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REPRODUCTIVE OUTPUT AND EGG MATURATION IN RELATION TO MATE-AVOIDANCE IN MONANDROUS FEMALES OF THE SMALL COPPER, *LYCAENA PHLAEAS* (LYCAENIDAE)

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ABSTRACT Females of the small copper, *Lycaena phlaeas*, were captured in the field and dissected to investigate their mating frequency and reproductive output. Spermatophore counts showed that most females were monandrous. A single spermatophore usually occupied about half of the bursa copulatrix, though the size of the spermatophore decreased with the female's age. Young females had 250 immature eggs in their ovaries and laid an estimated 150 eggs throughout the course of their lives. Mate avoidance behavior was frequently observed in both mated and virgin females. In laboratory experiments, virgin females contained a few mature eggs immediately after eclosion, and the number of mature eggs gradually increased with age. Ovaries of females accepting copulation contained significantly larger numbers of mature eggs than ovaries of females avoiding mating. Copula duration was about 16 min (at 30°C) irrespective of male age. Egg maturation in the ovaries is thus seen as important in the mating behavior of such monandrous species.

Additional key words: bursa copulatrix, egg load, *Lycaena phlaeas*, mate-avoidance, monogamous, spermatophore.

In butterflies, ejaculates from males during copulation are used in female egg production and/or somatic maintenance. Females may, therefore, benefit from mating more than once (Boggs 1981, Boggs & Gilbert 1979, Watanabe, 1988). One potential benefit of repeated matings for the female butterfly is the substantial increase of sperm supply (Lederhouse 1981). As a rule, however, males transfer a number of sperm in excess of that needed to inseminate all eggs at a single mating (Watanabe et al. 1998). Consequently, in cases in which a spermatophore is used only as a reservoir of sperm to fertilize eggs, females should benefit from mating only once. Re-mating may decrease the time available to females for egg-laying and foraging activities, and may increase the risk of predation while *in copula*. In the evolution of such species, therefore, natural selection should favor females who avoid males after mating. The mate refusal posture displayed by unreceptive females has been reported in many butterfly species (Shapiro 1970, Watanabe et al. 1997).

The small copper, *Lycaena phlaeas* (Linnaeus), is a multi-voltine butterfly that flies from spring to autumn in central Japan. Adults inhabit sunny areas such as forest margins, clearings, open fields with low vegetation, banks and roadsides (Suzuki 1976). Suzuki (1978) reported mate-avoidance behavior in females and suggested that females generally mate only once. However, there are few data on spermatophore counts in the bursa copulatrix of wild females, and few studies have been conducted on oviposition behavior, nor have any data on lifetime reproductive schedules been gathered.

In this paper, we clarify the mating frequency and fecundity of *L. phlaeas* in the field. Lifetime egg production and reproductive success are discussed. Since female monandrous butterflies may solicit courtship before copulation and actively avoid males after mating in order to allow time for oviposition (Wiklund 1982), we examined the relationship between mate-avoidance behavior and fecundity in the laboratory.

MATERIALS AND METHODS

The data presented in this paper were obtained primarily from summer generations of the small copper, *L. phlaeas,* in Shirouma in Nagano Prefecture, which is located in a cool-temperate zone of Japan. The major habitat consists of rice-fields and margins of deciduous forests where adults may feed on nectar plants, and larvae on host plants (primarily *Rumex japonicus*, Polygonaceae), during the summer. During the day, large numbers of males fly near the ground to gather nectar and search for mates. Chasing behavior between males and females was sometimes observed over the course of the summer. Females engaged in various activities such as feeding, roosting, flying, copulating and ovipositing, were collected from late July to early August of both 1995 and 1996. To examine mating frequency of females flying in natural populations, sampling was done on windless, sunny days (a total of 13 days). When females were captured, their abdomens were amputated and immersed in a solution of 50% ethyl alcohol, and their wing condition and forewing length were recorded. We classified individuals into 5 age groups, on the basis of the degree of wing damage, using a ranking from O to IV, following Watanabe and Ando's method (1993). The five age groups were as follows: O, newly emerged females with wings having lustrous scales and no visible damage; I, fine tears and fewer lustrous scales; II, tears and frayed scales; III, notched tears and frayed scales; and IV, broken or extensive tears and frayed scales. All females were examined for the number of spermatophores in their bursa copulatrix and the number of eggs stored in their ovaries.

