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Effects of Aging, Temperature and Photoperiod on Testis Development of *Polygonia c-aureum* (Lepidoptera: Nymphalidae)

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Abstract. The effects of aging, photoperiod and temperature on testis development in the Asian comma butterfly *Polygonia c-aureum* were examined in connection with adult diapause and overwintering. Photoperiods during larval, pupal and imaginal stages did not exert pronounced influences on testis development, indicating that testis development is not influenced by diapause (i.e. controlled photoperiodically). Larval testes size increased with time until the end of the pupal stage, and thereafter began to decrease, especially during the first month of adulthood. When adults were kept under short day length (SD) and three different temperatures (15℃, 21℃ or 25℃) after adult eclosion, the rate of testis shrinkage was lowest at 15℃. Irrespective of timing (15 or 45 days of adult age) and duration (1, 2 or 3 months) of chilling at 5℃, the size of shrunken testis remained nearly constant throughout the post-chilling period at 21℃. Field examination also showed that testis size after overwintering as well as in late autumn before overwintering remained small. These results suggest that testis shrinkage ceases before or during overwintering. After ecdysed to the final (fifth) larval instar, a yellow membrane covered the testes and began to degenerate at the end of the pupal stage. The timing of yellow membrane degeneration coincided with the commencing of testis shrinkage, apyrene sperm movement from testis to post-testicular organs, and reduction of apyrene spermiogenesis. Therefore, it is suggested that there is a distinct possibility of the yellow membrane being involved in these reproductive events of the testis.

Key words: testis, sperm, reproduction, diapause, Lepidoptera, Nymphalidae.

Introduction

A cessation of reproductive function is characteristic for reproductive diapause in adult females (Beck, 1980; Tauber *et al.*, 1986; Pener, 1992; Kim, 1995). In contrast, reproductive diapause in adult males has not been adequately defined because the relationships between diapause and reproductive development in adult males vary considerably from species to species (Pener, 1992). In lepidopteran, it has been reported in two nymphalid butterfly species, i.e. *Danaus plexippus* (Herman, 1975, 1981) and *Vanessa cardui* (Herman & Dallmann, 1981), that the development of the accessory reproductive glands, tubular gland and ejaculatory duct is suppressed during imaginal diapause. Although it has been shown that males of *D. plexippus* have mature sperm during the winter (Hill *et al.*, 1976) and injections of JH and/or ecdysterone into *D. plexippus* do not bring about the significant change in the testes, unlike the three male reproductive organs mentioned above (Herman & Barker, 1976; Herman, 1981), it is still unclear whether imaginal diapause affects testis development.

A nymphalid butterfly, *Polygonia c-aureum*, has imaginal diapause and seasonal diphenism, i.e. summer form and autumn form, which show the distinct differences in morphology and reproduction. The summer form butterflies that emerged in summer begin to reproduce shortly after adult eclosion and are polyvoltine at least in the warmer regions of Japan. In contrast, the autumn form ones that emerged in autumn enter diapause and reproduce after overwintering. Both diapause induction and seasonal forms are determined mainly by photoperiod and temperature during the larval and pupal stages (Hidaka & Aida, 1963; Endo *et al.*, 1992). Long photoperiods and/or high temperatures favor the production of the summer form without diapause, whereas short photoperiods and low temperature favor the autumn form with diapause. My preliminary examinations revealed that imaginal diapause in both sexes of *P. c-aureum* was maintained under short photoperiods, but termi-

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nated under long photoperiods. Aging and temperature were also found to terminate diapause (Hiroyoshi, unpublished data).

The present study was undertaken to determine if aging, temperature and photoperiod affect testis development and to clarify the relationship between imaginal diapause and testis development in *P. c-aureum*.

Materials and Methods

Insects

A laboratory colony of *P. c-aureum* was established from summer form butterflies collected in the Tokyo Metropolis and Saitama Prefecture, central Japan in 1989, and maintained under LD (long day length; 15 : 9 h) and 21±1℃, where only summer forms were produced (Hiroyoshi, 1992).

To compare with the results obtained in the laboratory, field examinations were performed at Akigase Park, Saitama Prefecture in 1992. The results were described in the Discussion section.

Rearing

Immature insects were reared by the methods previously described (Hiroyoshi, 1997) unless otherwise stated. After adult eclosion, females and males were kept separately in wood-framed cages (17 cm×16.5 cm×46 cm) covered with net, in groups of 30 to 40. Ten percent sugar solution absorbed into cotton held in a glass dish (9 cm diameter) was provided for butterflies *ad libitum*, and replaced once a week. However, butterflies were not given a diet during the chilling periods at 5℃.

