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Ejaculation Timing of Eupyrene and Apyrene Sperm in the Cabbage White Butterfly *Pieris rapae* (Lepidoptera: Pieridae) during Copulation

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Abstract. During copulation, the male small white, *Pieris rapae,* fills a single spermatophore in the bursa copulatrix of the female. Artificial interruption of copulation showed that the male filled first white gel after which the spermatophore was structured. No sperm were found in the gel. Both eupyrene sperm bundles and free apyrene sperm were ejaculated into the structured spermatophore immediately before the completion of copulation. A male transferred about 11,000 eupyrene sperm, whereas about 46,000 apyrene sperm were found in a complete spermatophore just after copulation. The sperm density in the spermatophore also indicated that the velocity of transferring both eupyrene and apyrene sperm increased torward the end of copulation. The significance of timing in sperm ejaculation is discussed from a view point of sperm competition.

Key words: Aapyrene sperm, bursa copulatrix, eupyrene sperm, interrupted copulation, *Pieris rapae.*

Introduction

In butterflies, a spermatophore is transferred during copulation. It contains two types of sperm (eupyrene and apyrene sperm) and accessory substances. In the cabbage white butterfly, *Pieris rapae,* the shape and size of both types of sperm were described for the first time by Tsukaguchi & Kurotsu (1922), and thereafter, the two types of sperm were recognized in many lepidopteran insects by several investigators (e.g. Tschudi-Rein & Benz, 1990; Gage, 1994). During copulation, bundles of eupyrene sperm are transferred to the female. After copulation, the bundles are disintegrated and eupyrene sperm migrate together with or after apyrene sperm from the spermatophore to the spermatheca (Katsuno, 1977a). Apyrene sperm were produced in large numbers in *P. rapae,* compared with eupyrene sperm (Watanabe *et al.*, unpublished). However, the function of apyrene sperm is poorly understood in the polyandrous males (Cook & Wedell, 1996) while a role in sperm competition has been suggested in some species (e.g. Silberglied *et al.*, 1984).

Spermatophores remain in the reproductive tract of female butterflies. A large amount of accessory secretion in the spermatophore may be of advantage in sperm competition or serve to increase the male fertilization success, because females that receive larger spermatophores would have a longer period of sexual unreceptivity (Kaitala & Wiklund, 1994). Several potential costs that males incur in producing spermatophores have been suggested to be the time of forming a spermatophore itself, the energy to replenish accessory gland materials to form a spermatophore, and so on (Dewsbury, 1982). Spermatophores contain significant quantities of nitrogen (Marshall, 1985; Bissoondath & Wiklund, 1995, 1996) and sugars (Watanabe & Sato, 1993). With an increased risk of sperm competition, males should invest more in their ejaculate as well as sperm quantity to ensure better fertilization. The present investigation was carried out to determine the relationship between male investment and the timing of sperm ejaculation in *P. rapae rapae* by artificially interrupting copulation.

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Materials and Methods

Adults of *P. rapae rapae* were collected in the campus of Stockholm University, Sweden, June 1996, and females were allowed to oviposit on the horse radish, *Armoracia rusticana* (Brassicaceae) which is one of the many natural host plants in Sweden. Larvae were reared in the laboratory under a 18h light: 6h dark regime and 25℃ to avoid diapause. In mid July, all the pupae were put in the cage with a sheet of paper at the bottom to ensure normal eclosion. Adults were weighed on the day of eclosion and given individual marks on the hind wing with a felt tipped pen. Then, each sex was separately held in wood-frame cages ($56 \times 56 \times 56$ cm^3) one side of which was covered with black mesh and the other sides with hyaline vinyl sheet. The butterflies were fed on flowers of the *Cirsium arvense* intermittently sprayed with 10% sucrose solution.