The individuals of *L. phlaeas* used in the mating experiments were the offspring of females collected in the wild. They laid eggs in cages, and the hatching larvae were reared on the host plant, *R. japonicus,* in small chambers at 25°C, with 18 hours of light per day. A newly emerged virgin female was placed in a mating cage (30 × 40 × 45 cm) along with 10 virgin males (less than 10 days old) to allow matings. The cage was set in a greenhouse for two hours around noon (at ca 30°C). In the present study, 451 females emerged in the laboratory. Their average forewing length was 16.5 ± 0.6 mm (SD), which is not significantly different from that of the captured wild females (16.3 ± 0.9 mm, *U*-test, Z = 0.825, n.s.). Out of the 451 females, 147 were randomly selected for mating experiments.

The mating pairs were kept in small cages (24 × 30 × 38 cm) until they separated. The duration of copulation was recorded. After copulation terminated, females were dissected to examine the spermatophore and to count the number of eggs in ovaries. As a control, virgin females of various ages in stock culture were also dissected.

In the present study, many females introduced into the cages avoided or refused to copulate with males displaying courtship behavior, although the males repeatedly approached females in the course of two hours. These females were taken out of the cage and supplied with sugar solution in another small cage at ca. 25°C. On the following day, they were then placed back in the mating cage. In this way, the copulation trials for virgin females were repeated every day until the female engaged in copulation.

Since both the spermatophore and the bursa copulatrix are oval, their respective volumes were calculated as an ellipsoid. Although eggs in the ovaries could be classified into three groups (mature, submature, immature), those in the oviducts were primarily of the mature type. These oviduct eggs were large, pale green and sufficiently well-formed (with a semi-spherical shape and pronounced ribbing) to be ready for oviposition. The vitellarium of the ovaries contained mainly submature eggs; these were large but more lightly colored than the mature eggs. Immature eggs, which included oocytes, were found in the terminal filament, the germarium and part of the vitellarium of the ovarioles. Eggs in the ovarioles decreased in size toward the tip of the terminal filament filled with oocytes. Because oocytes do not contain yolk, they appear white, and we were able to count them using tweezers and a light microscope (40×).

RESULTS

Over the course of the two-year study period, we examined a total of 99 wild females from the study area. In 1995, more than half of the females captured were young (ages O and I), and no females of the oldest age class (age IV) were captured (Table 1). Five of 36 age-O and 2 of 20 age-I females were virgins, and 2 additional virgin females were captured at age I. In 1996, 3 age-I females were virgin. In both years, there were a few females that contained both an intact full-sized spermatophore and a spermatophore fragment, thus showing evidence of having mated twice as shown in Table 1. No females contained mating plugs blocking their genital openings.

TABLE 1. The frequency of spermatophore distribution in the bursa copulatrix of female *Lycaena phaeas* caught in the wild for 5 age classes (0 ~ IV) in the summer generation of 1995 and 1996.

Years	Number of spermatophores present	Age class				
		0	I	II	III	IV
	0	5	2	0	0	0
1995	1	29	16	7	1	0
	2	2	2	0	0	0
	0	2	1	0	0	0
1996	1	3	9	6	8	4
	2	0	0	1	0	1

In mated females, the spermatophore has been ejaculated into the bursa copulatrix and is filled with white secretion. The sperm sac is an elongated cone which occupies the bursal duct and has its opening at the end of the duct near the seminal duct. There is no appendix bursae in the female reproductive system.

In age-O females, the average volume of the spermatophore was about 0.05 mm^3, which occupied about 31% of the volume of the bursa copulatrix (Fig. 1). There was no significant difference in the size of the spermatophores among females of age O to III (F = 1.610, n.s.). Among females of these four age classes, the spermatophore occupied about 40% of the bursa copulatrix. In age-IV females, the spermatophore size significantly decreased ($p < 0.01$), compared with the spermatophore size of age-I ($U = 14$), age-II ($U = 6$) and age-III ($U = 3$) females, but still occupied about 45% of the bursa copulatrix.

