumura) (Sekita, 2002). However, there are species in which there is a close relationship and both seasonal form and diapause are expressed in the same imaginal stage, for example, in the lacewings *Chrysopa carnea* (Stephens) (Tauber & Tauber, 1970), *C. congrua* (Walker) (Winterton, 1999), and *C.* sp. (Canard, 2005), as well as the rape bug *Eurydema oleracea* (L.) (Fasulati, 1979), stink bug *Plautia crossota stali* Scott (Kotaki & Yagi, 1987; Kotaki, 1998a, b), brown stink bug *Euschis-*

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tus servus (Say) (Borges et al., 2001), the two-spotted spider mite *Tetranychus urticae* C.L. Koch (Kawakami et al., 2009), and elm leaf beetle *Xanthogaleruca luteola* (Müller) (Soudi & Moharramipour, 2011), although seasonal polyphenism occurs within the same population in these species. In the squinting bush butterfly, *Bicychus anynana* (Butler), the wet season females lay eggs immediately after mating, whereas the dry season females delay egg-laying (Prudic et al., 2011). In addition, there is a strong link between reproductive maturity, diapause, and wing colour in the red locust *Nomadacris septemfasciata* Audinet-Serville (Franc & Luong-Skovmand, 2009). Wing form is often strongly linked with diapause, as for example in the linden bug *Pyrrhocoris apterus* (L.) (Socha & Kodrik, 1999) and water strider *Aquarius paludum* (Fabricius) (Inoue & Harada, 1997). In the bean bug *Riptortus clavatus* (L.), there is a close relationship between diapause and seasonal form, but the link is not consistent at near critical day lengths (Kobayashi & Numata, 1993).

Many studies report that diapause in insects is determined by photoperiod, temperature, humidity, or food conditions (Tauber et al., 1986); for example, termination of diapause in the peach twig borer, *Anarsia lineatella* Zeller, occurs after chilling (Damos & Savopoulou-Soultani, 2010) and both temperature and photoperiod affect the induction and termination of diapause in the rice bug, *Leptocorisa chinensis* Dallas (Yamashita et al., 2010). It is suggested that a photoperiod close to the critical day length has a significant influence on the intensity of pupal diapause in the swallowtail butterfly *Sericinus montela* Gray (Wang et al., 2009), while in many insects and mites, diapause ends spontaneously when they are kept under diapause-inducing conditions, without chilling or any other change in regime (Tauber et al., 1986; Veerman, 1992). In the nymphalid butterfly *Polygonia c-aureum* L. there is a close relationship between adult diapause and seasonal form (summer and autumn). Summer form butterflies begin reproduction soon after emergence, whereas autumn form butterflies enter adult diapause and reproduce in spring. Both the seasonal form and adult diapause induction in this butterfly are controlled mainly by the photoperiod and temperature experienced during the larval stage: long photoperiods and high temperatures induce the summer-form, which do not diapause, whereas short photoperiods and relatively low temperatures induce the autumn-form, which enters diapause (Hidaka & Aida, 1963; Hidaka & Takahashi, 1967). Diapause in female *P. c-aureum* is characterized by suppression of ovarian development (Hidaka & Aida, 1963; Fukuda & Endo, 1966), inhibition of the accumulation of secretory substances in female accessory reproductive glands (Endo, 1973b), and reduced tendency to mate (Endo, 1973a). However, mating of autumn-forms is reported in the field in the autumn (September) (Fukuda et al., 1983). Furthermore, autumn-forms that emerge in early autumn are short-lived compared to those that emerge in late autumn, and most of the former are unable to overwinter under laboratory conditions (Hidaka & Aida, 1963; Matsui & Matsui, 1985). Shapiro (1976) reports that in *P. c-album*, a species that is closely related to *P. c-aureum*, a small proportion of the autumn form mate before overwintering. This indicates it is possible that adult diapause in a proportion of the autumn form of *P. c-aureum* is terminated in early autumn or not induced. This study examines the link between seasonal form and adult diapause in females of *P. c-aureum* by investigating the effects of combinations of photoperiod and temperature on the induction, maintenance, and termination of adult diapause.

MATERIALS AND METHODS

Insect source and rearing of a colony in the laboratory

A laboratory colony of *P. c-aureum* was established using larvae collected in the city of Tokyo (35°67´N, 139°48´E), Japan, in April and May of 1987. The offspring of the autumn-form of this butterfly are likely to develop into the summer-form, regardless of photoperiod (Hidaka & Takahashi, 1967). To avoid this, newly hatched larvae were reared under a long day length (15L : 9D photoperiod) at 21 ± 1°C, which induced them to produce only the summer-form.

Insect rearing for the experiments

Newly emerged larvae from the laboratory colony were reared under (1) a short day length (8L : 16D photoperiod) (SD) at either 21 ± 1°C or 25 ± 1°C, (2) a critical photoperiod (13.5L : 10.5D photoperiod at 21 ± 1°C, (3) 13L : 11D photoperiod at 25 ± 1°C), or (4) a long day length (LD) at 21 ± 1°C, following the methods described by Hiroyoshi (1992). After emergence, the adult butterflies were kept either under the same conditions (1–3) as the immature stages, transferred to SD (1) or LD (4) conditions, or (5) transferred from 25°C to 21°C. In the last experiment, larvae in the fourth day of the final (5th) instar that were previously reared under SD at 21 ± 1°C were transferred to LD conditions at 21 ± 1°C (6). After emergence, females were separated from males, and kept in separate cages (17 cm × 16.5 cm × 46 cm). A cotton wool ball soaked in a sucrose solution (10%) was provided ad libitum as food for adults and renewed weekly.

