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Different ejaculate delivery strategies in first versus subsequent matings in the swallowtail butterfly *Papilio machaon* L.

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Summary. Evidence suggests that males incur costs in producing ejaculates. Therefore some discrimination should be expected with respect to their pattern of allocating ejaculates. In a laboratory study, males of the European swallowtail butterfly *Papilio machaon* were mated with conspecific virgin females up to six times. The weight of the spermatophore and of the accessory substances delivered by males, the duration of mating, and the number of sperm in ejaculates were measured. The weight of the first spermatophore was related to male weight but that of accessory substances was not. The weight of the spermatophore delivered by males upon remating was always less than that of the first spermatophore. Second spermatophores produced by males were smallest when delivered on the day after the first mating and increased asymptotically to about half the initial size with time elapsed since the first mating. Copula duration was highest for males remating on the day after the first mating but decreased to the duration of the first mating for males that were remated on the third day after the first mating or later. The weight of accessory substances delivered by males and the number of sperm in ejaculates were also higher in first matings compared to all subsequent matings. Both of two males that were handpaired on three consecutive days after the first mating produced a cumulative ejaculate mass equalling that transferred at the first mating. Thus the fact that males delivered smaller ejaculates upon remating on the third day after the first mating or later requires explanation, especially in view of the fact that copulations were not prolonged. We argue that the male ejaculate delivery strategy in mildly polyandrous species should be to mate for the first time with virgin females, when a maximum size ejaculate should be delivered, and then to maximize the number of matings. The rationale behind the male shift towards smaller ejaculate sizes delivered at subsequent matings is that: (1) the size of the ejaculate that can be produced is dependent on the time elapsed since the last mating; (2) the abundance of virgin females decreases as the flight season proceeds; and (3) there is an inverse relationship between the size and number of ejaculates that a male can produce in his lifetime.

Introduction

In some butterflies nutrients contributed by males through mating may be used for egg production and/or somatic maintenance and so in these species females may benefit from mating more than once (Boggs and Gilbert 1979; Engebretson and Mason 1980; Walker 1980; Boggs 1981). In species where the male ejaculate is used for fertilization of eggs only, females may benefit from mating a second time if the sperm received at the first mating is not sufficient to fertilize all of the eggs (Lederhouse 1981). It is also conceivable that females may mate more than once to avoid male harassment at courtship. However, since copulation in butterflies is quite time-consuming and the number of eggs laid by females is likely to be positively related to the time they can allocate to egg-laying activities, females must have a refractory period between matings. In two species of butterflies observed under laboratory conditions the females mated again 6–9 days after the first mating (David and Gardiner 1961; Suzuki 1979). Available evidence strongly suggests that the length of the refractory period in females is dependent on the size of the first spermatophore (Obara et al. 1975; Sugawara 1979; Rutowski 1980; Rutowski et al. 1981).

From the male's point of view, the traditional contention has been that males can cheaply produce enough sperm to inseminate many females. However, recent evidence suggests that males "incur non-trivial costs in producing ejaculates, that the number of ejaculates they can produce is limited, and that therefore some discrimination should be expected with respect to their patterns of allocating ejaculates" (Dewsbury 1982; cf. also Rutowski 1979; Walker 1980; Marshall 1982; Svärd 1985).

Given that there are severe limits to how much sperm a male can produce in his lifetime, how should this influence the size of subsequently delivered ejaculates, and to what extent is ejaculate size dependent upon the time elapsed since the male's previous mating? In this study we address these questions by hand-pairing males of the swallowtail butterfly *Papilio machaon* L. up to six times with virgin females, allowing males to rest for 1–11 days between matings.

Methods

The individuals of *P. machaon* used in the experiments were the offspring of swallowtails collected in the larval stage in the wild at four different localities in southern and central Sweden in 1983. In 1984 these wild-collected butterflies were hand-paired and their offspring reared on the natural host plant *Peucedanum palustre*. The larvae pupated in the middle of June and the pupae were transferred on 2 July to a refrigerator maintained at 0 °C. After a chilling for some $4\frac{1}{2}$ months the pupae were brought to 23 °C and a 18 h light: 6 h dark cycle on 16, 21, and 23 November. The adults emerged between 31 November and 16 December.

After eclosion females were kept at 8 °C until used for hand-mating. Most females were mated on the day of eclosion but a few were mated 1–3 days later. Males were also kept at 8 °C, but also spent 4 h every day in a flight cage at 30°–35 °C, except on days when they were mated. Each day, and after each mating, males were given free access to a 1:3 sucrose: water solution presented in an artificial flower.