Environmental conditions

Hatchlings were reared at 21±1℃. They developed to summer form butterflies under LD (15 : 9 h) and to autumn form under SD (short day length; 8 : 16 h). In Experiments 1 to 4, hatchlings were reared under LD or SD photoregime and 21℃ until adult eclosion to determine the developmental periods and changes of body weight, coloration and size of testes followed by individual development. In the next series of experiments, newly emerged butterflies were incubated under the following environmental conditions. 1) In Experiment 5, summer form adults were kept under LD and 21℃, whereas autumn form adults were maintained under either LD or SD and 21℃. 2) In Experiment 6, autumn form adults were kept under SD and at 15, 21 or 25℃ for a month. 3) In Experiment 7, autumn form adults pre-incubated under SD and 21℃ for either 15 or 45 days were kept in complete darkness and at 5℃ for 2 months, and then returned to LD or SD and 21℃. 4) In Experiment 8, autumn form adults pre-incubated under SD and 21℃ for 30 days were kept in complete darkness and at 5℃ for either 1, 2 or 3 months, and then returned to LD or SD and at 21℃.

Individual development

In Experiment 1, hatchlings were individually reared in a plastic petri dish (9 cm dia. ×2 cm depth) under either LD or SD photoperiodic conditions and 21℃ until adult eclosion to determine the developmental period at each stage. The occurrence of molting was confirmed during the latter half of the photophase every day. Twenty males and 25 females under LD and 22 males and 21 females under SD were used in the experiments.

In Experiment 2, body weight was measured from day 0 of the fourth larval instar to emergence day of the adult using different individuals whose testes size were measured.

Measurement of testes size

In Experiments 3 to 6, a pair of testes or fused testis was dissected in a physiological saline consisting of 8.6 g NaCl, 0.1 g KCl, 0.33 g $CaCl_2$ and 1 *l* of distilled water. The length (L) and width (W) of each testis were measured with the aid of a calibrated ocular micrometer under a phase-microscope. Testes volume was calculated using the following formula: Volume $=\pi/6\times L_1\times W_1^2+\pi/6\times L_2\times W_2^2$ (L_1, W_1: left side testis, L_2, W_2: right side testis) (for unfused testes) or $\pi/6\times L\times W^2$ (for fused testis) assuming that the testis is an ellipsoid (Nishiitsutsuji-Uwo, 1959).

Results

Developmental period (Experiment 1)

Developmental periods from hatching to adult eclosion were compared for insects reared under a diapause-averting photoperiod (LD) and those reared under a diapause-inducing photoperiod (SD) at 21℃ (Table 1). In the first, second and third larval instar stages, there were no significant differences in the developmental periods of both sexes between the two photoperiods ($P>0.05$, by Mann–Whitney *U*-test). However, development times during the fourth and fifth larval instar stages under LD were significantly longer than those under SD in both sexes ($P<0.05$, by Mann–Whitney *U*-test). In contrast, the period during the pupal stage under LD was significantly shorter than that under SD in both sexes ($P<0.001$,

Table 1. Comparison of developmental periods at each stage in *P. c-aureum* between immatures reared under a long day length (LD) and under a short day length (SD) at 21℃.

Stage	Sex	Developmental period (Day)				*P* (LD vs SD)
		under LD		under SD		
		Range	Mean±S.D.	Range	Mean±S.D.	
Larva						
1st Instar	Male	3–4	3.5±0.5	3–8	3.7±1.2	0.7474
	Female	3–4	3.3±0.5	3–4	3.6±0.5	0.0901
	Subtotal	3–4	3.4±0.5	3–8	3.6±0.9	0.1639
2nd Instar	Male	2–4	2.9±0.7	2–4	2.9±0.4	0.8797
	Female	2–3	2.8±0.4	2–3	2.8±0.4	0.9361
	Subtotal	2–4	2.8±0.6	2–4	2.9±0.4	0.7939
3rd Instar	Male	2–4	3.3±0.6	3–4	3.4±0.5	0.5666
	Female	3–5	3.6±0.6	2–5	3.2±0.8	0.1180
	Subtotal	2–5	3.4±0.6	2–5	3.3±0.6	0.3846
4th Instar	Male	3–6	4.4±0.8	3–5	3.7±0.5	0.0045*
	Female	4–5	4.5±0.5	3–5	4.0±0.6	0.0107*
	Subtotal	3–6	4.4±0.7	3–5	3.9±0.6	<0.0001*
5th Instar	Male	5–7	5.6±0.6	4–5	4.8±0.4	<0.0001*
	Female	5–8	5.9±0.7	4–6	4.9±0.4	<0.0001*
	Subtotal	5–8	5.7±0.7	4–6	4.8±0.4	<0.0001*
Pre-pupa	Male	0–1	0.5±0.5	0–1	0.6±0.5	0.5592
	Female	0–1	0.6±0.5	0–1	0.6±0.5	0.6886
	Subtotal	0–1	0.5±0.5	0–1	0.6±0.5	0.5020
Pupa	Male	8–9	8.9±0.3	9–10	9.7±0.5	<0.0001*
	Female	8–10	8.8±0.6	9–11	9.9±0.5	<0.0001*
	Subtotal	8–10	8.8±0.5	9–11	9.8±0.5	<0.0001*
Total	Male	27–31	28.9±1.0	27–33	28.7±1.3	0.3885
	Female	27–31	29.4±1.3	26–30	28.7±1.2	0.0473*
	Subtotal	27–31	29.2±1.2	26–33	28.7±1.2	0.0457*