Experimental design

Exp. #1: Effect of the two photoperiods near to CD

To assess the effects of critical day length (CD) on diapause induction (See Hiroyoshi, 1992), summer- and autumn-forms were reared throughout their lifespan under either a 13.5L : 10.5D photoperiod at 21°C or a 13L : 11D photoperiod at 25°C. Virgin summer- and autumn-form adult females were dissected 10, 20 or 30 days after emergence.

Exp. #2: Change in temperature at CD

To assess the effects of temperature on diapause maintenance, summer- and autumn-forms that were reared under CD (13L : 11D) at 25°C were transferred after they emerged as adults to the same photoperiod (13L : 11D) at 21°C. Virgin females of summer- and autumn-forms were dissected 10, 20 or 30 days after emergence.

Exp. #3: Effect of a change from SD to LD (at 21°C) after rearing under CD (at 25°C)

To assess the effects of a change in both temperature and photoperiod on diapause maintenance or termination, adult butterflies reared under CD at 25°C were transferred to LD or SD conditions at 21°C after emergence. Virgin females of the summer- and autumn-forms were dissected 10, 20 or 30 days after emergence.

Exp. #4: Effect of changing from SD to LD conditions at two temperatures

To assess the effect of a change in photoperiod on diapause maintenance or termination, adult butterflies that were reared under SD conditions at 21 or 25°C were transferred to LD at 21°C. Virgin females of the autumn-form were dissected 10, 20 or 30 days after emergence.

Exp. #5: Same photoperiod (SD; 8L : 16D) at 21 or 25°C

To assess the effects of the combination of a short day photoperiod and different temperatures, adult butterflies reared under SD were kept under the same photoperiod at 21°C or 25°C throughout their entire life span. Virgin females of the autumn-form were dissected 10, 20 or 30 days after emergence.

Exp. #6: Transfer to another photoperiod during the larval stage

In the last experiment, larvae reared under SD at 21°C were transferred to LD at 21°C on the 4th day of the final instar (5th). Virgin females of the summer- and autumn-forms were dissected 2, 5 or 10 days after emergence. The mean duration of the final larval stage reared under SD was 4.9 ± 0.4 days (Hiroyoshi, 2000).

In all experiments, butterflies were dissected as described below, to record ovarian development in order to determine whether they were in diapause or not.

Ovarian development

To assess the effects of various rearing regimes on reproductive readiness, ovaries of insects were dissected in a Lepidoptera saline solution (consisting of 147.15 mM NaCl, 2.97 mM $CaCl_2$ and 0.57 mM KCl per liter distilled water) under a binocular microscope every 10 days (10, 20 or 30) after adult emergence unless otherwise stated. In this study, the incidence of diapause and the number of eggs in the ovaries were determined and classified based on the colour and size of the oocytes: females in diapause have opaque or white coloured oocytes, while females with yellow, yellowish green or green oocytes are not in diapause. Oocytes that were green were classified as mature. These experiments were repeated 8 to 39 times for adults of each age.

Oviposition did not occur during this experiment (thus all the eggs were in the ovaries) as *P. c-aureum* only laid eggs if leaves of their host plant were provided. If host plant leaves were provided, even unmated sexually mature females laid eggs, although they laid fewer eggs than mated females. In this study, neither host plant leaves nor mates were provided for the females used in the experiments.

Statistics

Diapause incidence was analyzed using logistic regression in order to estimate the effects of the two seasonal forms and age. To avoid the results of the analysis becoming unstable due to the presence of 0 data, the adjusted odds ratios were compared. Then, if necessary, pairwise comparisons using a G-tests weighted by Bonferroni correction were carried out. Whenever no or almost no summer-forms emerged, diapause incidence was analyzed using logistic regression in order to estimate the effect of age. Then, if necessary, pairwise comparisons using a G-tests weighted by Bonferroni correction were performed.

Because of the lack of normality and homoscedasticity of the data, the number of eggs were analyzed using two-factor-extended Kruskal-Wallis tests (Sokal & Rohlf, 1995) to estimate the effects of seasonal form and age. Then, if necessary, pairwise comparisons using Steel-Dwass test were performed. Whenever no or almost no summer-forms emerged, the number of eggs were analyzed using two-factor-extended Kruskal-Wallis tests (Sokal & Rohlf, 1995) in order to estimate the effects of age, because of the lack of normality and homoscedasticity of the data. Then, if necessary, pairwise comparisons using Steel-Dwass test were performed.

RESULTS

Exp. #1: The effect of rearing summer and autumn forms at or near the critical photoperiod

The effects of the two rearing regimes at or near the critical photoperiod (CD) on summer- and autumn-forms in terms of diapause and reproduction were examined by keeping each form under a 13.5L : 10.5D photoperiod at 21°C or a 13L : 11D photoperiod at 25°C throughout their life.

