Effects of Photoperiod and Age on the Initiation of Sperm Movement in Male *Polygonia c-aureum* LINNAEUS (Lepidoptera: Nymphalidae)

Satoshi HIROYOSHI

*Laboratory of Applied Entomology, Faculty of Agriculture,
Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan*

(Received 5 December 1995; Accepted 29 July 1996)

The time of sperm movement was examined in pupae of *Polygonia c-aureum* in relation to seasonal form and imaginal diapause. Although the duration of the pupal stage was one day shorter under long day-length than under short day-length, the initiation of sperm movement and the progression of adult development of summer-form pupae also occurred one day earlier than autumn-form pupae. Apyrene sperm movement started two days before adult eclosion and eupyrene sperm movement started the day before eclosion. No significant difference was observed in the pattern of sperm movement between the two types of pupae. It is concluded that the initiation of sperm movement is not influenced by the seasonal form and the presence or absence of imaginal diapause.

Key words: photoperiod, *Polygonia c-aureum*, sperm movement, adult development, seasonal form

INTRODUCTION

The process of sperm movement from the testis to the duplex via the vas deferens has been previously studied from various aspects in Lepidoptera. For example, it has been reported that sperm movement starts before adult eclosion in *Anagasta kuhniella* (RIEMANN et al., 1974), *Bombyx mori* (KATSUNO, 1977 a, b), *Lymantria dispar* (GIEBULTOWICZ et al., 1988) and *Mamestra brassicae* (SHIMIZU, 1989). Sperm is released from the testis in a daily rhythmic fashion (RIEMANN et al., 1974; LACHANCE et al., 1977; THORSON and RIEMANN, 1977; RIEMANN and THORSON, 1978) and this rhythm is of an endogenous circadian nature (GIEBULTOWICZ et al., 1988, 1989). In some species (LACHANCE et al., 1977; PROSHOLD, 1991), the number of eupyrene sperm bundles stored in the duplex increases linearly with age. However, no study has examined sperm movement in relation to imaginal diapause.

The nymphalid butterfly, *Polygonia c-aureum*, has a seasonal diphenism, i.e. summer form and autumn form, in the adult stage. This seasonal form is mainly determined by photoperiod and temperature during the larval stage: the summer form is produced under long day-length and/or high temperatures, whereas the autumn form is produced under short day-length and relatively low temperatures (HIDAKA and AIDA, 1963). Summer forms reproduce shortly after eclosion, whereas autumn forms enter imaginal diapause and reproduce the following spring (FUKUDA and ENDO, 1966). However, we know little about male reproduction in this species, except that mating behavior is suppressed in diapause males of the autumn form and their corpora allata are inactive, as in the case of diapausing females (ENDO, 1973), and that specific sperm movement occurs at mating (HIROYOSHI, 1995). It is unknown whether males of the two seasonal forms differ in reproductive characteristics, such as spermatogenesis and sperm movement.

HIROYOSHI (1992) reported that adults of *P. c-aureum* took approximately one day less to eclose from pupae destined for summer form butterflies (S-pupae) than from pupae destined for autumn form butterflies (A-pupae). A similar result has also been obtained for *P. c-album*, a species closely related to *P. c-aureum* (NYLIN, 1992). These findings suggest that the two forms are physiologically different in certain developmental traits even before adult eclosion.

The present investigation was carried out to determine the relationship between adult development and the timing of initiation of sperm movement in male *P. c-aureum* by comparing pupae of the two seasonal forms.

MATERIALS AND METHODS

Animals. Larvae of *P. c-aureum* were collected in Tokyo Metropolis and Saitama Prefecture in 1989 and successively reared in the laboratory. Summer form adults were obtained under long-day conditions (15L–9D) at $21 \pm 1^\circ\text{C}$ and their offspring were used for experiments. Pupal ecdysis was checked every hour in the latter half of the photophase and only those individuals which pupated during that period were used for observations 6 to 9 days after pupal ecdysis.