The asterisk shows a significant difference between LD and SD by Mann–Whitney's *U*-test.

by Mann–Whitney *U*-test). The total developmental period in males did not differ significantly between the two photoperiods ($P>0.05$, by Mann–Whitney *U*-test), but significant differences in females were observed ($P<0.05$, by Mann–Whitney *U*-test).

Body weight (Experiment 2)

The changes of body weight from the fourth larval instar to the day of adult eclosion were compared between the two photoperiods at 21℃ (Fig. 1). During day 0 to 2 of the fourth larval instar, body weight under LD and SD increased in the same way. Between the pharate fifth instar larval stage and day 6 of the pupal stage, except for day 2 of fifth larval instar ($P>0.05$, by Mann–Whitney *U*-test), body weights under LD were significantly heavier than those under SD ($P<0.05$, by Mann–Whitney *U*-test). On the day of adult eclosion, however, there was again no significant difference in body weight between the two photoperiods ($P>0.5$ by Mann–Whitney *U*-test). This seems likely to be caused by differences of the examination time. Adult eclosion concentrated on the first half of photophase, and body weight of two seasonal forms was measured at around 8 : 00 p.m., whereas light-on times under LD and SD conditions were different, i.e. 9 : 00 a.m. and 4 : 00 p.m., respectively. Consequently, some newly emerged autumn form adults had a great deal of meconium in their gut at the time of examination, whereas a majority of summer form adults had already excreted it. As the fore-wings of summer form males (N=19, Mean±S.D.=26.3±1.3) were significantly longer than those of autumn form males (N=11, Mean±S.D.=22.7±1.4) ($P<0.001$, Mann–Whitney *U*-test), it appears that body weight of the summer form just at adult eclosion was heavier than that of the autumn form.

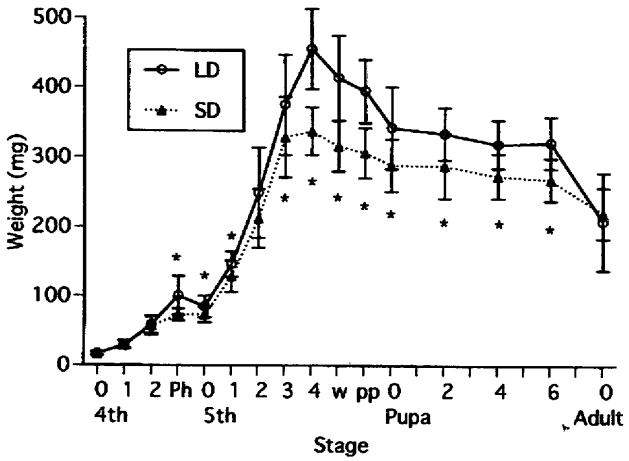

Fig. 1. Effects of photoperiod on body weight (mean± S.D.) of *P. c-aureum* during the larval, pupal and day 0 of adult stages at 21℃. The asterisk shows a significant difference at the 5% level by Mann–Whitney *U*-test for each day between LD (long day length) and SD (short day length). Each point represents 10 to 23 samples. In stages, numerals, Ph, W and PP indicate days, pharate fifth larval instar, wandering and prepupa, respectively.

Color changes of testis (Experiment 3)

The color changes of the testis were observed during the fourth and fifth larval instars, pupal and imaginal stages. The testis had two epitherial membranes: the outer membrane was transparent, while the inner membrane was pale red during the fourth larval instar. After ecdysed to the fifth larval instar, the inner membrane of the testis gradually turned red and the yellow membrane began to cover the testis. This yellow membrane was distinct, especially from the later phase of the fifth larval instar to the end of the pupal stage. When the yellow membrane was removed from the testis using forceps, the red inner membrane of the testis could be exposed easily. The yellow membrane began to degenerate from the end of the pupal stage. This membrane still remained in newly emerged males, then disappeared completely 10 days after adult eclosion (see Fig. 2), and never appeared again throughout their imaginal life. Such a color change of the testis occurred in the same way in both seasonal forms.