The mean number of eggs (±SE) in wild-caught virgin females of age O was 41.7 ± 13.3 (n = 7), 19.1 ± 3.8 (n = 7) and 242.1 ± 39.3 (n = 7) for mature, submature and immature eggs, respectively. The mated females of age O contained means (±SE) of 261 ± 15 immature eggs (n = 29), which number was similar to that for virgin females. Therefore, the potential fecundity of *L. phlaeas* was estimated at about 300. Figure 2 shows the lifetime changes in the number of immature eggs in monandrous females. The number of immature eggs decreased with age. Since there were less than 20

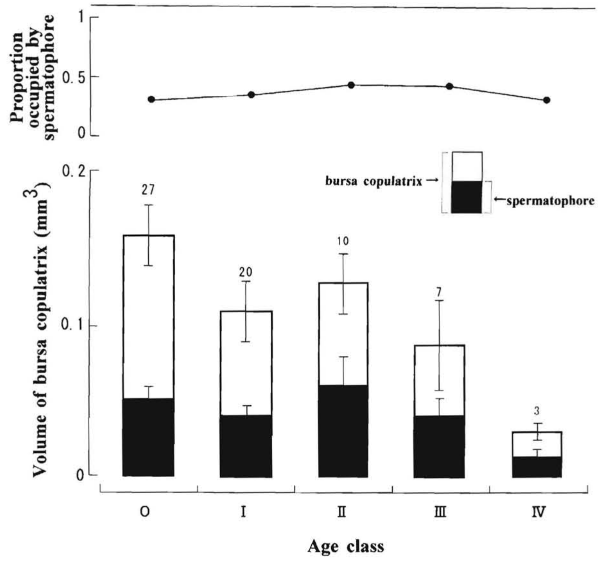

FIG. 1. Change in the volume of spermatophore in the bursa copulatrix and its proportion in wild monandrous females of *Lycaena phlaeas* for 5 age classes (O, I, II, III and IV). Each bar represents SE. The numbers above the bars show sample size.

mature eggs in the ovaries of females of age IV, the decrease in immature eggs resulted from resorption or oviposition. However, no fused eggs were observed in the ovaries, suggesting that few eggs were consumed for somatic maintenance. In such cases, in which there is no addition of immature eggs during the adult stage, the decreasing number of immature eggs in the ovaries is thought to be due to oviposition. A monogamous female of age IV may thus have laid 150 eggs.

In the mating cages, all *L. phlaeas* males were seen perching, and would presumably mate with the females. We observed a total of 605 unsuccessful courtships, which can be described in terms of five distinct behavioral events. After approaching a female, the male tried to make initial contact. In 43% of unsuccessful courtships, females remained perched and ignored approaching males. Walking or flying away from males was seen in 29% and 14% of approaches, respectively. In some cases, females turned face-to-face with the male (9%) or fluttered (4%) until the male flew away. The latter two behaviors were considered to constitute mate-refusal behavior rather than mate-avoidance, although no females showed the pierid mate-refusal posture in which the wings are spread and the abdomen elevated (Shapiro, 1970). There was no significant difference between the forewing length of males failing to copulate (wing length = 15.2 ± 0.2 [SE] mm, n = 24) and that of males succeeding in copulation (wing length = 15.6 ± 0.2 mm [SE], n = 9) (*U*-test, Z = 0.788, n.s.).

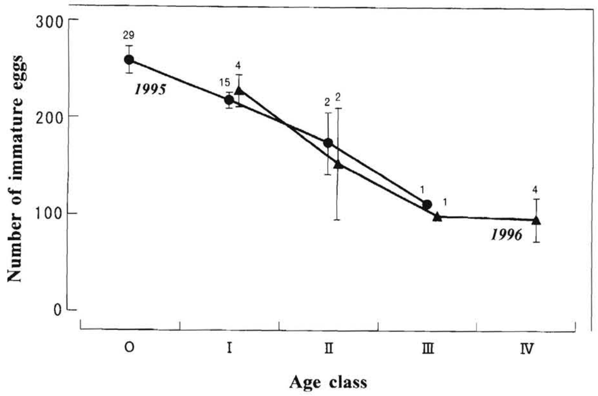

FIG. 2. Changes in the number of immature eggs of age O, I, II, III and IV wild monandrous females of *Lycaena phlaeas* in 1995 (circle) and 1996 (triangle). Each bar represents SE. The numbers above the symbols show sample size.

