*MATING FREQUENCY IN NATURAL POPULATIONS OF SKIPPERS AND BUTTERFLIES AS DETERMINED BY SPERMATOPHORE COUNTS**

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Communicated by Curt Stern, September 6, 1968

In the course of copulation, a male lepidopteran typically leaves a spermatophore—rather than an amorphous ejaculate—in the bursa copulatrix of the female. This attribute can readily be turned to an investigator's advantage in diverse evolutionary and behavioral studies of Lepidoptera: spermatophore counts in dissected wild females are useful in analyzing genetic polymorphism, preferential mating, and mating frequency.

Genetic analysis can start directly with wild-caught females: when eggs have been secured, a wild female is sacrificed, and larvae are reared only from those females found to contain but one spermatophore. Their progeny necessarily represent single-pair matings. In isozyme studies, the genotype of a wild female can normally be inferred from electrophoretic mobility patterns obtained from tissue homogenates when the female is sacrificed. Only the lone male parent remains unknown, so that at worst much valuable information and at best a complete genetic interpretation can be extracted from the first generation reared. Since Lepidoptera are often difficult and time-consuming to rear and upon continued rearing in the laboratory are often devastated by microbial and viral infections, the ability to short-circuit one generation can be crucial. This elliptic approach has been successful in recent genetic analyses of complex esterase polymorphisms in natural populations of *Colias eurytheme* (Pieridae)[1] and *Hemiargus isola* (Lycaenidae),[2] in which one to two dozen esterase alleles occur at single loci in single natural populations. In a different though related way, spermatophore counts have facilitated genetic analysis of sex-limited wing-color dimorphism in *Poanes hobomok* (Hesperiidae).[3]

Preferential mating can be detected and its magnitude estimated where males are presented with a choice, as, for example, in diurnal lepidopterans exhibiting color-pattern polymorphism sex-limited to the female. In *Papilio glaucus* (Papilionidae), a species in which males are always tiger-striped (black on yellow), whereas females are either malelike (and nonmimetic) or totally dark (and mimetic of a distasteful species), the mean number of spermatophores per wild female was about the same (1.72 and 1.75) in samples from two different populations. However, on an average, the nonmimetic morph consistently exceeded the mimetic morph (by 0.34 and 0.39 spermatophores per female) in these samples. Relative to the nonmimetic morph, the mimetic morph was mated only about 81 per cent as frequently.[4] Such a mating preference must be counted among the selective forces influencing the relative proportions of mimetic and nonmimetic morphs in natural populations.[4, 5]

Mating frequency is of evolutionary interest because it is a factor affecting the speed with which genetic variants can be shuffled in a population.[6] Interacting with other factors in a variety of contexts, mating frequency may signifi-

cantly accelerate or retard the process of population differentiation. Although the tools are at hand, it has not been systematically studied in a comparative way in natural populations of diurnal Lepidoptera.

Two assumptions are made in using spermatophore counts in wild-caught females to determine mating frequency: (1) that the male deposits but one spermatophore per successful mating, and (2) that the spermatophore can always be recognized, even though subject to erosion, collapse, and considerable dissolution with time.[4] As will be shown, these assumptions are generally valid.

With respect to the first assumption (one spermatophore per mating), reared females—known virgins—have been mated in the laboratory and subsequently dissected for spermatophores. Altogether, several hundred females have been tested in this way in certain species of moths, e.g., *Carpocapsa pomonella* (Olethreutidae),[7] *Grapholitha molesta* (Olethreutidae),[8] *Pectinophora gossypiella* (Gelechiidae),[9] and *Atteva punctella* (Yponomeutidae).[10] In no female has the number of spermatophores present ever exceeded the number of times she has mated. Although correspondence is usually one to one, the count is sometimes low because some copulations are unsuccessful and no spermatophore is passed. (In highly artificial experiments inordinately maximizing mating opportunities for males of *Grapholitha molesta*, females sometimes were successfully inseminated without receiving a spermatophore. This happened when a male engaged in a relatively long or rapid succession of matings and the female in question came late in the series.[11]) In diurnal Lepidoptera, virgin females used in successful single-pair matings have always been found to contain one spermatophore: 12 females of *Poanes hobomok* (Hesperiidae),[3] 14 females of *Colias eurytheme* (Pieridae),[3] and a total of 41 females of *Limenitis arthemis* and *L. archippus* (Nymphalidae).[12] Wild-caught females of various butterflies and skippers commonly have but one spermatophore (Table 2).