On days when hand-pairings were carried out, males and females were allowed to fly freely in a 1 × 1 × 1-m flight cage at 30°–35 °C for about 1 h, after which they were mated by hand. The pair in copula was then immediately put in a 0.25 × 0.25 × 0.40-m cage and transferred to a cabinet kept at 25.0°–0.1 °C in full light. The pair was then observed every 10 min to estimate copula duration. After mating, males and females were weighed on a Sauter AR1014 electrobalance. Three males were mated for the first time on the day of eclosion, six after 1 day, two after 2 days, one after 3 days, two after 4 days and one after 6 days. Twelfe of the males were remated after 1, 2, 3, 5, or 10 days.

Immediately after copula termination females were decapitated and dissected under a stereo microscope. The bursa copulatrix and its content were then extracted and weighed on a Cahn 28 Automatic Electrobalance, after which the bursa was again put under the stereo-microscope and the spermatophore proper dissected out from the bursa and weighed. The average weight of the empty bursa copulatrix of *Papilio machaon* was assessed by dissecting out and weighing the empty bursae from three unmated females. Thus we obtained values of: (1) the whole male ejaculate (by subtracting the mean bursa weight from the weight of the full bursa); (2) the spermatophore proper; and (3) the accessory substances not contained within the spermatophore (by subtracting the spermatophore weight and the mean bursa weight from that of the full bursa).

After weighing, the spermatophore was homogenized in a 0.25 ml solution containing $NaHCO_3$ (50 g), 35% HCHO (10 ml), and distilled water (1000 ml). Two samples were taken from the homogenized spermatophore, and in each the number of sperm was counted in a hemacytometer. From the mean of the two samples, the total number of sperm in the spermatophore was calculated, taking into account the dilution procedure.

All values are given as mean ± standard deviations.

Results

The mean duration of the first copulation was 60 ± 8 min ($n = 12$). Taking into account the mean bursa weight of 2.09 ± 0.13 mg ($n = 3$) the mean weight of the first ejaculate was 8.59 + 1.15 mg ($n = 12$), representing 2.8% of the male body weight. The mean spermatophore weight was 5.24 ± 0.66 mg and that of the male accessory substances was 3.35 ± 0.78 mg.

The weight of the first spermatophore was dependent on male weight ($r^2 = 0.57$, $F_{15} = 17.07$, $P < 0.005$), whereas that of male accessory substances was not (Fig. 1). Neither the weight of the spermatophore nor that of accessory substances was dependent on days since eclosion.

When males were remated on the day after the first mating, copula duration increased 9-fold, and for males remated on the 2nd day after the first mating copula duration was still some 4 times that of the first mating (Fig. 2a). Males remated on

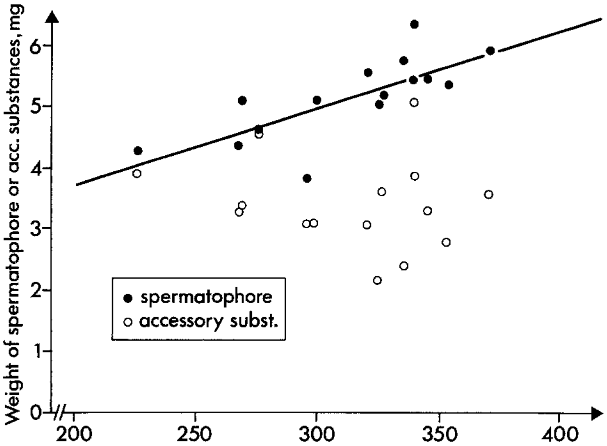

Fig. 1. Spermatophore weight (●) and weight of accessory substances (o) in relation to male body weight. The former relationship is statistically significant ($y = 0.013\ x + 1.212$, $r^2 = 0.57$, $F_{15} = 17.07$, $P < 0.005$) whereas the latter is not ($F_{15} = 0.12$, $P > 0.5$)