Mating experiments were done in $50 \times 56 \times 56$ cm^3 flight cages in a green house under 400 W mercury-vapour lights at about 25℃ and 10% sucrose solution was sprayed on the flowers of *C. arvense.* About 10 males (most of them were 1 day old after emergence) and several virgin females (0 day old) were placed together in each cage and allowed to copulate freely during the reproductively active period (0900–1300 hours). Butterflies were observed continuously so that the start of the copulation could be recorded. Soon after copulation had

started, the mating pairs were isolated in plastic cups (ϕ4.5×3.5 cm) to avoid harassment by other males in the flight cage.

Spermatophore transmission to the female was examined by artificial interruption of copulation. We allowed pairs to engage in copulation for 10, 20, 30, 40, 50 or 60 min before interruption (total 20 pairs) or uninterrupted (16 pairs). The male and female in each pair were separated with a pair of tweezers to interrupt copulation. All females were decapitated and dissected within 5 min of separation before sperm would migrate from the ejaculate to spermatheca (cf. Cook & Wedell, 1996). The weight of the bursa copulatrix containing the ejaculate from the male was measured to the nearest 0.001 mg. The weight of bursa copulatrix of virgin females (12 individuals) was also measured in order to estimate the weight of ejaculate, because it is difficult to measure the weight of gel-like ejaculate at the early period of copulation (Watanabe & Sato, 1993).

In the spermatophore immediately after copulation, the eupyrene sperm are packed in bundles, whereas the apyrene sperm bundles have already been dissolved so that apyrene sperm can be observed individually (e.g. Katsuno, 1977a). Eupyrene sperm bundles are clearly visible at ×40 and are apparently uniform in size. Therefore, we counted the eupyrene sperm bundles in the ejaculate using a stereoscopic microscope. Then, the ejaculate was thoroughly washed out into a small vial with a known volume of Ringer's solution for insects. The vial was gently stirred for 1 min. A total of six 10 μl subsamples were removed from each sample using a Gilson autopipette and allowed to dry on slides under dust covers. The dry slide was dipped for about 3 sec in distilled water and allowed to dry again. Each subsample was examined under dark-field phase-contrast microscopy (×100) to count apyrene and eupyrene sperm.

There are 256 eupyrene sperm in each bundle (P. A. Cook, unpublished). The number of bundles was multiplied by 256 to give the total number of eupyrene sperm, because few single eupyrene sperm were found. The total number of apyrene sperm per spermatophore was calculated by multiplying the average 10 μl sperm count by the dilution factor.

Results

Out of 37 pairs examined for mating, one female showed the mate refusal posture when a male approached. This female was discarded. The other females accepted males instantly and copulated. Few males failed to copulate with virgin females.

The weight of the bursa copulatrix in virgin females was 0.84 ±0.100 mg (mean±SD, n=12). The weight of the ejaculate can be assessed by subtracting the weight of the bursa copulatrix of virgin females from the whole weight of the bursa copulatrix of mated females. There was no detectable weight of ejaculate in males 20 min after the beginning of copulation (Fig. 1). The weight thereafter began to increase. The ejaculate weight after 60 min of copulation was not significantly different from that in uninterrupted copulation ($F=2.244$, $P>0.05$). During copulation, the male secretions from the ductus

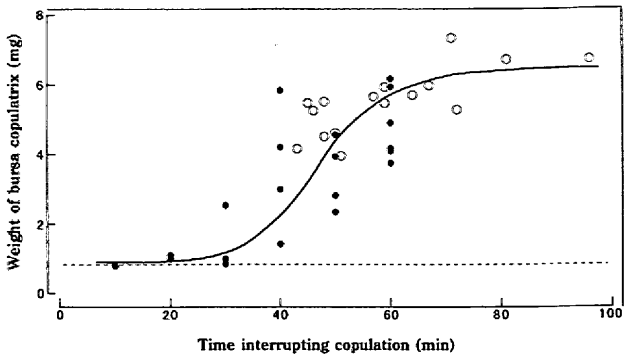

Fig. 1. Weight of bursa copulatrix of females after interrupted (closed circles) and uninterrupted (open circles) copulations. The dotted line shows the mean weight of the bursa copulatrix of virgin females.