Rearing. Eggs collected from a laboratory culture were surface-sterilized with ca. 3% formaldehyde solution for 30 min, washed with tap water, air-dried, and placed in a plastic Petri dish (9 cm dia. $\times$ 2 cm depth) until hatching. Newly emerged larvae were placed on filter paper in a glass Petri dish (15 cm dia. $\times$ 4 cm depth or 12 cm dia. $\times$ 3 cm depth), and fed fresh leaves of *Humulus japonicus* (MORACEAE) in groups of 30 to 40 individuals. Rearing density was gradually reduced after ecdysis to the penultimate (4th) larval instar. Each pupa was placed vertically to facilitate adult eclosion in a plastic Petri dish (9 cm dia. $\times$ 2 cm depth).

Environmental conditions. Hatchlings were held at either long day-length (15L–9D) or short day-length (8L–16D) and $21(\pm 1)^\circ\text{C}$. Only summer forms were produced at the former photoperiod and autumn forms at the latter.

Sperm movement. The quantity of sperm in each part of a pair of the vasa deferentia (upper, middle, or lower part) and in the duplex was determined to monitor the initial process of sperm movement. Lepidopteran insects have two distinct types of sperm: a eupyrene sperm (a large nucleate spermatozoa) and an apyrene sperm (a small anucleate spermatozoa). An apyrene sperm bundle dissociates into single sperm when it is released from the testis into the vas eferens, but eupyrene sperm remains as a bundle within the male reproductive organs (KATSUNO, 1977 a). The number of eupyrene sperm bundles can be counted easily, but it is difficult to count the number of apyrene sperm. In this study, apyrene sperm was quantified using an index by observing three to five randomly selected fields under a phase contrast microscope: 0, no sperm; 1, sperm can be seen in the field selected; 2, a few sperm can be seen in every fields; 3, more sperm are present and the number can be counted; 4, sperm are too numerous to count unless the sample is diluted.

Adult development. Development of wings was examined to stage pupae during the latter half of the pupal stage. Wing development was divided into five grades by the following features: 0, wings are small and white in color except for the upperside which is pale orange; 1, wings are large and fully occupy the wing cases. Ruggedness in a marginal portion of the wings becomes distinct; 2, orange color on the upperside of wings becomes

clear; 3, areas with dark orange color appear on the upperside of wings; 4, either reddish brown or light brown background color and black spots on upperside of wings become distinct.

Relationship between sperm movement and adult development. In order to clarify the relationship between sperm movement and adult development, the furthestmost regions to which the two types of sperm moved from the testis were compared at each stage of wing development. The movement of sperm was followed by dividing the sperm pathway into five regions: testis, upper vas deferens, middle vas deferens, lower vas deferens, and duplex.

RESULTS

Sperm movement

The timing of initiation of sperm movement was compared between S-pupae and A-pupae. Results on apyrene sperm movement are shown in Table 1. In S-pupae, no apyrene sperm were seen in the vas deferens or the duplex on the 6th day of the pupal stage. On the 7th day, apyrene sperm was found in the vas deferens, but the median value of the apyrene sperm index tended to decrease with increased distance from the testis. On the 8th day, all or most males had apyrene sperm in the upper and middle vas deferens, but only 50% and 60% of pupae had apyrene sperm in the lower vas deferens and the duplex, respectively. In A-pupae, apyrene sperm appeared one day later than in S-pupae, but the pattern of apyrene sperm movement was similar in the two types of pupae.

The results on eupyrene sperm movement are shown in Table 2. In S-pupae, no eupyrene sperm bundles were observed in the post-testicular organs on the 6th and 7th days of the pupal stage. On the 8th day, however, bundles were seen in the upper and middle vas deferens as well as in the duplex. In A-pupae, eupyrene sperm bundles were first found in the post-testicular organs on the 9th day, one day later than in S-pupae.

Table 1. Progression of apyrene sperm movement with the advance of pupal age under two photoperiodic regimes at $21(\pm 1)^{\circ}\text{C}$

Photoperiod	Age	No. animals used	Median of index number of apyrene sperm ^a (% of males with apyrene sperm)				
			Vas deferens			Duplex	
			Upper	Middle	Lower		
Long day-length	6	10	0 (0)	0 (0)	0 (0)	0 (0)	
	7	10	2.0 (60)	0 (40)	0 (30)	0 (10)	
	8	10	2.0 (100)	2.0 (90)	0.5 (50)	2.5 (60)	
Short day-length	6	10	0 (0)	0 (0)	0 (0)	0 (0)	
	7	10	0 (0)	0 (0)	0 (0)	0 (0)	
	8	10	0 (40)	0 (30)	0 (10)	0 (0)	
	9	10	2.5 (100)	2.0 (100)	0.5 (50)	0 (30)	

^a The index number was determined by observing three to five randomly selected fields under a phase contrast microscope as follows: 0, no sperm; 1, few; 2, a few; 3, many; 4, too many to count.

