# Effects of Environmental Conditions and Aging on Eupyrene Sperm Movement in Male Adults of Polygonia c-aureum (Lepidoptera: Nymphalidae)

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# Effects of Environmental Conditions and Aging on Eupyrene Sperm Movement in Male Adults of *Polygonia c-aureum* (Lepidoptera: Nymphalidae)

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**ABSTRACT**—The effects of temperature, photoperiod and aging on eupyrene sperm movement from the testis to the duplex in *Polygonia c-aureum* male adults were examined in relation to seasonal form and imaginal diapause. In males of both summer and autumn forms obtained under long-day and short-day conditions, respectively, the number of eupyrene sperm bundles in the duplex increased linearly with age during the early stage of adult life at 21°C, and no marked difference was observed between the two seasonal forms. Photoperiod during the adult stage did not influence the rate of sperm movement in autumn forms with diapause. The lower the temperature, the smaller the number of sperm bundles moved from the testis to the duplex. Sperm movement did not appear to occur during a period of chilling at 5°C. Males of the autumn form which were pre-incubated for 30 days and chilled for 60 days failed to resume rapid sperm movement at the final incubation temperature of 21°C. Those which were pre-incubated only for 15 days at 21°C had much fewer sperm bundles in the duplex after chilling than those pre-incubated for 45 days. These results suggest the possibilities that eupyrene sperm movement is suppressed strongly during and after overwintering in the field, and that the eupyrene sperm to be used for reproduction in spring are transferred from the testis to the duplex before overwintering.

## INTRODUCTION

Imaginal diapause in male insects has not been adequately defined in contrast with female reproductive diapause characterized by the cessation of oocyte development (Pener, 1992). In males, the relationship between development of the reproductive organs and imaginal diapause is not consistent. In *Draeculacephala crassicornis* (Reissig and Kamm, 1975), *Chrysopa carnea* (Macleod, 1967) and *Pyrrhocoris apterus* (Žďárek, 1970) the testes grow in size or sperm mature during diapause, whereas the testes are underdeveloped in *Hypera postica* (Ascerno *et al*., 1978), and spermiogenesis is suppressed partially or completely in *Anthonomous grandis* (Brazzel and Newsom, 1959), *Sepedion fuscipennis* (Barnes, 1976) and *Brucus rufimanus* (Tran and Huignard, 1992) during diapause.

Many studies have been made on spermiogenesis and sperm movement in lepidopteran insects. Spermiogenesis starts at either a late larval instar or the pupal stage depending on the species. As a result, newly emerged adults have already many sperm in their testes (Dumser, 1980). Mature sperm are transferred from the testis to the duplex via the vasa deferentia and stored there until mating (Holt and North, 1970; Riemann *et al*., 1974; LaChance *et al*., 1977; LaChance, 1984). This sperm movement is entrained by a circadian rhythm (Giebultowicz *et al*., 1988, 1989). These findings are based on non-diapause individuals or species, and relatively little attention has been paid to the species with diapause. Some lepidopteran species with larval or pupal diapause such as *Laspeyresia pomonella* (Friedländer and Benz, 1982) and *Papilio xuthus* (Numata and Hidaka, 1981) stop spermatogenesis during diapause and resume it after diapause. However, no information is available for species with imaginal diapause. Is spermatogenesis inhibited during diapause in those species? If so, when do they produce and store the sperm?

The Asian comma butterfly, *Polygonia c-aureum*, is polyvoltine in Japan except for northern regions and shows seasonal diphenism with respect to the wing shape, pattern of wing pigmentation and reproductive activity, i.e. summer form and autumn form. The summer form emerges as an adult in summer and begins to reproduce shortly after adult eclosion. The wings of this form are lighter in color with larger dark spots and weaker notch in margin than those of the autumn form which appears in autumn and enters diapause after adult eclosion. Adults of the autumn form, thus, do not reproduce until the following spring. The seasonal form and diapause of this species are determined mainly by photoperiod and temperature (Hidaka and Aida, 1963; Fukuda and Endo, 1966; Hidaka and Takahashi, 1967). Endo (1973) re-

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ported that males of the autumn form do not copulate soon after adult eclosion under laboratory conditions, but it is not known when they produce and store the sperm which are used for copulation after overwintering. Are they different in reproductive manner from males of the summer form?

