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Effects of photoperiod on the testis fusion in the Asian comma butterfly, *Polygonia c-aureum* Linnaeus (Lepidoptera: Nymphalidae)

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ABSTRACT: The progress of testis fusion in the immature stages ranging from the 4th larval instar larvae to the pupae in *Polygonia c-aureum* was compared between two photoperiods (short-daylength and long-daylength). In this butterfly, imaginal diapause induction is controlled mainly by photoperiod and temperature during the immature stages. The study investigated the relationship between the imaginal diapause and testis fusion. The results showed that photoperiod did not exert significant effects on the process of testis fusion, indicating that testis fusion does not relate to the imaginal diapause. A pair of testes fused to a single testis during the prepupal stage and testis torsion occurred shortly after pupation. However, though in rare cases, a few male adults which had been reared in the laboratory and caught in the field had two testes, suggesting no occurrence of testis fusion during the prepupal stage.

KEY WORDS: Imaginal diapause, male, reproduction, testis fusion, torsion

INTRODUCTION

Numbers and morphology of testis in males considerably vary among insect species. In Lepidoptera, males have a pair of testes during the larval stage, and testes generally fuse to a single testis during the prepupal stage or the pupal stage as listed in Table 1.For example, in *Ostrinia nubilalis* (Parker and Thompson, 1926) and *Boarmia slenaria* (Scheepens and Wysoki, 1985), testis fusion occurs in prepupal stage, whereas in *Corcyra cephalonica* (Deb and Chakravorty, 1981) and *Papilio xuthus* (Numata and Hidaka, 1981), testis fusion occurs in pupal stage. However, in several moth species such as some Saturnids (Szöllösi, 1982) and *Bombyx mori* (Ômura, 1936), the testes do not fuse through their life span so that an adult has two testes.

P. c-aureum exhibits seasonal diphenism on their morphology, coloration of wings and reproductive manner, i.e. the summer form and autumn form. The summer form butterflies emerged in summer begin to reproduce shortly after emergence, while the autumn forms emerged in autumn induce an imaginal diapause and reproduce in spring followed by overwinter. Both the seasonal form and diapause induction are determined mainly by photoperiod and temperature during the immature stages: long daylength and/or high temperature favour the developing summer form, whereas short daylength and relatively lower temperature diapausing autumn

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form (Hidaka and Aida, 1963; Hidaka and Takahashi, 1967).

The relationship between diapause and testis fusion is less understood. In the case of pupal diapause, testis fusion in the diapausing pupae of *Papilio xuthus* is delayed as compared to the direct developing pupae (Numata and Hidaka, 1981). However, it is unknown whether or not imaginal diapause affects the timing of testis fusion. Pupal diapause is controlled by ecdysteroids, whereas imaginal diapause is controlled by juvenile hormones. As pupal periods of *Polygonia c-aureum* differ between short and long photoperiods (Hiroyoshi, 1992), it is possible that the process of testis fusion may differ between the prepupa or pupa destined to the developing adults and those destined to imaginal diapausing adults. In the present study, the process of testis fusion was examined in detail and compared between short and long photoperiodic conditions in imaginal diapausing species for the first time, and the existence of unfused testes both in the laboratory-reared and wild male adults of *P. c-aureum* was revealed in the butterfly.

MATERIALS AND METHODS

P. c-aureum larvae were collected at Tokyo Metropolis and Saitama Prefecture, Japan in 1989 and had been maintained as a laboratory stock colony under long daylength (15L9D) at 21±1^{o}C in their life span. The feral male adults were collected at Saitama Prefecture in the various seasons of 1991 and 1992. To examine the relationships between testis fusion and diapause, immatures were reared under either short daylength (8L16D) or long daylength (15L9D) at 21±1^{o}C. Adults were reared under the various combinations of photoperiods (short daylength, long daylength or constant darkness) and temperatures (5^{o}C, 21^{o}C or 25^{o}C) differing in the period and timing of incubation. After dissection of adults used in various experiments, they were counted the testis number.

The number of testis was examined in the summer and autumn form adults reared under the laboratory and caught in the field. A pair of testes or a single testis was dissected out in a lepidopteran physiological saline (consisting of 8.6g NaCl, 0.33g $CaCl_2$ and 0.1g KCl and made up to 1 liter with distilled water) under a binocular stereomicroscope. The coloration of the lateral sides of which testes closely located each other was observed. More intense reddish area than its surrounding area on the lateral sides of testes were regarded as the part of testes contacted. The progress of testis fusion was divided into five grades by the following features due to the rate of the reddish area occupied on the lateral sides of testes: 1) nothing, 2) less than one third, 3) ranging from one third to less than half, 4) ranging from half to almost complete, 5) complete. The data on the degree of testis fusion were scored from 1 to 5 and analyzed by Mann Whitney's *U*-test to compare between short and long daylength.