Testis development during larval and pupal stages (Experiment 4)

Testicular sizes in volume from the fourth larval instar to the day of adult eclosion were compared between males reared under LD and SD at 21℃ (Fig. 3). The testes under LD tended to be larger than those under SD from the wandering stage onwards, but the developmental pattern of testes under both

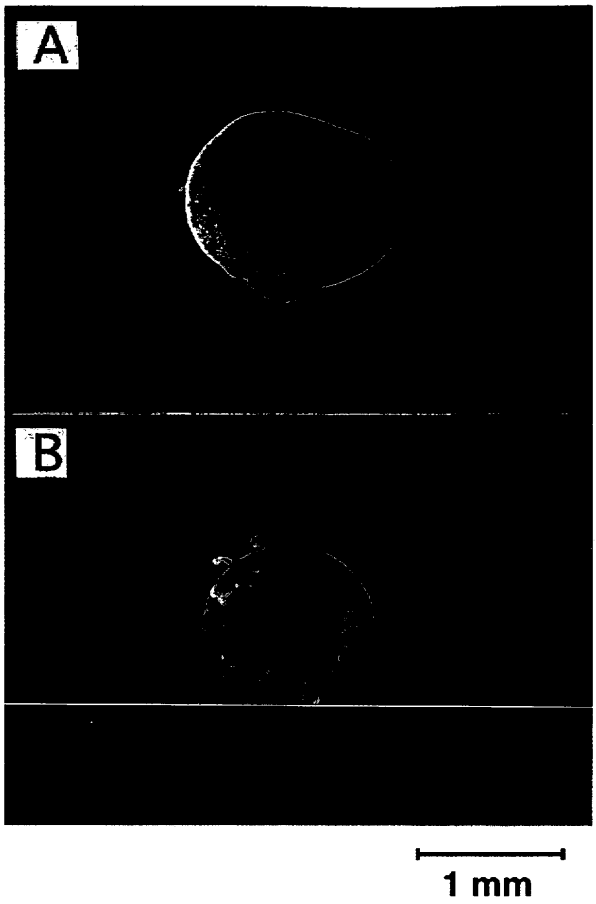

Fig. 2. Comparison of size and coloration of testis between day 0 (A) and day 10 (B) of the adult stage in the autumn form adult reared for SD (short day length) and 21℃.

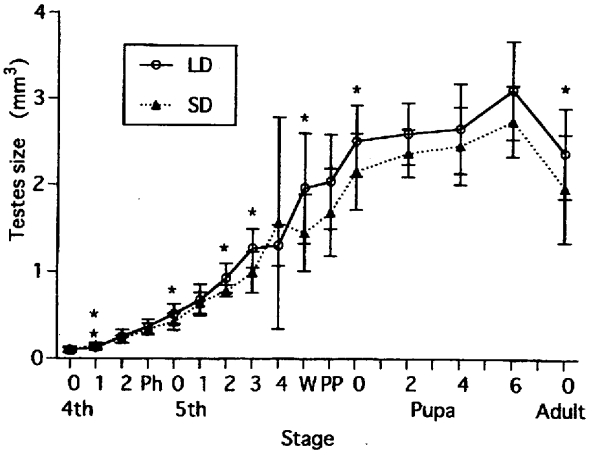

Fig. 3. Effects of photoperiod on the testis size (mean± S.D.) of *P. c-aureum* during the larval, pupal and day 0 of adult stages at 21℃. The asterisk shows a significant difference at the 5% level by Mann–Whitney *U*-test at each day between LD (long day length) and SD (short day length). Each point represents 10 to 23 samples. In stages, numerals, Ph, W and PP indicate days, pharate fifth larval instar, wandering and prepupa, respectively.

photoperiods was similar: testes size increased with age until the end of the pupal stage and thereafter began to decrease.

Testis development during adult stage (Experiment 5)

In Experiments 5 to 8, testis size in the adult stage was examined in relation to diapause and overwintering under various combinations of photoperiod and temperature. In Experiment 5, the changes of testis size of summer form adults kept under LD and autumn form adults under LD and SD at 21℃ were examined (Fig. 4). Testis size of both seasonal forms showed a marked decrease during the first 30 days of adulthood, continuously from the end of the pupal stage. Although testes of summer form adults tended to be larger than those of autumn form adults, the patterns of testis shrinkage were similar. Except for day 30 of the adult stage, no statistical differences were found in testis size between autumn form adults reared under LD and SD ($P>0.05$, by Scheffe's method after ANOVA). Also, there was no significant difference in testis size of summer form adults between LD and SD on day 30 ($P>0.05$ by Mann–Whitney U-test) (data not shown).