Out of 77 virgin females aged 2-days at the first trial, only 22 engaged in copulation (28.6%). The other females were re-introduced to the mating experiment on the following day and 35.0% of them (7/20) copulated. In the third trial, 28.6% of the virgin females (2/7) copulated. The remaining virgin females did not copulate with males.

The copulation duration was about 16–17 min, regardless of the number of previous trials (Table 2). When the copulation terminated, most females were

TABLE 2. The number of eggs loaded in accepting and avoiding females during male courtship behavior (mean ± SE).

Females of 1st trial	Accepting	Avoiding	*U*-test
Number of females dissected	22	6	
Copula duration (min)	15′ 33″ ± 41″	—	
Number of mature eggs	62.6 ± 8.9	2.8 ± 2.6	Z = 3. 191, p < 0.01
Number of submature eggs	39.1 ± 3.8	20.8 ± 4.4	Z = 2. 715, p < 0.01
Number of immature eggs	254.7 ± 8.6	228.7 ± 20.1	Z = 1. 225, n.s.
Females of 2nd and 3rd trials	Accepting	Avoiding	*U*-test
Number of females dissected	9	6	
Copula duration (min)	16′ 45″ ± 1′ 22″	—	
Number of mature eggs	60.9 ± 12.8	30.3 ± 11.9	*U* = 8, 0.05 > p > 0.01
Number of submature eggs	32.8 ± 5.0	21.5 ± 5.0	*U* = 13.5, n.s.
Number of immature eggs	227.3 ± 12.3	241.7 ± 7.8	*U* = 18, n.s.

dissected, and their bursa copulatrix and the number of eggs in their ovaries were examined. Every female had a single spermatophore in the bursa copulatrix, though it was not completely solidified in any of the females. The average volume of the bursa copulatrix among mated females was 0.195 ± 0.017 [SE] mm^3 (n = 25), which was significantly larger than that of wild females of age O (*U*-test, Z = 3.182, $p < 0.01$). The average volume of the spermatophore was 0.145 ± 0.015 [SE] mm^3 (n = 20), which occupied 74% of the bursa copulatrix compared with 40% in wild females. Spermatophore volume was also significantly larger than that of wild-caught females of age O (*U*-test, *U* = 37, $p < 0.01$).

After the trial, a number of virgin females who had consistently avoided mating were also examined to determine the number of eggs in the ovaries. As shown in Table 2, females accepting mating at the first trial contained significantly more mature and submature eggs than those who avoided mating at the first trial. The number of immature eggs did not differ among female mating outcomes. At the second and the third trials, females with immature eggs were older and must therefore have further developed their eggs in the ovaries. However, mated females loaded about 60 mature eggs, which was significantly greater than that of the females avoiding mating (30 mature eggs) in Table 2. There was no significant difference in the numbers of submature or immature eggs between females accepting and avoiding mating in either the second or third trials.

Figure 3 shows the change in the number of mature eggs in females reared in the laboratory. Very few mature eggs were found in the ovaries of newly emerged females (0-day-old). It follows that maturation of eggs occurs with aging. About 50 mature eggs had accumulated one week after emergence. Figure 3 also shows that the number of mature eggs in mating females was above the mean number of mature eggs of virgin females, and that the number of females avoiding mating was below it. An increase in the number of mature eggs thus appears to facilitate female willingness to mate.

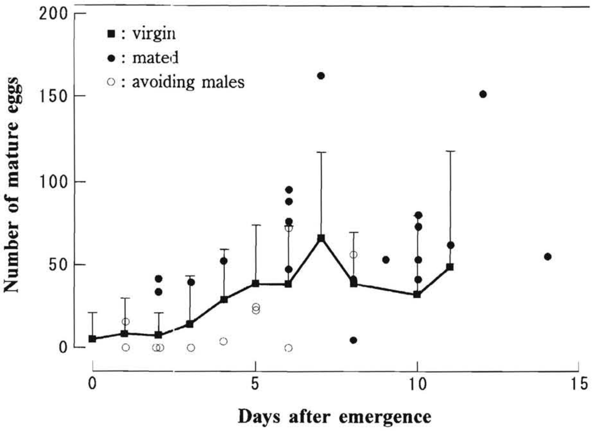

FIG. 3. Daily change in the number of mature eggs of virgin *Lycaena phlaeas* females (squares, + SD) and the number of mature eggs of females accepting copulation (closed circles) and of those avoiding males (open circles) in laboratory experiments.