At 21°C / CD, the incidence of diapause recorded in summer-form butterflies was very low, but a few individuals entered diapause on day 20 of their adult life. There were significant differences in diapause incidence only between the seasonal forms, but not among ages (result of logistic regression; form: df = 1, G-value = 36.512, $P < 0.0001$; age: df = 2, G-value = 0.764, $P = 0.6826$; interaction: df = 2, G-value = 3.181, $P = 0.2038$) (see Table 1-1) (Fig. 1A). Although the number of eggs in the ovaries of the summer-form increased with adult age (Fig. 1B), there were significant differences in the number of eggs in the ovary only between seasonal forms, but not among ages (result of logistic regression; form: df = 1, H-value = 48.398, $P < 0.0001$; age: df = 2, H-value = 1.752, $P = 0.4164$, interaction, df = 2, H-value = 1.281, $P = 0.5271$) (Table 2-1). On the other hand, for autumn-forms there was a decrease in diapause incidence and slowing in ovarian development during the adult stage (Fig. 1A, B).

Table 1-1. The results of the logistic regression analysis of the adjusted odds data on the effects of seasonal forms and ages on the incidence of diapause.

Factor	d.f.	*G*-value	*P*-value
CD at 21°C			
Seasonal form	1	36.512	<0.0001*
Age	2	0.764	0.6826
Seasonal form × Age	2	3.181	0.2038
CD at 25°C			
Seasonal form	1	41.402	<0.0001*
Age	2	1.974	0.3726
Seasonal form × Age	2	0.759	0.6843
CD at 25°C to 13L : 11D at 21°C			
Seasonal form	1	61.791	<0.0001*
Age	2	0.689	0.7083
Seasonal form × Age	2	1.327	0.5151
CD at 25°C to LD at 21°C			
Seasonal form	1	115.488	<0.0001*
Age	2	0.943	0.6241
Seasonal form × Age	2	1.166	0.5583
CD at 25°C to SD at 21°C			
Seasonal form	1	115.488	<0.0001*
Age	2	0.943	0.6241
Seasonal form × Age	2	1.166	0.5583
SD to LD at 5th larval instar			
Seasonal form	1	11.938	0.0005*
Age	1	0.451	0.5020
Seasonal form × Age	1	0.644	0.4223

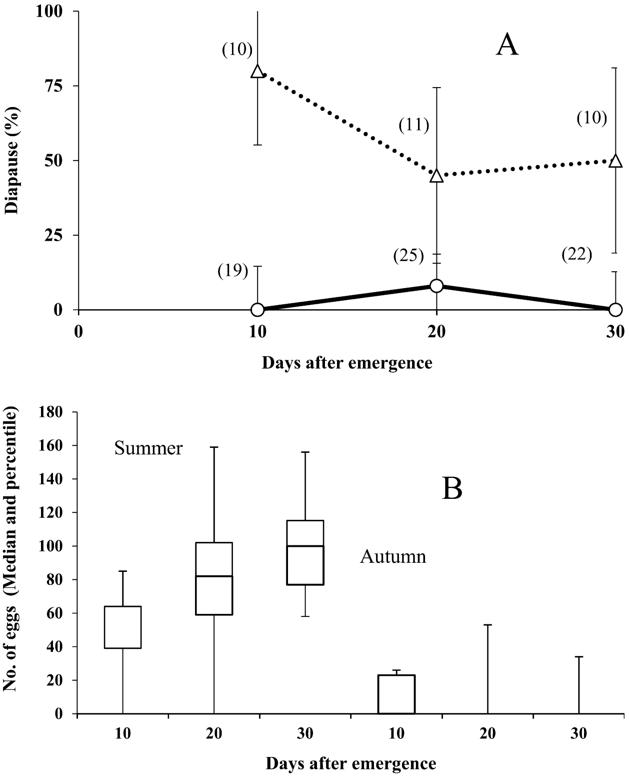

Fig. 1. The incidence of diapause (error bars are 95% CI of expression level classes) based on the colour of the oocytes (see Materials and Methods) (A) and the number (median and percentiles) of eggs in the ovaries (B) of *Polygonia c-aureum*, when each seasonal form was reared under 13.5L : 10.5D at 21°C throughout their life. Circles and solid line indicate summer-forms and triangles and broken line indicate the autumn-forms. Figures in the parenthesis indicate the number of animals used.

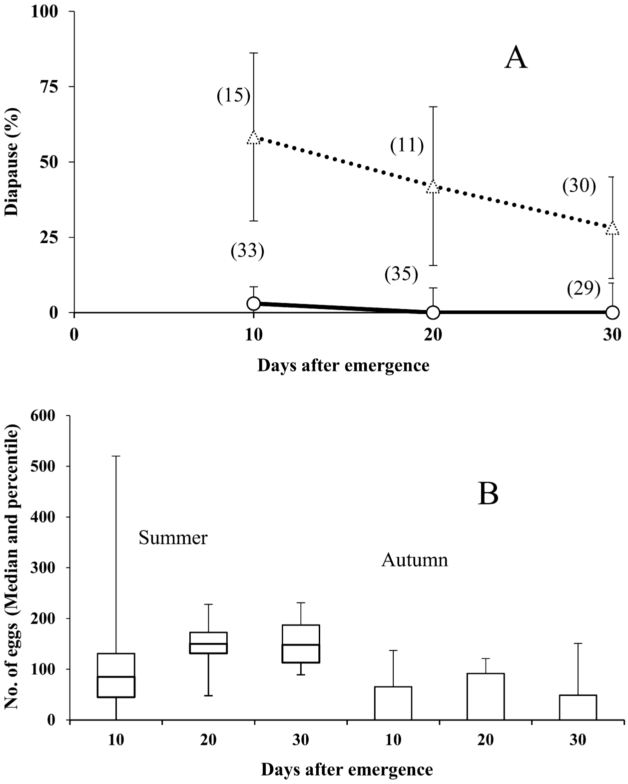

Fig. 2. The incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries of each of the seasonal forms (B) of *Polygonia c-aureum*, when each of seasonal forms were reared under 13L : 11D at 25°C throughout their life. Circles and solid line, and triangles and broken line indicate summer- and autumn-forms, respectively. Figures in the parenthesis indicate the number of animals used.