Effect of temperature on testis development (Experiment 6)

To determine the effect of temperature on the rate of testis shrinkage during the early adult stage, the newly emerged autumn form males were kept under SD and either 15, 21 or 25℃ for 30 days, and then testis size was examined (Table 2). Testes were largest at 15℃ among the three temperatures. Testes at

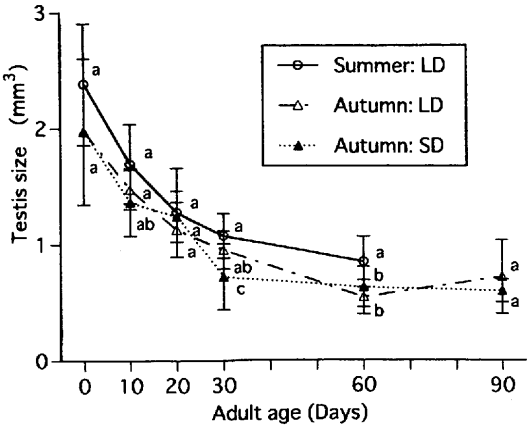

Fig. 4. Effects of photoperiod on testis size (mean± S.D.) of *P. c-aureum* with adult age in relation to seasonal forms. Means followed by different letters indicate a significant difference at the 5% level by Scheffs method after ANOVA. Each point represents 3 to 26 samples.

Table 2. Effects of temperature on testis development of *P. c-aureum* during the early adult stage under a short day length.

Temperature (℃)	No. of animals used	Testis size (mm^3) (Mean±S.D.)
15	19	1.158±0.272
21	19	0.720±0.284
25	6	0.823±0.212

all temperature regimes 30 days after adult eclosion were much smaller than on the day of adult eclosion as shown in Fig. 4. These results indicate that the rate of testis shrinkage in the early adult stage was reduced by low temperature.

Effect of timing of chilling on testis development (Experiment 7)

To examine the effect of timing of chilling on testis development in relation to overwintering, autumn form adults pre-incubated under SD and 21℃ for either 15 or 45 days after adult eclosion and chilled at 5℃ in darkness for a subsequent 60 days were then transferred to LD or SD at 21℃ (Fig. 5). From the results in Fig. 4, testis sizes on days 15 and 45 of the adult stage before chilling were supposed to be 1.2 mm^3 and 0.6 mm^3, respectively. After chilling, testis sizes of males pre-incubated for 15 and 45 days were 0.65 mm^3 and 0.5 mm^3, respectively. This indicates that testis size of the former decreased remarkably during chilling periods, whereas that of the latter decreased only slightly. In males pre-incubated for 15 days and chilled for 60 days, testis size did not change significantly over the next 30 days at 21℃ under both photoperiods (LD: $N=62, r^2=0.101, P=0.43$; SD: $N=75, r^2=0.013, P=0.34$), and there were no significant differences in testis size between the two photoperiods except 10 days after chilling ($P>0.05$ by Mann–Whitney *U*-test). In males pre-incubated for 45 days and chilled for 60 days, however, testis size showed no significant differences between LD and SD for any days during the post-chilling periods at 21℃ ($P>0.05$, by Mann–Whitney *U*-test). Although there was a weak significant correlation between testis size and days after the transfer to 21℃ under SD ($N=81, r^2=0.118, P<0.01$), the increase of testis size between days 0 and 30 during the post-chilling period was small.

Effect of chilling duration on testis development (Experiment 8)

To examine the effect of the chilling duration on testis development, autumn form adults pre-incubated

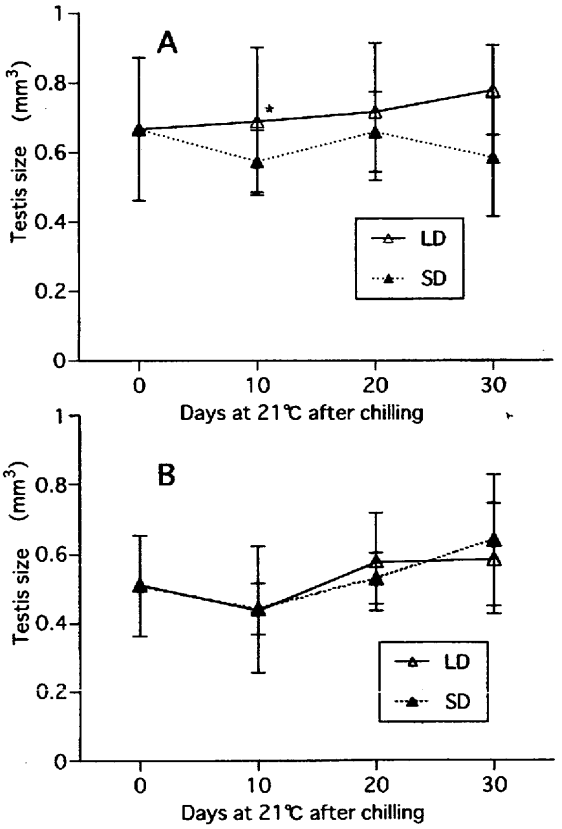

Fig. 5. Effects of chilling timing on testis size (mean± S.D.) of *P. c-aureum* upon transfer to 21℃. Male adults of the autumn form were pre-incubated at 21℃ for either 15 (A) or 45 (B), chilled at 5℃ for 60 days and then returned to 21℃. The asterisk shows a significant difference at the 5% level by Mann–Whitney *U*-test at each day between LD (long day length) and SD (short day length). Each point represents 10 to 28 samples.