Table 2. Progression of eupyrene sperm movement with the advance of pupal age under two photoperiodic regimes at $21(\pm 1)^{\circ}\text{C}$

Photoperiod	Age	No. animals used	No. of average eupyrene sperm bundles (% of males with eupyrene sperm)				
			Vas deferens			Duplex	
			Upper	Middle	Lower		
Long day-length	6	10	0 (0)	0 (0)	0 (0)	0 (0)	
	7	10	0 (0)	0 (0)	0 (0)	0 (0)	
	8	10	0.7 (10)	0.7 (10)	0 (0)	0.9 (20)	
Short day-length	6	10	0 (0)	0 (0)	0 (0)	0 (0)	
	7	10	0 (0)	0 (0)	0 (0)	0 (0)	
	8	10	0 (0)	0 (0)	0 (0)	0 (0)	
	9	10	0 (0)	0 (0)	0 (0)	0.8 (10)	

Table 3. Progression of wing development with the advance of pupal age under two photoperiodic regimes at $21(\pm 1)^{\circ}\text{C}$

Photoperiod	Age	No. animals used	Grade of wing development ^a					Median
			0	1	2	3	4	
Long day-length	6	10	0	9	1	0	0	1.0
	7	10	0	5	2	2	1	1.5
	8	10	0	0	0	1	9	4.0
Short day-length	6	10	10	0	0	0	0	0
	7	10	10	0	0	0	0	0
	8	10	0	2	8	0	0	2.0
	9	10	0	0	2	7	1	3.0

^a Wing development was graded ranging from the immature stage 0 to the mature stage 4. See the classification of grades in Materials and Methods.

Adult development

In S-pupae, the first sign of wing development was noticed on the 7th day of the pupal stage (Table 3). Most pupae reached grade 4 on the 8th day when the seasonal form could be identified by coloration. In A-pupae, on the other hand, wing development appeared to occur slightly later than in S-pupae. Only one individual attained grade 4 by the 9th day.

Relationship between sperm movement and adult development

Apyrene sperm reached the upper, middle or lower vas deferens even when adult development was still at an early phase in both S- and A-pupae (Table 4). As adult development proceeded, the median values of the furthestmost region where apyrene sperm reached from the testis increased significantly in S-pupae (KENDALL's $\tau = 0.98$, $z = 5.28$, $p < 0.001$) and in A-pupae (KENDALL's $\tau = 0.85$, $z = 4.64$, $p < 0.001$). On the other hand, eupyrene

Table 4. Relationship between wing development and sperm movement in the late pupal stage of males under two photoperiodic regimes at 21(± 1)°C

Photoperiod	Grade ^a	No. animals used	No. of individuals with apyrene (eupyrene) sperm ^b in				
			T	UV	MV	LV	D
Long day-length	1	14	12 (14)	2 (0)	0 (0)	0 (0)	0 (0)
	2	3	2 (3)	0 (0)	0 (0)	1 (0)	0 (0)
	3	3	0 (3)	1 (0)	1 (0)	1 (0)	0 (0)
	4	10	0 (7)	0 (0)	1 (1)	2 (0)	7 (2)
Short day-length	0	20	20 (20)	0 (0)	0 (0)	0 (0)	0 (0)
	1	2	1 (2)	0 (0)	1 (0)	0 (0)	0 (0)
	2	10	5 (10)	1 (0)	2 (0)	2 (0)	0 (0)
	3	7	0 (7)	0 (0)	4 (0)	1 (0)	2 (0)
	4	1	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)

^a Wing development (See in Materials and Methods).

^b Numerical values indicate the number of individuals with sperm in the furthestmost region from the testis where sperm reached; T, testis; UV, upper vas deferens; MV, middle vas deferens; LV, lower vas deferens; D, duplex.

sperm bundles were first recognized in the post-testicular organs at the maximum grade 4 of adult development in both types of pupae.