At 25°C / CD, the incidence of diapause in summer-forms was very low, with just one out of 15 butterflies entering diapause on day 10 (Fig. 2A). There were significant differences in diapause incidence only between summer- and autumn-forms (df = 1, *G*-value = 41.402, $P < 0.0001$) (Table 1-1). The numbers of eggs in the ovaries of summer-forms was high and increased with adult age (Fig. 2B), and there were significant differences only in the number of eggs in the summer- and autumn-forms (df = 1, *H*-value = 56.568, $P < 0.0001$) (Table 2-1). On the other hand, the incidence of diapause in autumn-forms decreased greatly with adult age (Fig. 2A), and there was some degree of ovarian development throughout the adult life of the autumn-forms (Fig. 2B).

Table 1-2. The results of the logistic regression analysis of the effect of age on the incidence of diapause.

Factor	d.f.	*G*-value	*P*-value
SD at 21°C to LD at 21°C Autumn form			
Age	2	11.959	0.0025*
SD at 25°C to LD at 25°C Autumn form			
Age	2	9.381	0.0092*
SD at 25°C Autumn form			
Age	2	1.6145	0.4461
SD at 21°C Autumn form			
Age	2	0.349	0.839

Table 2-1. The results of two-factor-extended Kruskal-Wallis test (Sokal & Rohlf, 1995) of the effects of seasonal forms and ages on the number of eggs in the ovaries.

Factor	d.f.	*H*-value	*P*-value
CD at 21°C			
Seasonal form	1	48.398	<0.0001*
Age	2	1.752	0.4164
Seasonal form × Age	2	1.281	0.5271
CD at 25°C			
Seasonal form	1	56.568	<0.0001*
Age	2	2.475	0.2901
Seasonal form × Age	2	1.960	0.3753
CD at 25°C to 13L : 11D at 21°C			
Seasonal form	1	59.688	<0.0001*
Age	2	1.412	0.4936
Seasonal form × Age	2	1.283	0.5265
CD at 25°C to LD at 21°C			
Seasonal form	1	62.070	<0.0001*
Age	2	2.613	0.1354
Seasonal form × Age	2	0.894	0.3198
CD at 25°C to SD at 21°C			
Seasonal form	1	81.653	<0.0001*
Age	2	1.364	0.5056
Seasonal form × Age	2	2.367	0.3062
SD to LD on 5th larval instar			
Seasonal form	1	3.502	0.0371*
Age	1	16.238	0.0108*
Seasonal form × Age	1	3.503	0.0370*

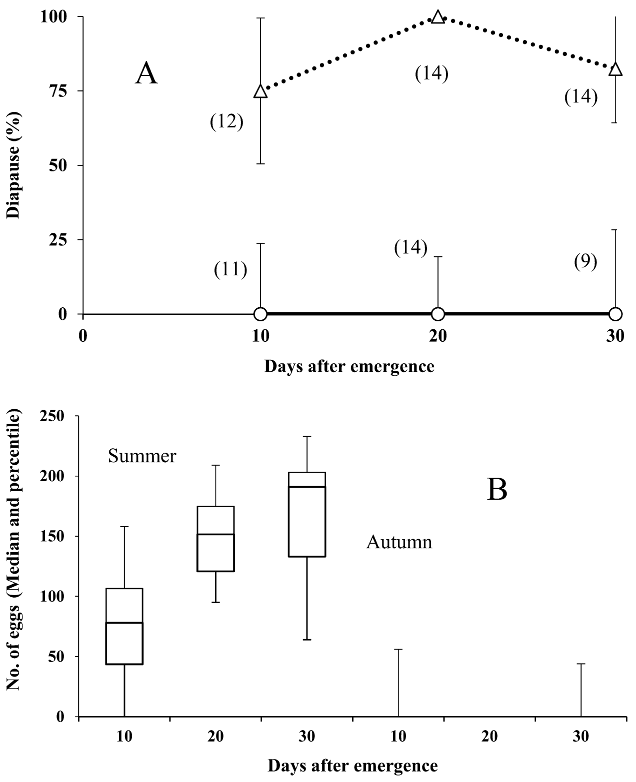

Fig. 3. The incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries (B) of each of the seasonal forms of *Polygonia c-aureum*, when both seasonal forms were reared under 13L : 11D at 25°C and then transferred to 21°C under the same photoperiod after adult emergence. They were examined 10, 20, or 30 days after adult emergence. Circles and solid line, and triangles and broken line indicate summer- and the autumn-forms, respectively. Figures in parenthesis indicate the number of animals used.