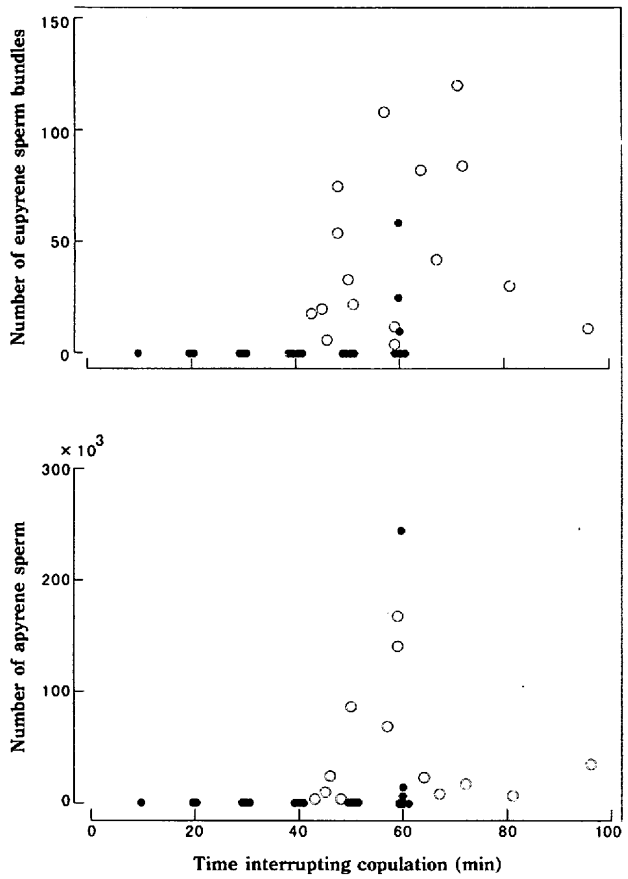

Fig. 2. Number of eupyrene sperm bundles and the estimated number of apyrene sperm in a spermatophore transferred from males. Closed and open circles refer to the interrupted and uninterrupted copulations, respectively.

ejaculatorius and accessory glands were transferred serially to the bursa copulatrix. The ejaculate was white gel mainly observed on the tip of the penis 10 min after the beginning of copulation. There was no spermatophore capsule in the bursa copulatrix after 20 min in copulation.

Successful copulations lasted 59.8±3.68 min (mean±SE), by which time the spermatophore has been structured in the bursa copulatrix and filled with white secretion and sperm. It seemed that the surface of the white gel solidified and became the spermatophore capsule. The spermatophore increased in volume until 60 min after the beginning of copulation. The

Table 1. Sperm density for eupyrene and apyrene sperm in a spermatophore transferred from males at the interrupted copulation for 50–60 min after the start of copulation and at the termination of copulation until 60 min (mean number per mg±SE), using Mann–Whitney *U*-test.

	Interrupted	Termination	
Eupyrene sperm density	483± 296 (n=10)	2,386± 831 (n=7)	$U=10$, $P<0.01$
Apyrene sperm density	4,711±4,580 (n=10)	11,000±4,521 (n=7)	$U=8$, $P<0.01$

spermatophore was elongated-shape and occupied the bursal duct with its opening at the end of the duct near the seminal duct. Mean wet mass of the spermatophores was about 5 mg. The mean body weights of virgin males and females were 78.0 ±2.18 mg (mean±SE, n=37) and 77.8±2.52 mg (mean±SE, n=37), respectively. The mass of a spermatophore, plus appendix bursa contents, represents 6.4% of body mass in both sexes.