DISCUSSION

The present study demonstrated the time of initiation of eupyrene and apyrene sperm movements occurring between the testis and the duplex via paired vasa deferentia in the two seasonal forms of *P. c-aureum*. Most individuals usually emerge as adults on the 8th or the 9th day after pupation under long-day conditions, and on the 9th or the 10th day under short-day conditions (HIROYOSHI, 1992). This, together with the present results, suggest that apyrene sperm started moving from the testis to the post-testicular organs two days before adult eclosion in both seasonal forms, whereas eupyrene sperm bundles were not seen until the day before adult eclosion. This result indicates that sperm movement starts before adult eclosion, although some individuals started eupyrene sperm movement after adult eclosion (HIROYOSHI, unpublished data), and that apyrene sperm movement commences earlier than eupyrene sperm movement in this species. A similar pattern has also been reported for *A. kuhniella* (THORSON and RIEMANN, 1982) and for *B. mori* (KATSUNO, 1977 a, b). No example is known in which apyrene sperm movement has been demonstrated to initiate in the adult stage. Thus, the initiation of apyrene sperm movement late in the pupal stage appears to be common in Lepidoptera.

Although A-pupae commenced sperm movement one day later than S-pupae (Tables 1 and 2), sperm movement occurred almost at the same phase of wing development in both types of pupae (Tables 3 and 4). Thus, it is concluded that the difference in the time of initiation of sperm movement between the two forms was caused by the difference in rates of morphogenesis.

It is known that imaginal differentiation of the vas deferens is induced by the molting hormone, ecdysone or 20-hydroxyecdysone in several lepidopteran species such as *A. kuhniella* (NOWOCK, 1973), *Samia cynthia* (SZÖLLÖSI and LANDUREAU, 1977) and *Mamestra brassicae* (SHIMIZU, 1989). The maturity of the vas deferens coincides with sperm release from the testis in *A. kuhniella* (RIEMANN and THORSON, 1976). SHIMIZU (1989) reported that sperm movement from the testis to the vas deferens in *M. brassicae* occurred the day before adult emergence, after the ecdysone titer decreased drastically, and then remained at a low level. In *A. kuhniella*, application of 20-hydroxyecdysone into pupae and pharate adults prevented eupyrene sperm release from the testis (THORSON and RIEMANN, 1982). These results suggest that the pattern of fluctuation in ecdysteroid titer may be important in triggering the initiation of sperm movement. If this is the case, it is possible that the pattern of temporal changes in ecdysteroid titer in *P. c-aureum* may differ between the two types of pupae. However, MASAKI et al. (1989) demonstrated that pupae of this species held under long- and short-day conditions showed almost the same patterns of fluctuation in hemolymph ecdysteroid titer, although the temperature conditions under which the two types of pupae had been maintained were not specified.

No apparent difference was found in sperm movement between the two seasonal forms of *P. c-aureum* except that sperm started moving one day earlier in S-form than in A-form pupae. Spermatogenesis during the larval and pupal stages proceeds in the same way in both seasonal forms (HIROYOSHI, unpublished data): Like S-form individuals, even newly emerged A-form butterflies have their testis filled with sperm in spite of the fact that they do not copulate until they terminate diapause. In conclusion, spermatogenesis and sperm movement are influenced neither by the presence or absence of imaginal diapause nor by the seasonal form.

ACKNOWLEDGEMENTS

I thank Prof. J. MITSUHASHI, Tokyo University of Agriculture and Technology, for his critical reading of this manuscript. Thanks are also due to Mr. T. MIYATAKE and Dr. T. SHIMIZU, Okinawa Prefectural Agricultural Experimental Station, for their invaluable suggestions and criticism.