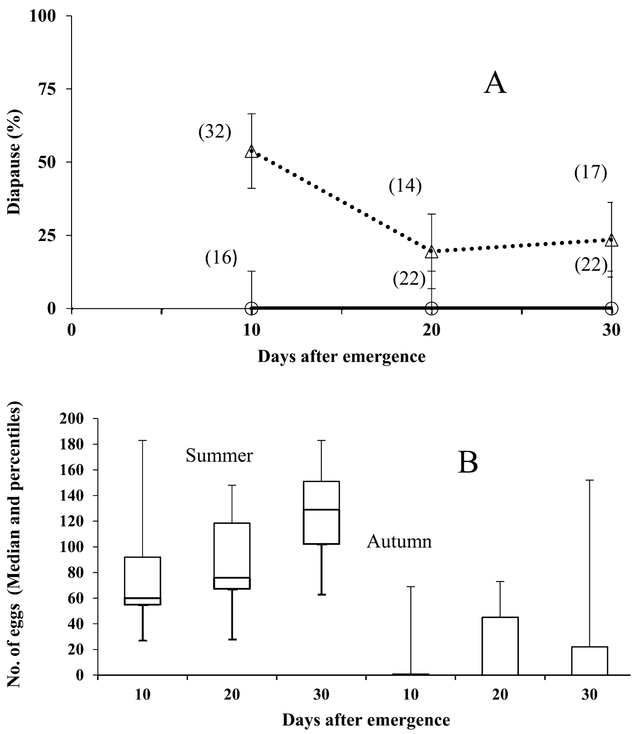

Fig. 4. The incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries (B) of each seasonal form of *Polygonia c-aureum*, when both were reared under 13L : 11D at 25°C during their immature stages and transferred to 15L : 9D at 21°C at adult emergence. They were examined 10, 20, or 30 days after emergence. Circles and solid line, and open triangles and broken line indicate summer- and autumn-forms, respectively. Figures in parenthesis indicate the number of animals used.

Exp. #2: Effect of a change in temperature on maintenance of diapause

To assess the effects of a reduction in temperature at the critical day length, the temperature was decreased after the adults emerged. Individuals reared under CD at 25°C were transferred to 21°C, while keeping the same day length (13 : 11). We found that summer-forms treated in this way remained reproductively active (Fig. 3A) and the number of eggs in their ovaries increased with age (Fig. 3B). There were significant differences in diapause incidence only between summer- and autumn-forms (df = 1, *G*-value = 61.791, $P < 0.0001$) (Table 1-1). On the other hand, almost no ovarian development was recorded in autumn-forms (Fig. 3B). Significant differences were found in the number of eggs only between summer- and autumn-forms (df = 1, *H*-value = 59.688, $P < 0.0001$) (Table 2-1). These results are quite different from the results when butterflies were reared under CD at 25°C throughout their lives (Fig. 2).

Table 2-2. The results of Kruskal-Wallis test on the effect of age on the number of eggs in the ovaries.

Factor	d.f.	*H*-value	*P*-value
SD at 21°C to LD at 21°C Autumn form Age	2	10.145	0.0063*
SD at 25°C to LD at 21°C Autumn form Age	2	4.061	0.1313
SD at 25°C Autumn form Age	2	2.385	0.3035

Exp. #3: Effect of transferring from rearing under a CD at 25°C to LD at 21°C

To assess the effects of photoperiod on diapause in both seasonal forms, freshly emerged adults reared under a CD at 25°C were transferred to LD at 21°C. None of the summer-form butterflies entered diapause at any age and ovarian development proceeded with age, whereas at adult emergence there was a high incidence of diapause in autumn-form butterflies (data not shown), which decreased to a low level as they aged, at which time there was only a slight increase in oocyte maturation (Fig. 4). There were significant differences in diapause incidence recorded for the summer- and autumn-forms (df = 1, *G*-value = 115.488, $P < 0.0001$) (Table 1-1) and in the number of eggs in the two forms (df = 1, *H*-value = 62.070, $P < 0.0001$) (Table 2-1).

Exp. #4: Effect of a change from CD at 25°C to SD at 21°C

Adults reared under CD at 25°C were transferred to SD at 21°C shortly after emergence (Fig. 5). Almost no summer-forms entered diapause and their ovaries were well de-

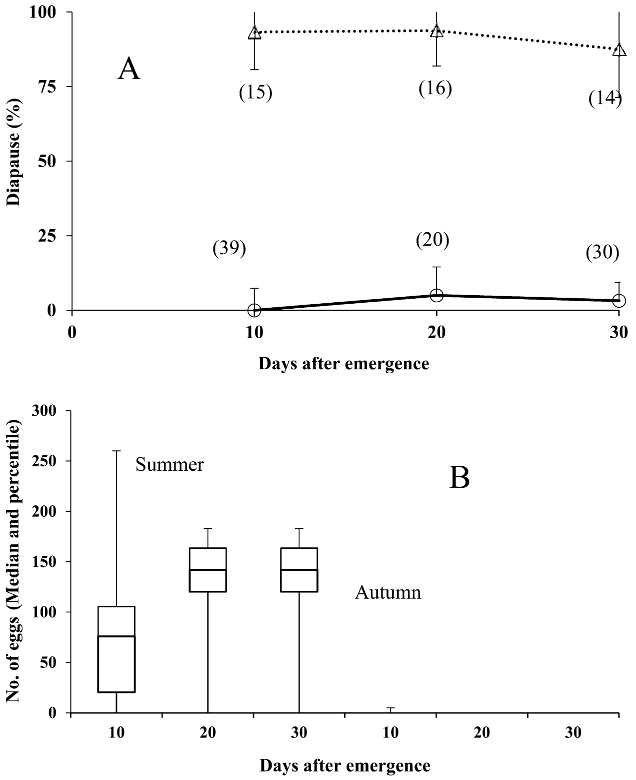

Fig. 5. The incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries (B) of each seasonal form of *Polygonia c-aureum*, when both were reared under 13L : 11D at 25°C and transferred to 8L : 16D at 21°C after adult emergence. Circles and solid line, and triangles and broken line indicate summer- and autumn-forms, respectively. Figures in parenthesis indicate the number of animals used.