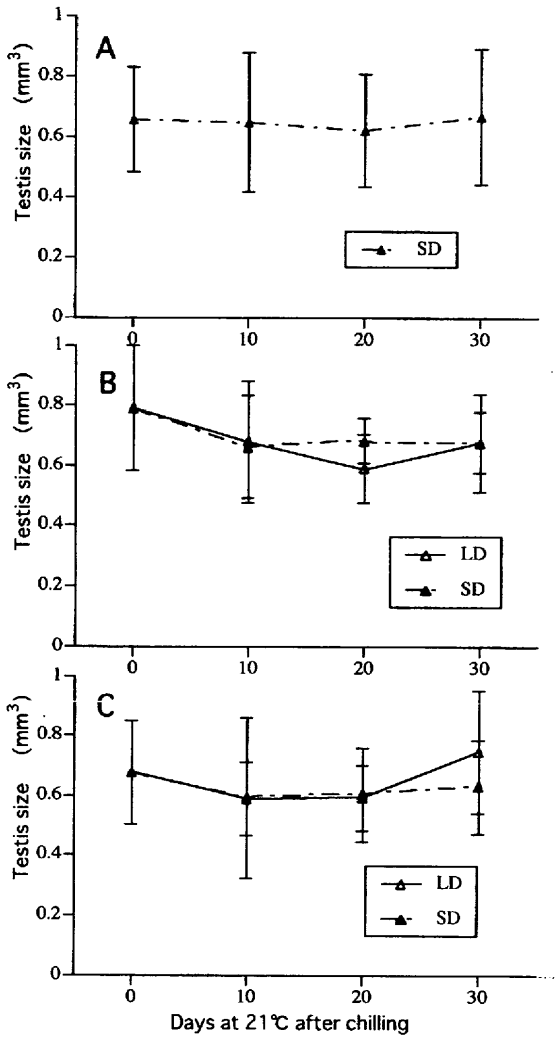

Fig. 6. Effects of periods of chilling on testis size (mean ±S.D.) of *P. c-aureum* upon transfer to 21℃. Male adults of the autumn form were pre-incubated at 21℃ for 30 days, chilled at 5℃ for either 1 (A), 2 (B) or 3 (C) months and then returned to 21℃. No significant differences were found between LD (long day length) and SD (short day length) by Mann–Whitney *U*-test in any experiments. Each point represents 9 to 21 samples.

under SD at 21℃ for a month after adult eclosion and chilled at 5℃ in darkness for either 1, 2 or 3 months were then transferred to LD or SD and 21℃ (Fig. 6). From the results shown in Figure 4, the testis size of autumn form adults under SD on day 30 of the adult stage before chilling was approximately 0.7 mm³. There were no significant differences among testis sizes before chilling and immediately after chilling for 1, 2 and 3 months (df=3, F=0.910, P=0.442, by ANOVA). This indicates that the testis did not shrink during the chilling period under these conditions. There were no correlations between testis size and days after the transfer to 21℃ after pre-incubation and chilling for a month under SD (N=48, r^2=0.0003, $P>0.05$), 2 months under LD (N=49, r^2=0.068, $P>0.05$) and SD (N=53, r^2=0.041, $P>0.05$), and 3 months under LD (N=65, r^2=0.032, $P>0.05$) and SD (N=58, r^2=0.007, $P>0.05$). Also, there were no significant differences between LD and SD for any day during the post-chilling periods at 21℃ after pre-incubation and chilling for 2 months ($P>0.05$ by Mann–Whitney *U*-test) and 3 months ($P>0.05$ by Mann–Whitney *U*-test).

Discussion

The present study has shown that the testes of summer form males in *P. c-aureum* tended to be larger than those of autumn form ones during the pupal and imaginal stages. This seems to be caused by the differences of body weight and size provided by the quantitative differences of food intake because the

duration of the final larval instar, during which they feed most voraciously, was one day longer under LD than under SD. Although there is no direct evidence, prolongation of this stage might result in heavy and large individuals as pupae and adults. Thus, one may argue that there is a relationship between body weight, photoperiod and seasonal forms, that is, short photoperiods produce light autumn form whereas long photoperiods produce heavy summer form. However, this relationship is altered depending on the length of photoperiod experienced during the larval stage; under intermediate photoperiodic conditions, no differences are found in body weight between pupae destined for either summer or autumn form (Hiroyoshi, 1992). In contrast, in *P. c-album,* closely related to *P. c-aureum,* the situation is somewhat different: development time of both larvae and pupae under SD is longer than that under LD, and pupae under SD are heavier than those under LD (Nylin, 1992). The ecological significance of modification in durations of the final larval instar and pupal stages due to the photoperiod in *P. c-aureum* is unclear.