In lepidopteran insects, it is known that after testis fusion the whole testis twists where this phenomenon is called as testis torsion. If the testis experienced the testis torsion, a single testis could not be separated into two testes with a pair of forceps. Therefore, separation of testis was attempted to determine if testis torsion occurred.

RESULTS

The progress of testis fusion was examined during the later larval and pupal stages. A series of the process of testis fusion was identified between short and long photoperiods (Table 2). There were no significant differences in testis fusion between two photoperiods at any stages and substages ($p>0.05$,by Mann Whitney's *U* test). The 4th instar larvae had a pair of testes that was closely located on the dorsal midline of the 8th abdominal segment. The rate of individuals having intense reddish areas with the shape of long circle on the central part of lateral side of testes increased with advancing the stage after the ecdysis to the fifth instar larvae. The intense reddish areas of each side of testis abruptly increased in size around at the wandering stage and then expanded to the whole lateral sides of both testes, contacting each other at the prepupal stage. Immediately after pupation, all pupae showed the complete fusion of the testes, indicating that testis fusion occurred during the prepupal stage.

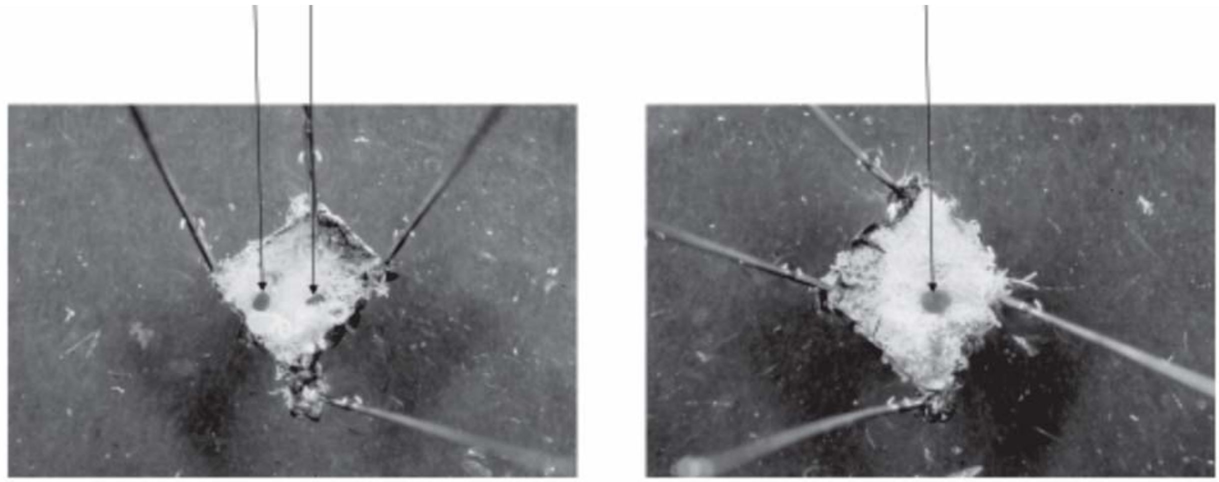

Fig. 1 Photographs of testis in *P. c-aureum* adult. Unfused testes in summer form collected from the field (left side) and fused testes in autumn form reared in the laboratory (right side). Arrows indicate the testis.

On the day of pupation, the testis was easily separated each other with an artificial manipulation, while on day 2 of pupae the testis was no longer separated. This reveals that testis torsion occurs between day 0 and day 2 after pupation.

In laboratory-reared insects, 5 out of 1390 adults (0.4%) examined had two testes. Also, 1 out of 143 wild adults (0.7%) showed two testes (Fig. 1). Since any larvae and pupae never had four testes, the existence of two testes seen in the adult implies that testis fusion did not occur in such individuals during the prepupal stage.

Most fused testes took the shape of sphere or like that, but some testes long and slender. On the other hand, the morphology of the testes that did not fuse differed among individuals and either right or left side of the testis: most testes took the shape of semi-sphere, but a few showed the shape of sphere as if it fused. All adults without testis fusion normally, at least seemingly, emerged and exhibited the normal development of the reproductive organs except for the testis fusion. One out of two adults without testis fusion examined showed the normal progress of spermiogenesis (data not shown): In the other individual, one testis had many two types of sperm, i.e. eupyrene and apyrene sperm, whereas the other one had a relatively less amount of apyrene sperm and few eupyrene sperm, indicating the abnormal spermatogenesis.