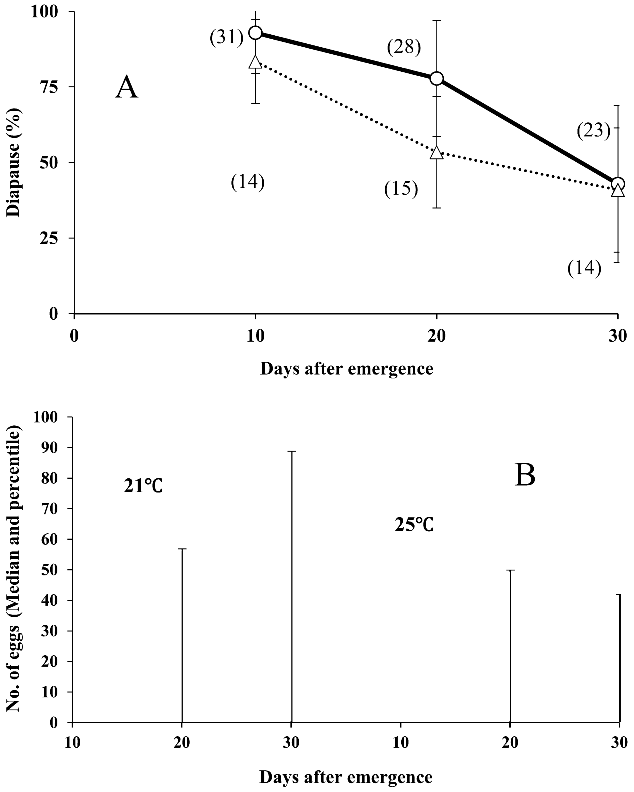

Fig. 6. Incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries (B) of *Polygonia c-aureum*, when reared under 8L : 16D at 21°C or 25°C during the larval and pupal stages and transferred to 15L : 9D at 21°C upon adult emergence. Circles and solid line, and triangles and broken line indicate autumn-form reared under 8L : 16D at 21°C and those reared at 25°C, respectively. Figures in parenthesis indicate the number of animals used.

veloped, whereas a high percentage of the autumn-forms remained in diapause and their ovaries did not develop. There were significant differences in the percentages of the summer- and autumn-forms in diapause (df = 1, *G*-value = 115.488, $P < 0.0001$) (Table 1-1), and in the number of eggs in their ovaries (df = 1, *H*-value = 81.653, $P < 0.0001$) (Table 2-1). These results together with the results in Figs 1–4 show that the temperature adults experience affects the incidence of diapause.

Exp. #5: Effects of temperature on the incidence of diapause when transferred from SD to LD at 21 or 25°C or constant SD at 21 or 25°C

To assess if the temperatures experienced by larvae and pupae affect the incidence of diapause, autumn-forms reared under SD at 21 or 25°C at the larval and pupal stages were transferred to LD at 21°C shortly after emergence. Significant differences were recorded at different ages in the incidence of diapause (21°C, df = 2, *G*-value = 11.959, $P < 0.01$; 25°C, df = 2, *G*-value = 9.381, $P < 0.01$) (Table 1-2) (Fig. 6). The incidence of diapause decreased with adult age in both treatments and was less than 50% on day 30 of adult life. There was no significant difference in the number of eggs in the ovaries at 25°C (df = 2, *H*-value = 4.061, $P > 0.05$) (Table 2-2), although a significant difference was recorded at 21°C (df = 2, *H*-value = 10.145, $P < 0.01$) (Table 2-2). These results indicate that the temperature experienced before emergence affected the induction and maintenance of diapause.

In another experiments, individuals were reared under SD at 21 or 25°C throughout their development. There were no significant differences in the incidence of diapause at different ages (21°C, df = 2, *G*-value = 0.349, $P > 0.05$; 25°C, df = 2, *G*-value = 1.6145, $P > 0.05$) (Table 1-2) or in the number of eggs in their ovaries at 25°C (df = 2, *H*-value = 2.385, $P > 0.05$) (Table 2-2) (Fig. 7). There were no eggs in the ovaries at 21°C. Unlike the data in Fig. 6, these results indicate that temperatures experienced by pupae and adults did not affect the incidence of diapause in this butterfly reared under the short day conditions.