The relationship between imaginal diapause and testis development differs greatly among insect species. For example, in the boll weevil *Anthonomus grandis* and the alfalfa weevil *Hypera postica,* the testes are small and spermatogenesis is suppressed during diapause in comparison with nondiapausing males (Brazzel & Newsom, 1959; Ascerno *et al.,* 1978). Moreover, in the green lacewing *Chrysopa carnea,* mature sperm are present in the testes, but no sperm are accumulated in the seminal vesicle during diapause (Macleod, 1967). In carabid beetle *Carabus yaconinus,* however, the testes mature irrespective of the occurrence of diapause (Sota, 1986). In *Draeculacephala crassicornis,* testis size increases during the aestival diapause, comparable to non-diapausing males (Reissig & Kamm, 1975). In Lepidoptera, it has been shown that testis development including spermatogenesis is suppressed during larval and pupal diapauses (Santa & Otuka, 1955; Cloutier & Beck, 1963; Numata & Hidaka, 1981; Friedländer & Benz, 1982), but no information is available for imaginal diapause except for a brief description in *D. plexippus* as mentioned above. In *P. c-aureum,* testis size during the larval, pupal and imaginal stages exhibited similar developmental patterns between diapausing and developing males (Figs. 3 and 4). Similar results were obtained also for spermatogenesis of this species (Hiroyoshi, 1999; Hiroyoshi, unpublished data). Therefore, I conclude that testis development of *P. c-aureum* is not affected by diapause.

Larval testes of *P. c-aureum* enlarged with the advance of individual development until the end of the pupal stage at 21℃, and then considerably shrank towards the earlier phase of adulthood (Figs. 3 & 4). Many reports reveal that a marked decrease of volume and weight of testis occurs during the pupal and adult stages in other lepidopterans, e.g. *Helicoverpa virescens* (Chase & Gilliland, 1972), *H. assulta* (Jeong & Kown, 1996), *Trichoplusia ni* (Holt & North, 1970), *Spodoptera litura* (Sridevi *et al.,* 1989), *Ostrinia nubilalis* (Chaudhury & Raun, 1966), *Manduca sexta* (Reinecke *et al.,* 1983), *Lymantria dispar* (Salama, 1976), *Boarmia selenaria* (Scheepens & Wysoki, 1985), *Papilio xuthus* (Nishiitsutsuji-Uwo, 1959), and *D. plexippus* (Herman *et al.,* 1989), although the timing of commencing testis shrinkage differs among species. Thus, testis shrinkage may be common in lepidopterans.

The progression of testis shrinkage in *P. c-aureum* adults exhibited complicated responses to aging and temperature. First, when autumn form adults were kept under SD and three different temperatures for the first 30 days of adulthood, testis shrinkage at a lower temperature (15℃) proceeded slowly compared with that at higher temperatures (21℃ and 25℃) (Table 2). Presumably, testis would be further shrunken at 15℃ if the length of incubation was further extended. However, I cannot rule out the possibility that a temperature of 15℃ was too low to feed sufficiently, leading to a failure of complete testis shrinkage. Second, the timing of chilling at 5℃ in darkness after pre-incubation at 21℃ did not affect testis size immediately after chilling (Fig. 5). Whenever chilling was started sooner or later, testis sizes immediately after chilling were similar to each other, but the rate of testis shrinkage was greater in males pre-incubated for 15 days than in those pre-incubated for 45 days. This indicates that testis shrinkage occurred even at 5℃ in darkness for a 60-day exposure, but ceased during the chilling period after reaching a peak. Third, the testis size of *P. c-aureum* did not change between before and after chilling, when the chilling was initiated 30 days after adult eclosion irrespective of the length of chilling, which ranged from 1 to 3 months (Fig. 6). This indicates that testis shrinkage ceased before chilling in this case and aging during the pre-incubation period acted intensively against testis shrinkage. Thus, the influence of temperature might be limited to the early adult stage. Finally, aging, photoperiods and the transfer to 21℃ during the post-chilling periods did not exert pronounced influences on testis size (Figs. 5 & 6). The data presented here suggest that both aging