All spermatophores of the pairs that normally completed copulation contained both types of sperm, irrespective of the copula duration (Fig. 2). The number of eupyrene sperm bundles observed in spermatophores just after copulation was 47.8±9.61 (range, 6–120). Thus, multiplying by the number of eupyrene sperm in each bundle (256), a male transfers 11000± 2000 (mean±SE) eupyrene sperm by mating. On the other hand, the estimated number of apyrene sperm transferred by the male was much higher. A spermatophore contained 46000 ±15000 apyrene sperm. Therefore, apyrene sperm outnumbered eupyrene sperm in the spermatophore. At mating, virgin males transferred apyrene sperm of about 4-fold of eupyrene sperm.

Neither eupyrene nor apyrene sperm was found in the ejaculate in bursa copulatrix before 60 min of copulation, indicating that males did not transfer both types of sperm during this period. We observed both types of sperm in the spermatophore 60 min after the start of copulation, when some pairs had already completed copulation. The ejaculate in copulation interrupted after 50 min contained neither eupyrene nor apyrene sperm and had not structured a spermatophore. Therefore, males seem to ejaculate both sperm types just before the completion of copulation, and just after the spermatophore has been structured.

The sperm density, i.e. the number of sperm of the two types per mg of spermatophore, is shown in Table 1. Since we observed no free eupyrene sperm but eupyrene bundles in each spermatophore, we calculated the number of free eupyrene sperm, multiplying by 256 as mentioned above. The eupyrene sperm density in spermatophore in copulation interrupted after 50–60 min was about 500, while that of copulation completed in 60 min was about 2,400. There was also a significantly lower apyrene sperm density in the spermatophore in interrupted copulations than in the completed copulation. Therefore, the velocity of transferring both eupyrene and apyrene sperm increased toward the end of copulation period.

Discussion

Mating costs time. Although Oberhauser (1989) showed that monarchs spend several hours in copula (mainly at night), many butterfly species spend much less time in copula (e.g. Rutowski & Gilchrist, 1986). During copulation *P. rapae* males continuously transferred the accessory gland material (Watanabe & Sato, 1993). The duration of copulation of a male and a female was about 60 min for *P. rapae* in Sweden (Watanabe *et al.*, unpublished).

Rutowski & Gilchrist (1986) suggested that the duration of copulation is relatively long because of mechanical problems in filling the bursa copulatrix. However, in the silkworm, *Bombyx mori*, ejaculation of seminal fluid into the spermatophore terminated 20 min after the beginning of copulation (Osanai *et al.*, 1986). In *P. rapae*, a little amount of ejaculation was observed already 10 min after the beginning of copulation. Most of the ejaculated substances were passed to the female 50 min after the start of copulation. The pattern of transferring materials to the female reproductive tract by the male in *P. rapae* was similar to that of other Lepidoptera. For example, sperm were the last material to be transferred in *Colias eurytheme* (Rutowski & Gilchrist, 1986). In the present study, when copulation was prematurely terminated (before 50 min from the start), the male had passed some nutritious material, but not sperm.

The mass of ejaculate corresponds to 1.4%–15.5% of the male body weight in 25 butterfly species (Svard & Wiklund, 1989). Ejaculates transferred by males of *P. napi* constitute on average 15.0% of the male body weight (Wiklund & Kaitala, 1995). However, Watanabe & Sato (1993) reported that during copulation, a *P. rapae crucivora* male from Japan fills the appendix bursa with a white substance containing sugars, and the deposition represents approximately 7% of the male's body mass. In this study with a different subspecies of *P. rapae*, about 6% of male weight was the spermatophore mass, suggesting that the ejaculate does not affect the production of subsequent ejaculates. Marshall (1985) reported that 6 to 7% of the male's body mass was ejaculated in *Colias philodice* and *C. eurytheme*.