DISCUSSION

The mating frequency of *L. phlaeas* females was examined in terms of the number of spermatophores in the bursa copulatrix, because each mating generally results in the deposition of a single spermatophore. It is well known that the number of spermatophores in several butterfly species increases with female age (Lederhouse 1981, Watanabe & Nozato 1986, Watanabe & Ando 1993). However, there is little information on the mating frequency of female lycaenid butterflies (Burns 1968, Suzuki 1978). In the present study, most females (94%) of the small copper, *L. phlaeas*, had a single spermatophore in the bursa copulatrix irrespective of their age class. We found a few polyandrous females (6%) that had two spermatophores, one of normal size lying near to the site of sperm migration in the bursa copulatrix and the other being very small or consisting of fragments. Although we did not find any signs that the spermatophore had been absorbed by the female, the spermatophore just after copulation of laboratory-reared young females was larger than that of females of age class O, suggesting that females quickly absorbed the spermatophore. This means that wild-caught females had retained the remnants of the spermatophore. Therefore, the fragments of spermatophore must be derived from a small spermatophore, which might be transferred by old males (e.g., Lederhouse et al. 1989) or by recently mated males (Watanabe et al. 1997). When the first spermatophore is too small for females to avoid re-copulation, females must be exceptionally polyandrous.

Female reproductive success will depend on the amount of three potential resources available to female somatic maintenance or offspring. One is the nitrogenous reserves accumulated during their own larval feeding and stored in the fat body, which is usually depleted with egg development (e.g., Dunlap-Pianka et al. 1977). Another is nectar feeding during the adult stage (e.g., Boggs 1986, Watanabe 1992), though the nectar contains little nitrogen. The third resource is the contributions of the male ejaculate during copulation (e.g., Boggs & Watt 1981). Boggs and Gilbert (1979) showed that the ejaculates were used for egg development by females. In the present study, however, females seemed not to use the ejaculates to increase their fecundity. Bissoondath and Wiklund (1995) stated that both relative ejaculate mass and protein content in the spermatophore are low in monandrous species.

In this study, the small copper females had few mature eggs following eclosion, and they used their own energy or nectar to develop eggs without ejaculates from males. The lifetime reproductive output was estimated at about 150 eggs in the present study. Females feed on nectar as an energy source. On the basis of our experiments in which virgin females were kept in a flight cage and allowed to feed freely on nectar, such females live more than 3 weeks without mating. Boggs (1986) reported that females of the nymphalid butterfly, *Speyeria mormonia*, which is monandrous, survived for an average of 19 days in captivity, laying eggs for 14 days on average.

The present study showed that the ratio of spermatophore volume to the volume of the bursa copulatrix was stable, regardless of the spermatophore size. Sugawara (1979) showed that a certain volume of spermatophore is perceived by the stretch receptors which stimulate the mate refusal posture of the female white butterfly, *Pieris rapae*. The stretch receptors in the bursa copulatrix may also operate in the small copper to induce mate-avoidance behavior over the course of the insect's life, and thereby maintain female monandry.

The present study suggests that the number of mature eggs determines the mating behavior of the female butterflies. This means that there is a period of sexual immaturity and avoidance or refusal of males after eclosion. Therefore, females of this species have a pre-mating adult period, during which they mature initial eggs. One possible explanation for this phenomenon is that it allows females to be more particular in their choice of mate, since the small copper is a perching species, in which males spend part of the day sitting on some object waiting for passing females who are then pursued and courted. Such behavior may be particularly advantageous in a monandrous species. Wiklund (1977) showed that female monogamy in the pierid butterfly, *Leptidea sinapis*, is maintained by the females simply remaining quiescent during courtship, without any kind of mate-refusal posture. However, there is no quantitative information on the diurnal activity of females of the small copper or on their choosing of mates in the field. A detailed study of preferential mating will be required for the monandrous small copper.

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