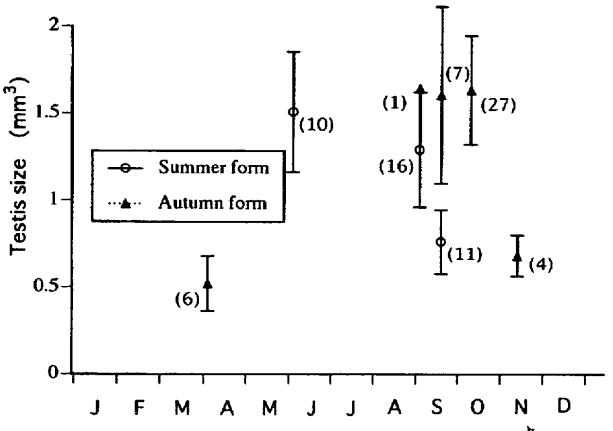

Fig. 7. Seasonal changes of testis size (mean±S.D.) of *P. c-aureum* collected in the field in 1992. The numerals in parentheses indicate the number of adults examined.

and temperature no longer affect testis size after overwintering because testis shrinkage is completed in the earlier phase of adulthood before and/or during overwintering. Thus, it appears that neither the season of adult emergence nor the timing of overwintering in autumn form males affects testis size after overwintering.

According to my field observations in Tokyo and Saitama, autumn form butterflies of *P. c-aureum* first appear as an autumn generation in September. Only a small number of summer form adults can be seen by the earlier days of October. Field examinations performed in 1992 (Fig. 7) showed that testes of summer form adults collected on 18 September tended to be smaller than those collected on 4 September. This suggests the possibility that the former was derived from old individuals that emerged in August or early September, but not those that emerged in mid-September, because the autumn form butterflies are likely to emerge in September due to short photoperiods and testis size decreases with adult age (Fig. 4). Similarly, testes of autumn form adults collected in November were much smaller than those collected in October. This also suggests that the former progressed in testis shrinkage due to aging. Although testis sizes of autumn form adults collected in September and October were nearly identical, these values in both populations were close to those in young males as shown in Fig. 4 and some proportions of those wild males possessed yellow or yellowish testis. This suggests that both populations were constituted with a young generation. Moreover, testes of autumn form adults collected in April after overwintering were small and similar to those collected in November, although the former originated from a previous year of population. In conclusion, the results obtained in the present laboratory study are in harmony with the results in the changes of testis size in the wild males collected during various seasons. Thus, testis size can be a good indicator for estimating ages in wild males.

In *P. c-aureum*, it was found that the yellow membrane began to cover the surroundings of the testis after ecdysed to the final larval instar. As the yellow membrane degenerated, the testis turned from yellow to red. However, it is evident that this color change of the testis is due to the disappearance of the yellow membrane, but it dose not provide any substantial modification in the testis because the testis itself was red if the yellow membrane was removed off. Such a false color change of the testis from yellow to red after adult eclosion is also reported in a hesperid butterfly *Calpodes ethlius* (Lai-Fook, 1982). The existence of the yellow membrane in distant taxon such as Hesperidae or Nymphalidae may reveal the possibility that the color change of the testis due to the disappearance of the yellow membrane is common among butterfly species, although the coloration of the testis differs among butterfly species (Hiroyoshi, unpublished observation).

The mechanism controlling testis shrinkage is still unknown. Interestingly, however, the timing of starting the testis shrinkage in *P. c-aureum* coincides well with commencing apyrene sperm movement (Hiroyoshi, 1997), reduction of apyrene spermiogenesis (Hiroyoshi, 1999), and disappearance of the yellow membrane (this study). This raises an interesting hypothesis that the yellow membrane is involved in these reproductive events because the disappearance of the yellow membrane would allow the influences of certain factors in hemolymph for the testis.

Physiological events in the testis are known to relate to ecdysteroids (Dumser, 1980; Raabe, 1986). Application of ecdysteroids *in vivo* and *in vitro* promotes spermatogenesis (Schmidt & Williams, 1953; Takeuchi, 1969; Kambysellis & Williams, 1971, 1972; Takeda, 1972; Dumser & Davey, 1975; Kiss & Williams, 1976; Rungger-Brändle, 1976) and testis fusion (Nowock, 1972), although many other studies indicate that spermatogenesis progresses without relation to ecdysteroid in hemolymph (Mitsui *et al.*, 1976; Shimizu *et al.*, 1988, 1989). Yagi and Tanaka (1992) suggest that testes atrophy of the armyworm by parasitization of an endoparasitoid wasp is caused by the loss of responsiveness to ecdysteroids. Furthermore, a decline in hemolymph ecdysteroid levels is considered to be essential for initiating sperm movement from testis to post-testicular organs during the late phase of

the pupal stage (Thorson & Riemann, 1982; Shimizu, 1989; Giebultowicz *et al.*, 1990). It is possible, therefore, that the rise and fall of ecdysteroids titer in hemolymph during the pupal stage may be a trigger for the reproductive events in the testis as mentioned above. The validity of this hypothesis will be verified with a future experiment that designs an *in vitro* culture of testis with or without ecdysteroids and the yellow membrane.

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