Exp. #6: Transfer to a different photoperiod during larval development

In the final experiment, insects reared under SD at 21°C were transferred to LD on the 4th day of the final instar, and then the incidence of diapause in the adults was assessed. There were significant differences in the incidence of diapause only in the summer- and autumn-forms (df = 1, *G*-value = 11.938, $P < 0.001$) (Table 1-1) and significant differences in the number of eggs in the ovaries of the two seasonal forms (df = 1, *H*-value = 3.502, $P < 0.05$) and at

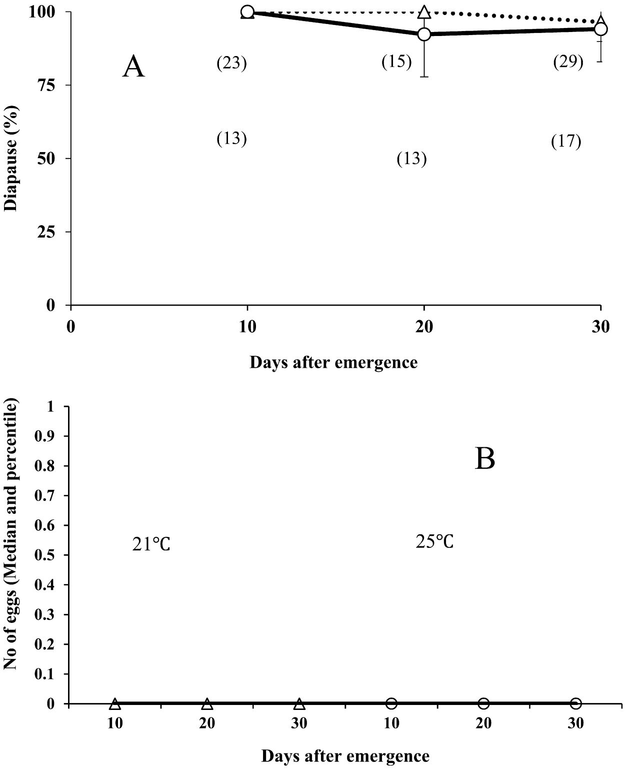

Fig. 7. The incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries of *Polygonia c-aureum* (B) at adult emergence, when reared under 8L : 16D at 21°C or 25°C throughout their life. Circles and solid line, and triangles and broken line indicate autumn-forms reared at 21°C and at 25°C, respectively. Figures in parenthesis indicate the number of animals used.

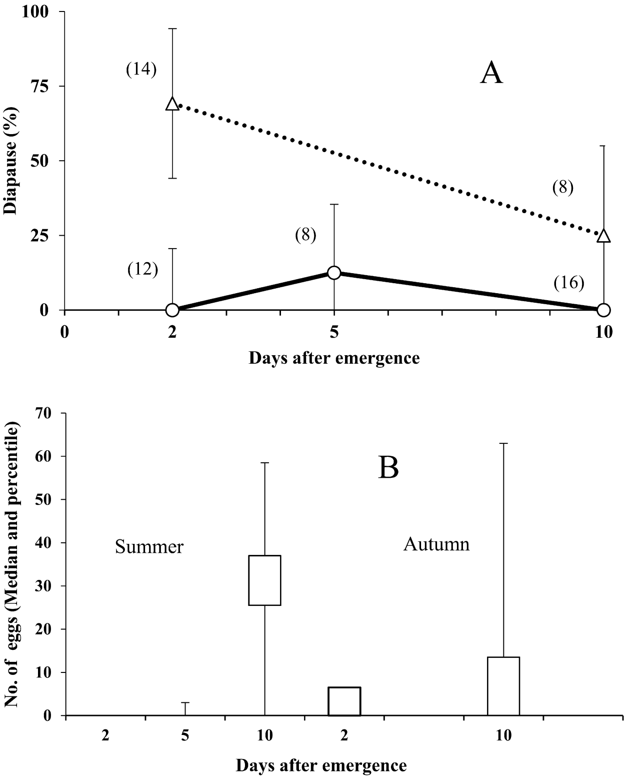

Fig. 8. Incidence of diapause (error bars are 95% CI of expression level classes) (A) and number (median and percentiles) of eggs in the ovaries of *Polygonia c-aureum* (B) of both seasonal forms, when reared under 8L : 16D at 21°C until the 4th day of the final (5) larval instar when they were transferred to 15L : 9D. Circles and solid line, and triangles and broken line indicate summer- and autumn-forms, respectively. Figures in parenthesis indicate the number of animals used.

different ages (df = 1, *H*-value = 16.238, $P < 0.05$) (Table 2-1) (Fig. 8). The incidence of diapause in autumn-forms on day 2 of adult life was less than 75%, indicating that 25% of these butterflies did not enter diapause.

DISCUSSION

The present study shows that more eggs matured in the ovaries of adults of the autumn-form of *P. c-aureum* that were reared under a critical photoperiod (Figs 1 and 2) but transferred to a long photoperiod at the adult stage, but not in the ovaries of those that were reared under short day conditions (Figs 4 and 6). Since there was almost no difference in the incidence of adult diapause in the autumn-forms reared at 21°C and at 25°C under the same short day conditions during first 30 days of adult life (Fig. 7), these results indicate that environmental conditions experienced before emergence, especially photoperiod, determine the intensity of diapause in the autumn-form of this butterfly. Thus, during the photosensitive stage when diapause is induced (Košťál, 2006), short photoperiods induced a strong diapause, whereas critical photoperiods induced only a weak (or shallow) diapause or no diapause. Thus, the relationship between seasonal forms and diapause in *P. c-aureum* is not absolute as it is dependent on environmental conditions. Furthermore, decreasing day lengths above the critical day length induce diapause in the autumn form of *P. c-album* (L.) (Nylin, 1989), indicating that the response to photoperiod is complicated.