In the present study, we examined the effects of photoperiod, temperature and aging on sperm movement from the testis to the duplex in *P. c-aureum* to answer the above questions.

## MATERIALS AND METHODS

### Insects

Larvae of *P. c-aureum* were collected in Tokyo Metropolis and Saitama Prefecture, Japan in 1989, and successively reared in the laboratory at a long photoperiod (15L9D) and 21 ± 1°C. Under such conditions, they developed to summer form butterflies. Only those individuals hatching from eggs deposited by summer forms were used for experiments, as offspring of autumn form females are likely to become the summer form under any photoperiodic conditions due to a maternal effect (Hidaka and Takahashi, 1967).

### Rearing

Eggs from the laboratory culture were surface-sterilized with ca. 3% of form-aldehyde solution for 30 min, washed in tap water, air-dried, and placed in a plastic Petri dish (9 cm dia. × 2 cm depth) until hatching.

Hatchlings were placed in groups of 30 to 40 on a piece of filter paper in a glass Petri dish, and supplied with fresh leaves of *Humulus japonicus* (Moraceae). The rearing density was gradually reduced after ecdysis to the penultimate (4th) larval instar. After pupation, each individual was placed in a Petri dish kept vertically to facilitate adult eclosion.

After adult eclosion, females and males were separately kept in wood-framed cages (17 cm × 16.5 cm × 46 cm) covered with Saran® net in groups of 30 to 40. Butterflies fed on 10% sugar solution absorbed into cotton held in a glass dish.

### Environmental conditions

Hatchlings were reared at 21 ± 1°C; they developed to summer form under a long day (LD: 15L9D) and to autumn form under a short day (SD: 8L16D). Newly emerged male butterflies were kept under the following environmental conditions: Experiment 1) summer and autumn forms were held at 21 ± 1°C under LD or SD throughout the imaginal life, Experiment 2) autumn forms were incubated at either 15, 21 or 25 ± 1°C for 30 days under SD, Experiment 3) autumn forms held at 21 ± 1°C under SD (simulating autumn conditions) for the first 30 days were chilled at 5 ± 1°C under continuous darkness (simulating winter conditions) for 60 days, and then returned to 21 ± 1°C under either LD or SD (simulating spring conditions), Experiment 4) autumn forms held at 21 ± 1°C under SD for the first 30 days were chilled at 5 ± 1°C under continuous darkness for either 30 or 90 days, and then returned to 21 ± 1°C under either LD or SD, Experiment 5) autumn forms kept at 21 ± 1°C under SD for either 15 or 45 days were chilled at 5 ± 1°C under continuous darkness for 60 days, and then returned to 21 ± 1°C under either LD or SD. During a period of chilling at 5 ± 1°C, most males of the autumn form had survived, whereas all those of the summer form died within a month. Thus, the effects of chilling on sperm movement were not examined for the summer form.

### Sperm movement

Lepidopteran males have two distinct types of sperm, i.e. nucleated eupyrene sperm and anucleated apyrene sperm. Eupyrene sperm migrate as bundles within the male reproductive organs, whereas apyrene sperm bundles dissociate into single sperm, when they migrate from the testis to the vas efferens (Riemann, 1970; Riemann *et al.*, 1974; Katsuno,1977). Only eupyrene sperm bundles were examined quantitatively in the present study.