When two or more spermatophores are present, it is often obvious that they have come from different matings at different times because (1) they hold sequential positions in the blind, saclike, posteriorly opening bursa copulatrix—with the oldest farthest anterad—and (2) they display distinct stages of erosion and/or collapse—with the newest (posteriormost) spermatophore the fullest. *Epargyreus clarus*, for example, is one of a number of species examined in which spermatophores are especially resistant to erosion; in every wild female I have dissected that has had more than one spermatophore (i.e., 22 ♀♀ with two and 2 ♀♀ with three; see Table 2), the older one or two spermatophores have been shoved, stalk and all, quite to the blind end of the bursa, whereas the newer spermatophore has been situated well caudad with its stalk still in the ductus bursae—a position the spermatophore typically is in when only one is present.

With respect to the second assumption (that spermatophores last), reared females kept alive for varying numbers of days after being mated show their lone spermatophore clearly. Similarly, wild-caught females sustained in the laboratory for varying periods before dissection still have spermatophores. Provided their abdomens are previously soaked in a 0.5 per cent aqueous solution of trisodium phosphate (which softens and restores desiccated tissues),[13] even females that have been dead and dry in collections for years can be dissected for sperma-

tophores. In most of the species I have so far sampled from nature, spermatophores are readily discernible, although they may be collapsed or eroded.

There are exceptions to spermatophore persistence, however. I have dissected seven spermatophoreless females that (1) were all quite worn (therefore well-flown) and (2) were caught in areas where males of their species were frequent enough that these females could scarcely have avoided mating: three ♀ ♀ *Atlides halesus* (Lycaenidae); and two ♀ ♀ *Erynnis horatius*, one ♀ *E. funeralis*, and one ♀ *Pyrgus communis* (three rather closely related pyrgine hesperiids). That three of the "empty" females had in fact mated was manifest: two females of *A. halesus* still had small but unmistakable fragments of the spermatophore stalk in the ductus bursae, although the bulk of the spermatophore was gone; in the lone female of *E. funeralis* the spermatophore had totally disintegrated, but an egg awaiting oviposition (past the point in the reproductive tract at which sperm enter the micropyle) contained a live, fully formed, first-instar larva. In contrast to such old, worn females secondarily lacking spermatophores, true virgins are, as a rule, in excellent condition (freshly emerged) and except in special situations are rarely encountered. Preliminary investigation to decide which species are "reliable" for spermatophore analysis of mating (i.e., which have suitably durable spermatophores) is mandatory.

A source of bias in sampling natural populations to determine average mating frequency requires comment. The older the female the more likely she is to have mated multiply. Hence, in a species with a discrete flight period in which the population of adults is more or less normally distributed in time, the mating frequency distribution can be shifted upward or downward, depending upon when the sampling is done. Under these circumstances, a satisfactory way of ascertaining the average condition in the population is to sample at regular intervals throughout the flight period and pool the data. On the other hand, taking large numbers in a short span in roughly the middle of the flight period is often both effective and accurate. The latter approach is especially appropriate for common and ecologically widespread species that emerge over a considerable period and for multivoltine species, whose adult temporal distribution after the first flight period in any year tends to become smeared,[14] producing a situation more favorable for sound "instant" sampling. There is evidence that in some species of moths mating frequency increases as the season progresses, and particularly that in multivoltine species, later generations show more multiple mating than the first.[8, 15] Thus it is advisable not only to specify whether sampling is done all at once or over an interval, but also to indicate at what time it is done. Moreover, it is well to state whether or not a sample is taken at a very restricted locality, i.e., whether or not it is drawn from a single population of presumably interbreeding individuals at essentially a "point" in space. Such "point" sampling, which is preferable whenever practical, was carried out for most of the skipper species and about half of the butterfly species investigated. Spatial and temporal specifications of the samples used in this study appear in Table 1.