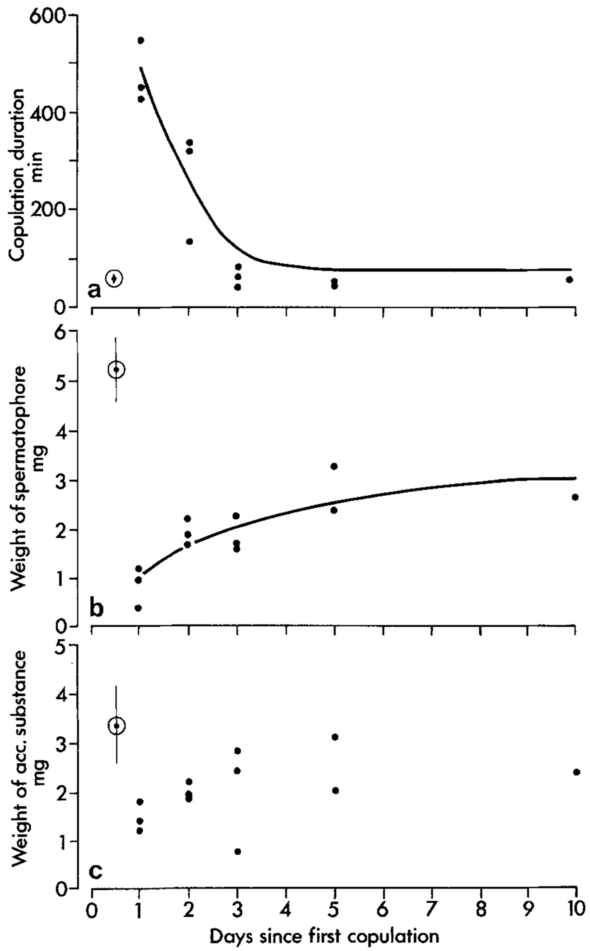

Fig. 2. a Copula duration of second matings by males in relation to time elapsed since the first mating ($y=427\ x^{-2}+64$, $F_{12}=45.54$, $r^2=0.82$, $P<0.001$). Mean duration of the first copulation ±1 SD is indicated by ⊙. **b** Spermatophore weight of second matings by males in relation to time elapsed since the first mating ($y=2.15\log x+1.04$, $F_{12}=26.98$, $r^2=0.73$, $P<0.001$). Mean spermatophore weight of first matings ±1 SD is indicated by ⊙. **c** Weight of accessory substances delivered by males at second matings in relation to time elapsed since the first mating ($F_{12}=3.87$, $P>0.1$). Mean weight of accessory substances delivered at the first mating ±1 SD is indicated by ⊙

the 3rd day after the first mating or later had copula durations similar to that of the first mating (Fig. 2a). Thus the duration of the second copulation was dependent on time elapsed since the first copulation ($r^2=0.82$, $F_{12}=45.54$, $P<0.001$).

The weight of the second spermatophore delivered by males upon remating was always less than that of the first spermatophore ($\bar{x}_{1\text{st}}=5.24\pm0.66$ vs $\bar{x}_{2\text{nd}}=1.88\pm0.78$; $t_{24}=11.39$, $P<0.001$). Spermatophore weight decreased 5-fold for males remated on the day after the first mating, and for males remated on the 2nd day after the first mating or later spermatophore weights increased asymptotically with time elapsed since the first mating to a value about half of that of the first spermatophore ($r^2=0.73$, $F_{12}=26.98$, $P<0.001$; Fig. 2b).

The weight of the accessory substances delivered by males upon remating was also always lower than that of the accessory substances transferred at the first mating ($\bar{x}_{1\text{st}}=3.35\pm0.78$ vs $\bar{x}_{2\text{nd}}=2.00\pm0.66$; $t_{24}=4.55$, $P<0.001$), but there was no orderly gradual increase in the accessory substances delivered after the first mating as was the case for spermatophores ($F_{12}=3.87$, $P>0.1$; Fig. 2c). The pattern of delivery of the whole ejaculate follows that exhibited by the spermatophores ($r^2=0.58$, $F_{12}=13.59$, $P<0.01$).

The number of sperm delivered at the first mating was significantly higher than that transferred at second matings ($\bar{x}_{1\text{st}}=1.9\times10^5\pm1.0\times10^5$ vs $\bar{x}_{2\text{nd}}=4.9\times10^4\pm2.8\times10^4$; $t_{24}=4.61$, $P<0.001$). This was mainly due to the smaller size of secondary spermatophores as evidenced by the fact that sperm density, i.e. number of sperm per mg, did not differ significantly between 1st and 2nd matings ($\bar{x}_{1\text{st}}=3.6\times10^4\pm2.0\times10^4$ vs $\bar{x}_{2\text{nd}}=2.6\times10^4\pm1.8\times10^4$; $t_{24}=1.32$, $P<0.2$).