The number of sperm bundles was counted in the following manner. The abdomen of butterflies was cut off and immersed in about 20 ml of saline solution (8.6 g NaCl, 0.33 g $CaCl_2$ and 0.1 g KCl per liter distilled water), and dissected under a stereomicroscope. The duplex was carefully taken out and raptured with a pair of forceps and a needle to release the sperm inside. Eupyrene sperm bundles entangled complicatedly were dissociated into single bundles with a needle. The number of sperm bundles in each sample was counted in a glass Petri dish (3 cm diameter × 1 cm height) placed on a latticed plastic Petri dish (5 cm diameter × 1 cm height) under a phase-contrast microscope.

## RESULTS

### Effects of photoperiod and age on sperm movement

To study the progress of eupyrene sperm movement after adult emergence, summer form adults were maintained at 21°C under LD, while autumn forms were kept at the same temperature under either LD or SD (Experiment 1). The results are shown in Fig.1. The number of sperm bundles in the duplex increased rapidly with age in all groups of adults examined under either photoperiod. The rate of increase was then reduced gradually. Summer form males normally lived less than two months on average at 21°C (Hiroyoshi, unpublished data), but 7 out of 8 summer form males which lived for 60 days had sperm bundles in their vasa deferentia, indicating the occurrence of sperm movement (data not shown). These results suggest that sperm movement continued throughout their life span. Numerous apyrene sperm were observed in the duplex at any age, though their sperm number was not counted.

In a separate experiment, we measured dry weight of the

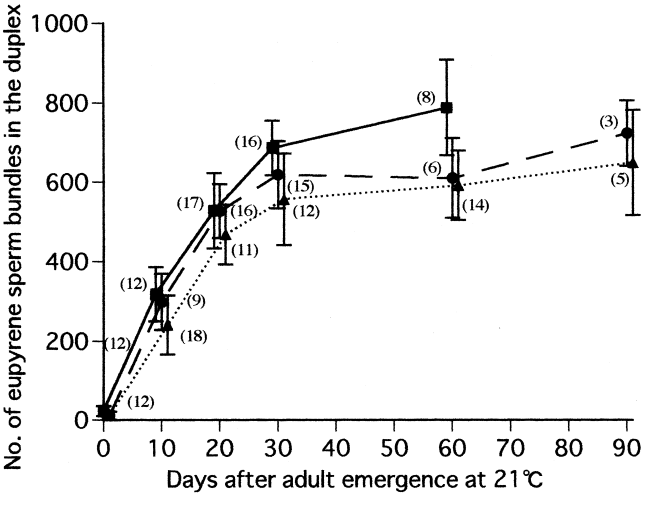


**Fig. 1.** Effects of photoperiod and aging on the accumulation of eupyrene sperm bundles (mean ± S.D.) in the duplex of *P. c-aureum* male adults at 21°C. Squares, circles and triangles represent summer forms under LD, autumn forms under LD, and autumn forms under SD, respectively. The numbers in parentheses indicate N.

duplex in the summer form under LD and SD, and it increased with age under either photoperiod, the pattern being consistent with that in Fig. 1. This may suggest that sperm movement in summer forms, as well as autumn forms, is not influenced by the photoperiod in the adult stage, although sperm movement in the summer form under SD was not examined in the present study.

Summer forms had a significantly larger number of eupyrene sperm bundles in the duplex than autumn forms at each day examined. As demonstrated earlier (Hiroyoshi, 1992), pupae grown under SD are smaller than those reared under LD, and thus autumn form adults are smaller than summer form ones. It is highly likely that the duplex is accordingly larger in summer forms than in autumn forms under these photoperiodic conditions. Therefore, summer forms may accumulate more sperm bundles in the duplex than autumn forms.

**Effect of adult temperature on sperm movement**

The effect of temperature on eupyrene sperm movement was examined in autumn form males which had been exposed to three different temperatures under SD for 30 days before they were dissected to count the number of sperm bundles in the duplex (Experiment 2). As shown in Fig. 2, the higher the temperature, the greater the number of sperm bundles in the duplex ($r = 0.897$; $p < 0.01$; $n = 23$).