Comparative data on mating frequency appear in Table 2. Mating frequency varies widely among species: females of some rarely mate more than once; females of others regularly do so; those of still others lie between these

TABLE 1. *Spatial and temporal specifications of lepidopteran population samples used in mating frequency determinations.*

Species	Where sampled	Sampled at one restricted locality	When sampled	No. days sampled	Voltinism where sampled*	Generation sampled in M species†
Thymelicus lineola	Mich.	Yes	6/26/67	1	U	—
Atalopedes campestris	Tex.	Yes	4/18/67–5/2/67	6	M	S
Poanes viator	Tex.	Yes	7/10 & 15/67	2	M	S
Wallengrenia otho	Tex.	Yes	4/20/67–5/27/67	12	M	F
Polites mystic	Va.	Yes	6/13–29/65	4	U	—
Hesperia sassacus	Va.	No	6/12–19/65	4	U	—
Euphyes vestris	Tex.	Yes	4/20/67–5/27/67	11	M	F
Lerema accius	S.C.	Yes	8/24–27/66	4	M	S
Epargyreus clarus	Va.	No	6/18/65–7/2/65	7	M	S
Cercyonis pegala	Va.	Yes	8/1 & 6/65	2	U	—
Speyeria cybele	Va.	No	7/23/65–8/15/65	9	U	—
"	Md.	Yes	8/24–31/65 8/30/66 & 9/3/66 9/1–4/67	11	U	—
Danaus gilippus	Tex.	No	6/22/67–8/11/67	10	M	S
Pieris rapae	Va.	No	8/2–16/65	9	M	S
Battus philenor	Va.	No	7/30/65–8/3/65	3	M	S
Papilio glaucus	Va.	No	6/13/65–8/19/65	18	M	S
"	Md.	Yes	8/24–31/65	6	M	S
"	Md.	Yes	9/1–4/67	3	M	S

* U = univoltine, M = multivoltine.
† F = first generation, S = subsequent generation.

extremes. Like the genitalia themselves, spermatophores are often species-specific in form; but unlike the genitalia, their form can change in time. Nevertheless, in fresh condition they can be of taxonomic value; and certainly their frequency in females constitutes a distinct ethologic taxonomic character.

Reproducibility of mating frequency data is critical in this connection and from the beginning of this study has been examined from diverse angles.

Influence of sample size was deliberately investigated in *Epargyreus clarus* and *Wallengrenia otho*. It appears that for species like these, with intermediate to low mating frequencies, an efficient sample is 30–45 females: above this range, each major increment (culminating in total samples of 62 *E. clarus* and 171 *W. otho*, Table 2) did not shift the frequency distribution nor alter the mean number of spermatophores per female by more than 0.01 or 0.02. For species averaging two or more spermatophores per mated female, in which not only the mean but also the variation in spermatophore counts among individuals is high, a larger sample is desirable.

Consistency of results both in space and in time is exemplified by *Papilio*

TABLE 2. *Female mating frequency in natural populations of skippers (Hesperiidae)*

Classification	Locality	Date (month and year)	No. females
Hesperiidae			
Hesperiinae			
Hesperia Group[16]			
Thymelicus lineola	Ann Arbor, Mich.	6/67	54
Atalopedes campestris	Austin, Tex.	4–5/67	55
Poanes viator	Clear Springs, Guadalupe Co., Tex.	7/67	79
Wallengrenia otho	Austin, Tex.	4–5/67	171
Polites mystic	MLBS, Va.*	6/65	49
Hesperia sassacus	MLBS, Va.*	6/65	31
Euphyes vestris	Austin, Tex.	4–5/67	46
Apaustus Group[16]			
Lerema accius	Galivants Ferry, Horry Co., S.C.	8/66	44
Pyrginae			
Urbanus Group[17]			
Epargyreus clarus	MLBS, Va.*	6–7/65	62
Papilionoidea			
Nymphalidae			
Satyrinae			
Cercyonis pegala	MLBS, Va.*	8/65	28
Nymphalinae			
Speyeria cybele	MLBS, Va.*	7–8/65	42
" "	Baltimore, Md.	8/65 8–9/66 9/67	26
Danainae			
Danaus gilippus	NW Travis Co., Tex.	6–8/67	50
Pieridae			
Pierinae			
Pieris rapae	MLBS, Va.*	8/65	49
Papilionidae			
Papilioninae			
Battus philenor	MLBS, Va.*	7–8/65	33
Papilio glaucus	MLBS, Va.*	6–8/65	84
" "	Baltimore, Md.	8/65	29
" "	Baltimore, Md.	9/67	92