Table 1 shows that both of the two males that were hand-paired on the three days following the first mating delivered a cumulative ejaculate mass equalling that transferred at the first mating (male no 6. refused to be hand-paired on the 3rd day as did male no. 36 the 4th). Thus from a physiological point of view males that did not remate until the 3rd day after the first mating should be physically capable of delivering an ejaculate mass equal to that transferred at the first copulation. Nevertheless, all of the six males that were remated on the 3rd day after the first mating, or later, i.e. males nos. 51, 53, 39, 17, 37, and 2, delivered a second ejaculate mass that was only 54% of their first ejaculate ($\bar{x}_{1\text{st}}=8.61\pm1.62$ vs $\bar{x}_{2\text{nd}}=4.70\pm1.31$). This indicates that male *P. machaon* adopt two different ejaculate delivery strategies, one for first matings and another for subsequent copulations.

This conclusion is corroborated by the observation that copula duration is down to 60 min for males remating on the 3rd day after the first copulation or later, i.e. the period typical of first copulations (Fig. 2a). Thus, although males are physically capable of copula durations up to 550 min, and increased copula durations should make it possible for males to deliver a larger ejaculate mass, males remated on the 3rd day or later ended copulation after some 60 min, when the ejaculate mass delivered was some 50% of the ejaculate delivered at the first mating. Because the two males that remated daily up to the 3rd day after the first

Table 1. Ejaculate (Eja), spermatophore (Spe), and accessory substance mass (Acc) delivered by male *P. machaon* at first and subsequent matings in relation to the time elapsed since the first mating on day 0, and duration of copulations in minutes (Tim). Values are mean weights in mg ±SD

Male no.	Substance	Days of mating						
		0	1	2	3	4	5	10
4, 6, 36	Eja	8.60±0.66	2.35±0.61	3.53±0.55	2.61±0.36	2.42±0.31	–	3.50
	Spe	5.06±0.64	0.87±0.41	1.50±0.30	1.52±0.02	1.41±0.11	–	2.39
	Acc	3.54±0.65	1.48±0.28	2.04±0.65	1.08±0.34	1.01±0.42	–	1.02
	Tim	52±3	476±65	316±157	335±49	198±67		50
		(*n*=3)	(*n*=3)	(*n*=3)	(*n*=2)	(*n*=2)		(*n*=1)
26, 27, 45	Eja	8.71±0.42	–	3.99±0.21	–	2.75±0.75	–	–
	Spe	5.52±0.27	–	1.94±0.26	–	1.73±0.42	–	–
	Acc	3.20±0.69	–	2.06±0.13	–	1.02±0.33	–	–
	Tim	62±3	–	263±111	–	83±58	–	–
		(*n*=3)	–	(*n*=3)	–	(*n*=3)		
51, 53, 39	Eja	7.94±0.92	–	–	3.91±1.35	–	–	–
	Spe	4.84±0.90	–	–	1.92±0.35	–	–	–
	Acc	3.09±0.02	–	–	1.99±1.09	–	–	–
	Tim	68±11	–	–	65±20	–	–	–
		(*n*=3)		–	(*n*=3)			
17, 37	Eja	9.41±2.97	–	–	–	–	5.42±1.38	–
	Spe	5.76±0.88	–	–	–	–	2.86±0.59	–
	Acc	3.64±2.09	–	–	–	–	2.56±0.79	–
	Tim	63±4	–	–	–	–	51±1	–
		(*n*=2)					(*n*=2)	
2	Eja	8.51	–	–	–	–	–	5.13
	Spe	5.11	–	–	–	–	–	2.72
	Acc	3.40	–	–	–	–	–	2.41
	Tim	55	–	–	–	–	–	60
		(*n*=1)						(*n*=1)

copulation, males nos. 4 and 36, had a mean copula duration of 335 min when remating on the 3rd day, compared to a mean of 65 min for males nos. 51, 53 and 39 when performing their first remating on the 3rd day after the first copulation (Table 1), we conclude that males maximize ejaculate mass at first matings but not at subsequent matings.

Discussion

Our results indicate that the cost of mating is high for male *P. machaon*, that they have different ejaculate delivery strategies for first relative to subsequent matings, and that they invest heavily in the first copulation but substantially less in all subsequent matings.

Traditional sexual selection theory predicts that females should be more discriminatory when choosing a mate than males, due to their greater investment in their offspring. Although we believe it to be futile to try and decide which sex exerts the strongest influence on mate choice, our results lead to the prediction that male butterflies should be very selective when choosing their first mate but should be less discriminatory when mating a second or third time. Wiklund and Forsberg (in press) have shown that male orange tip butterflies, *Anthocharis cardamines*, exhibit mate discrimination. They court virgin females very intensively (for up to 120 s), but immediately terminate courtship of females that exhibit the "elevated abdomen" as a sign that they have already mated (cf. also Rutowski 1982a, b). Evidence both from *Heliconius erato* (Gilbert 1976) and *Anthocharis cardamines* (Wiklund and Forsberg 1986) indicates that males transfer antiaphrodisiac pheromones to females at mating. In fact the ability of males to discriminate between virgin and mated females should benefit all three parties concerned: the mated female who is less harassed by courting males and accordingly can allocate more time to egg-laying; the mated male whose paternity of the eggs laid by the female is secured; and the unmated males who would not benefit from "wasting" their first ejaculate on a mated, and thus presumably older, female whose reproductive value is not as high as that of virgin females.