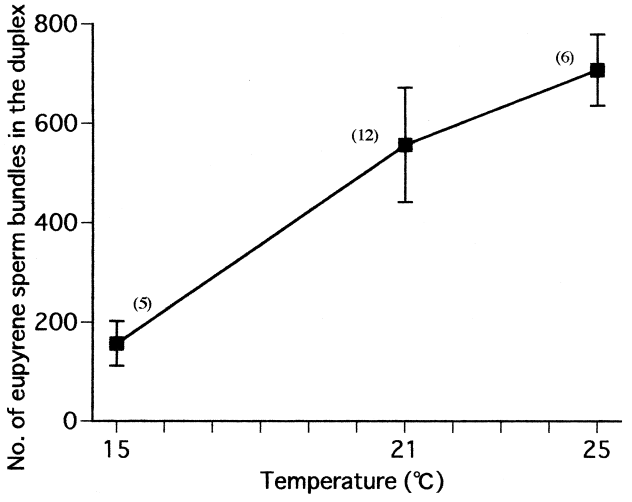


**Fig. 2.** Effect of temperature during the adult stage on sperm movement in *P. c-aureum*. Autumn form males exposed to different temperatures for 30 days were dissected to count the number of eupyrene sperm bundles in the duplex (mean ± S.D.). The numbers in parentheses indicate N.

**Sperm movement during chilling**

To examine the effect of low temperature on eupyrene sperm movement, the numbers of sperm bundles stored in the duplex of autumn form males before and after chilling were compared (Experiment 3). In this observation, males held under SD and 21°C for 30 days were dissected before chilling or after a 60-day exposure to 5°C in darkness. The numbers of sperm bundles before and after chilling (mean ± S.D.) were 556.3 ± 115.3 (n = 12) and 575.1 ± 127.8 (n = 12), respectively. No significant difference was obtained between the two means (Mann-Whitney's *U*-test, $p > 0.05$). This may indicate that eupyrene sperm movement did not occur during the chilling period.

**Sperm movement after chilling**

To examine the effects of temperature and photoperiod after chilling on the subsequent sperm movement, autumn form males held under SD and 21°C for 30 days were first chilled at 5°C for 60 days and then exposed to SD or LD at 21°C for various periods (Experiment 3, Fig. 3).

At the end of chilling, males had about 575 sperm bundles in the duplex on average. The number of sperm bundles did not change significantly over the following 30 days at 21°C under SD ($r = -0.016$; $p > 0.05$; $n = 47$) and LD ($r = -0.149$; $p > 0.05$; $n = 42$). Similar results were also obtained when males were treated similarly but the chilling lasted one or three months (Experiment 4, data not shown).

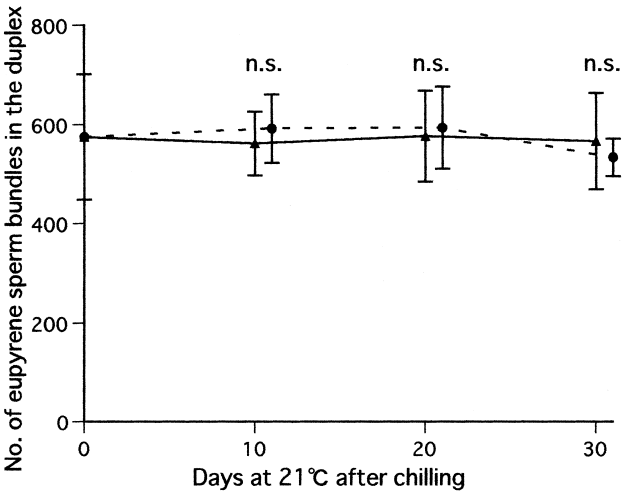


**Fig. 3.** The number of eupyrene sperm bundle (mean ± S.D.) in the duplex of *P. c-aureum* at 21°C after a 2-month exposure to 5°C. Triangles and circles indicate males of the autumn form kept under LD and SD, respectively. All test animals were incubated at 21°C and SD for 30 days before chilling. Each point represents 10 to 13 samples. n.s., not significant.