DISCUSSION

The present study demonstrated tha the paired testes of *P. c-aureum* fused into a single testis during the prepupal stage like other many lepidopteran species. However, the present study also showed that a few adults successively reared in the laboratory had a pair of testes that did not appear to fuse. It seems unlikely that the phenomenon that testes did not fuse may be caused by the abnormality of laboratory rearing conditions, because even an adult caught in the field had paired testes. Untestis fusion spreads over the butterfly species (Kato and Hiroyoshi, unpublished data) so that untestis fusion seen in *P. c-aureum* might have an evolutionary importance.

Testis fusion in *P. c-aureum* between short and long photoperiodic regimes occurs in the same way (Table 2). Thus, testis fusion has nothing to do with the seasonal form and diapause expressed in the adult stage, because the seasonal form and imaginal diapause are controlled by photoperiod and temperature. Butterflies with fused testis showed normal behavior including spermatogenesis and mating. This is the first report on the comparison of testis fusion between immatures destined for developing summer form and ones destined for diapausing autumn form adults in relation to imaginal diapause. Similarly, the phenomenon on

Table 1. Examples of testes fusion in various families of lepidopteran insects in the literature

Scientific name	No. of adult testis	Stage of testis fusion	Reference
Tineidae			
Corcyra cephalonica	1or2(rare)	pupa	Deb and Chakravorty (1981)
Gelechiidae			
Pectinophora gossypiella	1	?	LaChance et al. (1977)
Phthorimaea operculella	1	?	Brits (1978)
Hyponomeutoidea			
Acrolepiopsis assectella	1	?	Thibout (1979)
Xylorytinae			
Opisina arenosella	1	prepupa	Santhosh Babu and Prabhu (1989)
			Santhosh Babu (1995)
Tortricidae			
Choristoneura fumiferana	1	pupa	Outram (1970)
	1	prepupa	Retnakaran (1970)
Rhyacionia buoliana	1	prepupa?	Shen and Berryman (1967)
Gretchena bolliana	1	?	Tedders and Osburn (1970)
Laspeyresia caryana	1	?	Tedders and Calcote (1967)
L. pomonella	1	prepupa	Ferro and Akre (1975)
	1	prepupa	Giebultowicz and Brooks (1998)
Pyralidae			
Dioryctria abietella	1	?	Fatzinger (1970)
Ostrinia nubilalis	1	prepupa	Parker and Thompson (1926)
	1	prepupa	Jones et al. (1984)
	1	?	Chaudhury and Raun (1966)
Palpita unionalis	1	prepupa	Santorini and Vassilaina-Alexopoulou(1976)
Diatraea saccharalis	1	pupa?	Virkki (1963)
D. grandiosella	1	?	Davis (1968)
Ephestia kühniella	1	prepupa	Nowock (1972)
	1	prepupa	Musgrave (1937)
Bombycidae			
*Bombyx mor*i	2	no fusion	Ômura (1936)
Saturniidae			
Saturniidae0SÿPÿ.	2	no fusion	Cook (1910)
Platysamia cynthia	2	no fusion	Szçllçsi (1982)
Sphingidae			
*Manduca sext*a	1	prepupa	Reinecke et al. (1983)
Agrius convolvuli	1	pupa	Kubo-Irie et al. (2011)

Scientific name	No. of adult testis	Stage of testis fusion	Reference
Geometridae			
*Boarmia selenari*a	1	prepupa	Scheepens and Wysoki (1985)
	1	prepupa	Scheepens and Wysoki (1986)
Lymantriidae			
Lymantria dispar	1	prepupa	Rule et al. (1965)
	1	prepupa	Salama (1976)
Arctiidae			
Hyphantria cunea	1	prepupa	Sugai and Teramine (1970)
Noctuidae			
Acronycta sp.	1	pupa	Cook (1910)
Heliothis virescens	1	prepupa	Vinson et al. (1969)
	1	prepupa	Chen and Graves (1970)
	1	prepupa	Chase and Gilliland (1972)
Heliothis zea	1	?	Callahan (1958)
	1	?	Callahan and Chapin (1960)
Helicoverpa assulta	1	prepupa	Hoon et al.(2001)
Mamestra brassicae	1	prepupa	Santa and Otuka (1955)
Trichoplusia ni	1	pupa	Holt and North (1970)a
	1	pupa	Holt and North (1970)b
Spodoptera litura	1	prepupa	Sridevi et al. (1989)
	1	?	Etman and Hooper (1979)
S. littoralis	1	?	Haines (1981)
	1	prepupa	Gelbiè and Metwally (1981)
Achaea janata	1	prepupa	Sukumr (1985)
Plathypena scabra	1	prepupa	Buntin and Pedigo (1983)
Hesperiidae			
Calpodes ethlius	1	prepupa?	Lai-Fook (1982)
Papilionidae			
Papilio xuthus	1	pupa	Numata and Hidaka (1981)
P. cresphontes	1	pupa	Cook (1910)
Pieridae			
Pieris brassicae	1	prepupa	Junnikkala (1985)
Nympahlidae			
Danaus plexxipus	1	?	Herman (1975)
D. chrisippus	1	?	Screen and Sharna (1995)