In view of the heavy reproductive cost in males, wild-flying males in the field are likely to differ fundamentally in their capacity to produce large ejaculates, depending on whether they have mated (and in that case how recently) or not. Since receiving a full ejaculate is likely to be important for females it seems reasonable to assume that females would benefit from being able to discriminate between mated and unmated males. Rutowski (in press) has shown that female *Colias eurytheme* prefer highly UV-reflecting males to males exhibiting less UV reflectance and that old males reflect less ultraviolet from the dorsal wing surface than fresh males. Female preference for strongly UV-reflecting males may be viewed as means of maximizing the probability of mating with a fresh and unmated male. There would be a more certain way for females to distinguish between mated and unmated males if male courtship persistence were correlated with the ability to deliver a large ejaculate. Rutowski (1979) has shown this to be the case in *Pieris protodice* where males indeed behave as "honest salesmen", although the reason for this correlation is poorly understood from the male's point of view.

To what extent should male butterflies exhibit a predetermined pattern of ejaculate sizes delivered at first and subsequent matings, as do males of *P. machaon*? We believe that this question strongly relates to the degree of monandry vs. polyandry exhibited by females. In a mildly polyandrous species like *P. machaon* (Watanabe and Nozato, in press) we believe that a deterministic pattern should be favoured. The rationale is as follows: Because the reproductive value of females decreases with age, and the length of the refractory period in females probably increases with the size of the spermatophore, males should allocate most of their mating resources to copulations with virgin females. Since virgin females are more common early in the female flight period young males are more likely to encounter unmated females than are old males. Thus, it seems reasonable that the largest ejaculate should be allocated to the first mating of the male's life. In *P. aegeria* Svärd (1985) has shown that it takes 7 days after the first mating before males can produce a second spermatophore as large as the first one. Thus males have the option of producing a second ejaculate as large as the first, in which case recuperation time will be long, or to produce a second ejaculate that is smaller than the first, in which case recuperation time will be shorter. The rationale for why the option of a smaller second spermatophore may be the best alternative is as follows: First, the number of unmated females in seasonal butterflies decreases with the flight period so that the probability of finding a virgin female is inversely proportional to male recuperation time. Second, sperm precedence by the last male appears to be the rule in butterflies (Clarke and Sheppard 1962; Labine 1966; Sims 1979) and ensures that the latest male to mate with a female will father the majority of eggs laid by the female on the following days. Third, as a female has a relatively lower reproductive value when she mates a second time compared to when she mates for the first time, it may be unprofitable to males to invest in large ejaculates when mating with older females. Fourth, it is not unusual for females to remate before the previously received spermatophore(s) have been completely emptied in the bursa. Therefore it may be that males in species that meet the assumptions produce smaller second and third ejaculates because of the probable lack of room in the bursae of previously mated females (Rutowski, personal communication). Fifth, the number of matings that a male can perform is inversely correlated with the cumulative previous ejaculate mass, and in consequence it may be more effective to allocate the resources for ejaculate production after the first mating to a number of smaller, secondary, ejaculates.

However, in monoandrous species males should always maximize ejaculate size since all females that they mate with are likely to be unmated. Accordingly, in species where females are monogamous we expect ejaculate size to increase with time elapsed since the last mating performed by the male. Svärd (1985) has shown that this pattern is indeed observed in the monoandrous butterfly *Pararge aegeria* (Wickman and Wiklund 1983).

In general we believe that the pattern followed by males in strongly polyandrous species should resemble that of the mildly polyandrous species where males invest heavily in first ejaculates, but less heavily in subsequent ones. The reason for maximizing first ejaculates is identical to the reasons given above, and that for investing less heavily in subsequent matings is that the likelihood of mating with an already mated female must be great in polyandrous species, and the reproductive value of mated females is much lower than that of virgin females. However, since the probability that a female will remate several times is high in strongly polyandrous species, and the time during which the female is likely to remain unreceptive to subsequent male courtship is proportional to the size of the spermatophore delivered, we expect that males in strongly polygynous species will tend to deliver secondary ejaculates that are larger than those delivered by males in mildly polyandrous species.

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