**Effect of timing of chilling on sperm movement**

To examine the effect of timing of chilling on sperm movement, autumn form males pre-incubated at 21°C and SD for either 15 or 45 days were chilled at 5°C for 60 days, and then returned to 21°C and SD or LD (Experiment 5, Fig. 4).

In males pre-incubated at 21°C for 15 days and chilled for 60 days, the number of sperm bundles in the duplex was 345 on average immediately after chilling. The number did not change significantly over the next 30 days at 21°C when males were kept under SD ($r = 0.019$; $p > 0.05$; $n = 35$), but there was a significant correlation between the number of sperm bundles and the number of days after chilling in males

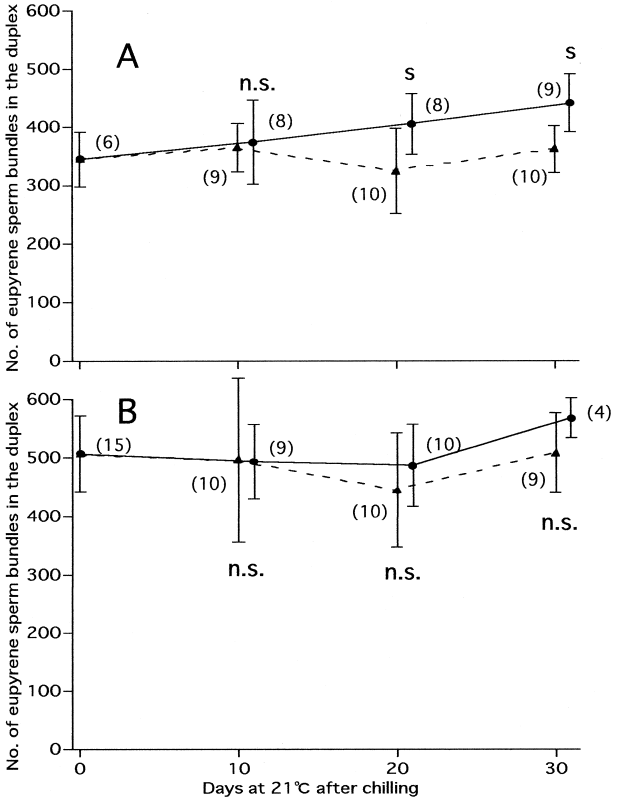


**Fig. 4.** Effect of chilling on the accumulation of eupyrene sperm bundles (mean ± S.D.) in the duplex of *P. c-aureum* upon transfer to 21°C. Male adults of the autumn form were incubated at 21°C for either 15 (**A**) or 45 days (**B**), chilled at 5°C for 60 days, and returned to 21°C. Triangles and circles indicate males of the autumn form kept under SD and LD, respectively. The numbers in parentheses indicate N. s, significant; n.s., not significant.

kept under LD ($r = 0.554$; $p < 0.01$; $n = 31$). Significant differences were also obtained in mean number of sperm bundles between SD and LD males kept at 21°C for 20 or 30 days after chilling (Mann-Whitney's *U*-test, $p < 0.05$ and $p < 0.01$, respectively). In males pre-incubated at 21°C for 45 days, the number of sperm bundles was not correlated significantly with age under SD ($r = -0.082$; $p > 0.05$, $n = 44$) or LD ($r = 0.115$; $p > 0.05$; $n = 38$). These males had significantly more sperm bundles in the duplex at any time after chilling than those pre-incubated at 21°C for 15 days (Mann-Whitney's *U*-test, $p < 0.05$).