Table 2. Comparison of testes fusion between short daylength and long daylength during the larval prepupal and pupal stages in male *P. c-aureum*

Stage	No. of animals used	Grade of testis fusion (%) Short daylenght I	II	III	IV	V	No. of animals used	Grade of testis fusion (%) Long daylength I	II	III	IV	V	U1 Value	U2 Value	W Value	SD vs LD P Value
Larva																
4th-0	16	62.5	37.5	0	0	0	16	81.3	18.8	0	0	0	280	232	368	0.4841
4th-1	14	50	50	0	0	0	13	84.6	15.4	0	0	0	122.5	59.5	150.5	0.0614
4th-2	18	5.6	94.4	0	0	0	23	0	100	0	0	0	195.5	218.5	366.5	0.2583
Sleeping	23	0	23	0	0	0	17	0	100	0	0	0	195.5	195.5	348.5	1
5th-0	22	0	100	0	0	0	21	0	85.7	9.5	4.8	0	198	264	495	0.0694
5th-1	18	0	100	0	0	0	21	0	90.5	4.8	4.8	0	171	207	342	0.1847
5th-2	21	0	85.7	9.5	4.8	0	22	0	90.9	9.1	0	0	244	218	475	0.57
5th-3	25	0	84	16	0	4	19	0	78.9	15.8	5.3	0	223.5	251.5	441.5	0.6204
5th-4	12	0	66.7	25	8.3	0	21	0	61.9	19	19	0	116	136	194	0.6614
Wandering	22	0	22.7	27.3	45.5	4.5	17	0	29.4	17.6	52.9	0	193.5	180.5	333.5	0.8423
Prepupa	21	0	0	14.3	61.9	23.8	18	0	0	11.1	44.4	44.4	151	227	398	0.2329
Pupa																
0	17	0	0	0	0	100	17	0	0	0	0	100	144.5	144.5	297.5	1
-2	13	0	0	0	0	100	10	0	0	0	0	100	65	65	120	1
-4	15	0	0	0	0	100	12	0	0	0	0	100	90	90	168	1
-6	14	0	0	0	0	100	10	0	0	0	0	100	70	70	125	1
4th instar	71	25.4	74.6	0	0	0	69	34.8	65.2	0	0	0	2976	3027	5442	0.9124
5th instar	120	0	72.5	12.5	10	0.8	121	0	74.4	12.4	13.2	0	7079.5	7440.5	14339.5	0.6574
prepupa	21	0	0	14.3	61.9	23.8	18	0	0	11.1	44.4	44.4	151	227	398	0.2329
pupa	59	0	0	0	0	100	49	0	0	0	0	100	1445.5	1445.5	2670.5	1

the testis, such as apyrene spermiogenesis (Hiroyoshi, 1999), testis shrinkage, disappearance of yellow membrane surrounding the testis (Hiroyoshi, 2000), and sperm movement (Hiroyoshi, 1997), did not differ between developing and diapausing individuals except for the quantitative differences: the testis of summer form grown under a long daylength is larger than that of autumn form grown under a short daylength (Hiroyoshi, 2000). These events on testis seem to have common endocrinological mechanisms, for example, the involvement of ecdysteroids, because the decrease of apyrene spermatogenesis, degradation of yellow membrane surrounding the testis, onset of testis shrinkage, and onset of apyrene sperm movement start with the same time, at the end of pupal stage.

Nowock (1972) demonstrates that the testis fusion was promoted by ecdysone in *Ephestia kuhniella*. In *P. c-aureum*, it does not seem likely that the occurrence of unfused testes does not appear to be provoked by the abnormality of synthesis and secretion of ecdysone in the prothoracic gland, because the individuals that did not fuse a pair of testes normally moulted, eclosed and developed the reproductive development except for the testis fusion. Probably, the occurrence of unfused testes may be induced by the abnormality of any step during the course of testis fusion. Alternatively, mutation on testis fusion might occur. Further study is needed to elucidate whether the ecdysteroid titer in hemolymph may affect the events around the testis or not.

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