REFERENCES

- ENDO, K. (1973) Hormonal regulation of mating in the butterfly, *Polygonia c-aureum* L. *Dev. Growth Differ.* **15**: 1–10.
- FUKUDA, S. and K. ENDO (1966) Hormonal control of the development of seasonal forms in the butterfly, *Polygonia c-aureum* L. *Proc. Jpn. Acad.* **42**: 1082–1087.
- GIEBULTOWICZ, J.M., R.A. BELL and R.B. IMBERSKI (1988) Circadian rhythm of sperm movement in the male reproductive tract of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* **34**: 527–532.
- GIEBULTOWICZ, J.M., J.G. RIEMANN, A.K. RAINA and R.L. RIDGWAY (1989) Circadian system controlling release of sperm in the insect testes. *Science* **245**: 1098–1100.
- HIDAKA, T. and S. AIDA (1963) Day length as the main factor of seasonal form determination in *Polygonia c-aureum* (Lepidoptera, Nymphalidae). *Zool. Mag.* **72**: 77–83 (in Japanese).
- HIROYOSHI, S. (1992) Effects of photoperiod and temperature on several pupal characters associated with imaginal polyphenism in *Polygonia c-aureum* (Lepidoptera: Nymphalidae). *Appl. Entomol. Zool.* **27**: 155–159.
- HIROYOSHI, S. (1995) Regulation of sperm quantity transferring to females at mating in the adult male of *Polygonia c-aureum* (Lepidoptera: Nymphalidae). *Appl. Entomol. Zool.* **30**: 111–119.
- KATSUNO, S. (1977 a) Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) I. The intratesticular behaviour of the spermatozoa at various stages from the 5th-instar to the adult. *Appl. Entomol. Zool.* **12**: 142–153.

- KATSUNO, S. (1977 b) Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) III. The post-testicular behaviour of the spermatozoa at various stages from the pupa to the adult. *Appl. Entomol. Zool.* **12**: 241–247.
- LACHANCE, L.E., R.D. RICHARD and R.L. RUUD (1977) Movement of eupyrene sperm bundles from the testis and storage in the ductus ejaculatoris duplex of the male pink bollworm: Effects of age, strain, irradiation, and light. *Ann. Entomol. Soc. Am.* **70**: 647–651.
- MASAKI, T., K. ENDO and K. KUMAGAI (1989) Neuroendocrine regulation of the development of seasonal morphs in the Asian comma butterfly, *Polygonia c-aureum*: Stage-dependent changes in the activity of summer-morph-producing hormone of the brain-extracts. *Zool. Sci.* **6**: 113–119.
- NOWOCK, J. (1973) Growth and metamorphosis in the testes of *Ephesia kuhniella* in vitro. *J. Insect Physiol.* **19**: 941–949.
- NYLIN, S. (1992) Seasonal plasticity in life history traits: growth and development in *Polygonia c-album* (Lepidoptera: Nymphalidae) L. *Biol. J. Linn. Soc.* **47**: 301–323.
- PROSHOLD, F.I. (1991) Number of sperm bundles in the duplex of tobacco budworms (Lepidoptera: Noctuidae) as a function of age. *J. Econ. Entomol.* **84**: 1485–1491.
- RIEMANN, J.G. and B.J. THORSON (1976) Ultrastructure of the vasa deferentia of the Mediterranean flour moth. *J. Morph.* **149**: 483–506.
- RIEMANN, J.G. and B.J. THORSON (1978) Adult Mediterranean flour moth: Effects of temperature on sperm production and periodicity of testis and vas deferens activity. *Ann. Entomol. Soc. Am.* **71**: 575–580.
- RIEMANN, J.G., B.J. THORSON and R.L. RUUD (1974) Daily cycle of release of sperm from the testes of the Mediterranean flour moth. *J. Insect Physiol.* **20**: 195–207.
- SHIMIZU, T. (1989) Development of spermiduct and seminal vesicle during pharate adult of the cabbage armyworm, *Mamestra brassicae* (Lepidoptera; Noctuidae). *Invert. Reprod. Dev.* **15**: 221–224.
- SZÖLLÖSI, A. and J.-C. LANDUREAU (1977) Imaginal cell differentiation in the spermiduct of *Samia cynthia* (Lepidoptera) responses in vitro to ecdysone and ecdysterone. *Biol. Cell.* **28**: 23–36.
- THORSON, B.J. and J.G. RIEMANN (1977) Abdominally entrained periodicities of testis and vas deferens activity in the Mediterranean flour moth. *J. Insect Physiol.* **23**: 1189–1197.
- THORSON, B.J. and J.G. RIEMANN (1982) Effects of 20-hydroxyecdysone on sperm release from the testes of the Mediterranean flour moth, *Anagasta kuhniella* (ZELLER). *J. Insect Physiol.* **28**: 1013–1019.