## DISCUSSION

The present results indicate that the number of sperm bundles in the duplex increases rapidly during the early stage of adult life in *P. c-aureum*. This rapid sperm movement occurred in the same way in both summer and autumn forms. The number of sperm bundles in the duplex later in the adult stage was slightly larger in the former, probably because of the difference in body size (Fig. 1). As mentioned above, only autumn forms enter imaginal diapause. The diapause males of *P. c-aureum* are characterized by abstinence from mating (Endo, 1973) and reduced development of the accessory glands and ejaculatory duct (Hiroyoshi, unpublished data). The diapause is induced and maintained under short day conditions, but terminated if they are exposed to long days and/or a high temperature (Hiroyoshi, unpublished data). In the present study, diapause males of the autumn form were kept under either short-day or long-day conditions. As a result, it was found that photoperiod experienced after adult emergence did not exert any significant influence on the number of sperm bundles in the duplex even after 60 or 90 days. Therefore, sperm movement appears to be independent on the diapause status.

The temperature has a strong effect on the progress of eupyrene sperm movement. During the early stage of adult life, the higher the temperature, the faster the accumulation of sperm bundles in the duplex (Fig. 2). No significant sperm movement took place at a low temperature. Riemann and Thorson (1978) also demonstrated that decreasing temperature considerably reduces the quantity of sperm transferred from the testis to the vasa deferentia or the duplex in *Ephestia kuehniella*. Because eupyrene sperm movement starts around at adult eclosion in *P. c-aureum* (Hiroyoshi, 1997), the cause for the fewer sperm bundles in the duplex at a lower temperature observed in the present study is likely to be due to reduced daily sperm release from the testis rather than a delayed onset of the release.

Chilling affects the subsequent sperm movement at a higher temperature. The number of sperm bundles in the duplex did not change significantly at 21°C over 30 days after chilling (Fig. 3). This was probably because the duplex was filled with sperm during the pre-incubation period of 30 days (Fig. 1) and few, if any, sperm could be transferred from the testis after chilling. Compared to those pre-incubated at 21°C for 30 or 45 days, males pre-incubated for only 15 days had much fewer sperm bundles in the duplex immediately after a 60-day chilling (Fig. 4). The number of sperm bundles in those males slightly increased upon transfer to 21°C under long-day conditions, but the rate of increase was much smaller than that for males continuously held at the same temperature. This indicates that sperm movement is strongly suppressed after chilling. Furthermore, the number of eupyrene spermatocysts is reduced remarkably during chilling and eupyrene spermiogenesis is suppressed after overwintering (Hiroyoshi, unpublished data), suggesting that little sperm release from the testis to the duplex would occur in spring. Therefore, it is likely that male adults fill the duplex with sperm before overwintering and use them for insemination in spring. In our preliminary observations, males of the autumn form chilled at 5°C for 2 months successfully mated with virgin females of the summer form, and their offspring hatched normally, indicating that the viability of sperm in the duplex was not impaired by chilling.

If the number of sperm that can be used for reproduction in spring is determined by the number of sperm stored in the duplex before overwintering, the timing of adult emergence would be of importance for males of the autumn form. If they emerge as adults too early, they would experience high tem-

perature and fail to maintain diapause until winter comes. In fact, a high temperature of 30°C can terminate diapause of males in a few days (Hiroyoshi, unpublished observation). If they become adults too late in the season, on the other hand, they would have little time to transfer sperm from the testis to the duplex.

Recently, Proshold and Bernon (1994) suggested that males of *Lymantria dispar* do not transfer all of the sperm stored in the duplex to females at one mating, contrary to the widely accepted theory. Hiroyoshi (1995) demonstrated that males of *P. c-aureum* regulate the sperm quantity to be transferred to females at mating. This suggests that the sperm quantity stored in the duplex before overwintering may determine the frequency of mating and thus the reproductive potential of the autumn form males in spring.

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