It is possible that slight differences between the seasonal forms might determine the response of the pupal stage to diapause induction and thus long photoperiods may avert or weaken diapause by shortening the diapause inducing critical photoperiod. This difference in the critical photoperiod inducing and terminating diapause is recorded in several insects, including the rush skeleton weed gall midge, *Cystiphora schmidti* (Rübsaamen) (Moore, 1987; Li et al., 2003). In fact, the mechanisms underlying the determination of seasonal forms and diapause induction in *P. c-aureum* differ; seasonal form is controlled by a summer-morph-producing hormone secreted from the pars intercerebralis 32 h after pupation (Endo & Funatsu, 1985; Endo et al., 1988; Masaki et al., 1989), while diapause is regulated by the corpora allata and brain (Endo, 1970), although seasonal diphenism in the map butterfly, *Araschnia levana* L. is controlled by the timing of ecdysteroid release (Koch & Bückmann, 1987). Furthermore, Endo et al. (1992) report that the period of photo-sensitivity for diapause induction continues to the middle pupal stage, while that for the seasonal form ends in the final larval instar of *P. c-aureum*, indicating that the photosensitive stages for seasonal form and diapause initiation may differ slightly.

The present study also shows that the relationship between the autumn-form and diapause is weakened by changing the photoperiod during the larval stage (Fig. 8), which is similar to the results recorded when the larvae and pupae were reared under the critical photoperiod (Figs 1 and 2). If the critical photoperiod is not precise then under a near critical photoperiod, the individual may respond by perceiving the photoperiod as either a short or long photoperiod, or both. However, this does not explain the results of other studies that indicate a quantitative measurement of photoperiodic time (Kimura, 1990; Spieth & Sauer, 1991; Vaz Nunes & Hardy, 1993; Numata & Kobayashi, 1994; Nakamura & Numata, 2000; Saunders, 2002; Goto, 2009, 2013; Goto & Numata, 2009; Tagaya et al., 2010). Several insects respond to photoperiod and/or thermoperiod quantitatively (Tauber & Tauber, 1972, 1973; Butterfield, 1976; Kimura et al., 1982; Taylor & Schrader, 1984; Horwath & Duman, 1986; Kim, 1996; Fantinou et al., 2004). In fact, there is a quantitative response in the pupal weight of *P. c-aureum* to photoperiod (Hiroyoshi, 1992) in which the longer the photoperiod the heavier the pupal weight of the autumn-form, which may even approach that of the summer-form. As the period of the final larval stage (active feeding) under a long photoperiod is one day longer than that under a short photoperiod (Hiroyoshi, 2000), pupal weight would naturally increase with the lengthening of the photoperiod.

In the present study the duration of diapause in most adult individuals of *P. c-aureum* reared under a short photoperiod was at least 30 days (Fig. 7), whereas if the adults are transferred to a long photoperiod after emergence diapause is quickly terminated (Fig. 6), which is similar to the results of Fujita et al. (2009). These results indicate that adults of the autumn-form respond to photoperiod after emergence and that under short photoperiods they remain in diapause. As short photoperiods induce the development of the autumn-form in the field in September and October in Tokyo and these butterflies mate the following March, it is unlikely that photoperiod plays a crucial role in the termination of diapause. Instead, low temperatures in winter and the subsequent increase in temperature in spring are more important in terminating diapause in this butterfly (Hiroyoshi & Reddy, 2018; Hiroyoshi et al., 2018). In addition to photoperiod, the temperatures pupae and/or adults experienced also affected diapause in *P. c-aureum*, as more mature eggs developed at 25°C than at 21°C under the critical photoperiods (Figs 1 and 2). More studies are needed, however, to determine the effects of the temperatures pupae and adults experience on adult diapause in this butterfly.

Diapause termination often requires certain periods of low temperatures and/or the changes in photoperiod, temperature or humidity (Beck, 1980; Tauber et al., 1986; Saunders, 2002). However, insects in adult diapause often emerge from diapause when exposed to long photoperiods in combination with high temperature in the laboratory, as is recorded for the shield bug *Eysarcoris ventralils* (Westwood) (Noda & Ishii, 1981) and monarch butterfly *Danaus plexippus* (L.) (Herman, 1973). At other times, some individuals in a population exhibit opportunistic diapause, such as in *P. c-album* (Shapiro, 1976) and *D. plexippus* (Herman, 1973). This strategy may result in an increase in the size of the overwintering population by enabling multivoltine species to continue reproducing late into a year.

The peak in the population of the autumn-form of *P. c-aureum* occurs in September or October in the fields around Tokyo, which coincides with when the host plant begins to bloom and the quality of its leaves decreases, which results in the larvae taking longer to complete their development and a decrease in adult size (Hiroyoshi & Reddy, 2018). As field temperatures and photoperiods gradually decrease in autumn, the risk of failing to complete their development before the onset of winter increases. Thus, opportunistic diapause, if it exists, in autumn-forms may be more advantageous than producing reproductive summer-forms in autumn. Although the various larval instars of *P. c-aureum* occur up to the middle of November in the fields around Tokyo, no reproducing summer-forms are recorded after the middle of October. While it is possible that the larvae found in late autumn are the offspring of autumn-forms, our field research indicates that all autumn-forms caught in autumn are in reproductive diapause (Hiroyoshi & Reddy, 2018). As the sample size and the number of assessments were both rather small, more detailed field studies are needed to clarify the possibility of autumn-forms reproduction in autumn.

In conclusion, as demonstrated in earlier and the present study, autumn-forms of *P. c-aureum* are produced under critical and short photoperiods, and those reared under critical photoperiods either do not enter diapause or enter a weak diapause. This explains why some of the autumn-forms reproduce in autumn. Further research is needed to elucidate the nature of the critical photoperiod underlying these mechanisms for measuring time.

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