Sperm quantity is an important determinant of fertilization success in sperm competition (e.g. Parker, 1970). He & Miyata (1997) reported that a larger spermatophore contained significantly more apyrene sperm than a smaller one. However, as a rule, one spermatophore contains enough (eupyrene) sperm to fertilize all eggs (Svard & Wiklund, 1989). In *P. rapae*, Watanabe & Ando (1993) estimated the lifetime output of the female to be about 250 eggs, whereas at least 10,000 eupyrene sperm were contained in a spermatophore after mating in this study. Males can thus transfer an excess number of eupyrene sperm to inseminate all eggs. Therefore, competition may select for sperm movement and vigor (e.g. Gage, 1994).

The importance of sperm size for fertilization success, either through sperm competition, or simply for reaching and pene-

trating an egg, is not clearly understood (Gage & Cook, 1994). Male *Polygonia c-aureum* could regulate the sperm quantity ejaculated into the female by a reversely directed sperm movement specific at mating (Hiroyoshi, 1995). Cook & Gage (1995) showed that male *Plodia interpunctella* moths may reduce eupyrene sperm numbers in mating with older virgin females of a lower reproductive value. In the present study, we used virgin females of 0 day old. If apyrene sperm functioned either as nutrient donations or a factor delaying the onset of female sexual receptivity, the males may benefit by ejaculating large numbers of apyrene sperm instead of large spermatophores.

Apyrene sperm started moving from the testis to the post-testicular organs before adult eclosion, whereas eupyrene sperm moved after adult eclosion in *Polygonia c-aureum* (Hiroyoshi, 1997). That is, apyrene sperm movement commences earlier than eupyrene sperm movement in the male development during the pupal stage in some lepidopteran insects (e.g. Katsuno, 1977b). On the other hand, eupyrene sperm were bundled to be inactive and transferred into the spermatophore. This may mean that apyrene sperm are ready to be ejaculated earlier than eupyrene sperm during copulation.

Eupyrene sperm bundles and apyrene sperm were ejaculated simultaneously in *P. brassicae* (Tschudi-Rein & Benz, 1990). In the silk moth, *Bombyx mori*, Katsuno (1977b) found that apyrene sperm bundles began to be released from the follicle 144 h after pupation and were separated when they passed through the basement membrane, while the eupyrene bundles separated in the copulatory pouch of the mated females. Cook & Wedell (1996) stated that the pattern of sperm precedence is not known in *P. rapae* but if sperm mixing or displacement occurs, then an increase in sperm number is advantageous in the competition both with prior and future rival male's ejaculates.

Sperm are generally transferred to the spermatheca during hours following copulation. Tschudi-Rein & Benz (1990) reported that sperm of *P. brassicae* was transported to the spermatheca 5.5 to 8 h after copulation. Eupyrene sperm bundles of *P. rapae* quickly disappear and do not stay in the spermatophore for long (unpublished). Cook & Wedell (1996) dissected females of *P. rapae* within 20 min after the termination of copulation because they believed that sperm migration from the spermatophore could take place after 20 min. Accessory gland products in spermatophores have been shown to function in sperm activation (e.g. Leopold, 1976). Sugar contained in the spermatophore might also contribute to sperm survival during this period (Watanabe & Sato, 1993). There are many hypotheses suggesting that apyrene sperm may function intimately with eupyrene sperm by mixing the spermatophore contents (Osanai *et al.*, 1986) and breaking up eupyrene bundles (Katsuno, 1977c), though there is no direct evidence (Silberglied *et al.*, 1984). However, if the shape and the size affect the energy content of each spermatozoon, a single apyrene spermatozoon may have a lower quantity of energy than a single eupyrene sperm. Gage (1994) stated that longer spermatozoa are likely to generate greater flagellar forces and swim faster. The longevity and the activity of apyrene sperm after ejaculation would be lower than the eupyrene sperm. If so, it may be advantageous that apyrene sperm must be ejaculated later than eupyrene sperm bundles, in order to keep their activity. Comparative work examining reproductive tract dimensions and sperm energetics is needed to determine the relationship between the timing of ejaculation of the two types of sperm and the sperm competition.

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