* Mountain Lake Biological Station, Giles County, Virginia, and vicinity.

glaucus (Table 2). In 1965, the mean number of spermatophores per female was 1.75 in mountains of southwestern Virginia and 1.72 at Baltimore, Maryland, 250 air miles away. At Baltimore, when the same limited area[4] was sampled two years later (1967) at the same season, the mean was 1.73. Similarly, in *Speyeria cybele*, almost identical mean female mating frequencies (1.05 and 1.04) were obtained for a sample taken in 1965 in southwestern Virginia and a Baltimore sample pooled from small subsamples, all taken in the same restricted area in 1965, 1966, and 1967 (Table 2).

In skippers, and especially in butterflies, the efficiency with which the sexes establish contact—as evidenced by the rarity of virgins—is impressive. I find that a male lepidopteran has almost always beaten me to any given female (Table 2). In favorable seasons—i.e., seasons that are neither too cold nor too dry—virgins of most species are apparently receptive soon after emerging from a pupa. Once mated, females of most species seem to reject males, at least for a considerable period. In general, then, virgin females are likely to lose their virgin status rapidly, whereas once-mated females are much less likely to mate

and butterflies (Papilionoidea) as determined by spermatophore counts.

Number of Spermatophores											Mean no. spermatophores per mated female
0	1	2	3	4	5	6	7	8	9	10	
Hesperiidae											
	49	5									1.09
1	50	4									1.07
	74	5									1.06
3	149	17	2								1.13
	37	9	2	1							1.33
2	21	6	2								1.34
2	28	12	4								1.45
8	17	7	9	1	1	1					2.03
3	35	22	2								1.44
Papilionoidea											
	27	1									1.04
	40	2									1.05
	25	1									1.04
1	17	14	10	2		1	2	2		1	2.63
2	12	7	16	9	3						2.66
	17	11	3	1	1						1.73
	39	32	10	1	2						1.75
	12	13	4								1.72
	34	49	9								1.73

again within a short time. The best procedure in calculating average number of spermatophores per female is to exclude virgins and base the calculation strictly on mated females.

Particularly in skippers, it appears that mating frequency may be inversely correlated with population density. In high-density populations, mating frequency is notably low: each of the first four species listed in Table 2 (*Thymelicus lineola, Atalopedes campestris, Poanes viator,* and *Wallengrenia otho*) was very common in the limited area in which it was sampled; and females of each of them rarely mated more than once. Populations of *P. viator* and *T. lineola* were especially dense. *P. viator* is one of a number of North American hesperiine skippers that are obligate marsh dwellers; rarely if ever encountered beyond the borders of their special habitat, these species sometimes abound within it. *P. viator* was intentionally sampled toward the end of a flight period, when both sexes were still abundant but all specimens were somewhat worn to very worn. Despite this attempt to bias the data in favor of multiple mating, only 5 of 79 females sampled had mated a second time, and the average mating fre-

quency was the lowest obtained for any skipper. *T. lineola*, an introduced Palearctic species currently colonizing much of North America, is not yet in ecologic adjustment in its New World holdings and frequently attains population densities enormous for a hesperiid.[18] It was sampled in mid-flight period when it was extraordinarily common.[19] Virgin females were not found in *P. viator* or *T. lineola* and were scarce in *A. campestris* and *W. otho* (< 2% of the sample).

At the other extreme is *Lerema accius*, which occurred in a relatively low density population and mated far more frequently. This suggests that in less dense populations, in which males presumably encounter females less often, females mate more readily when the opportunity does arise. The fact that many more virgins (18% of the sample) as well as more multiple matings were found indicates that females are mated less efficiently: because females are more receptive, males mate more frequently in lower-density populations but often mate with already mated females, thereby leaving a larger proportion of the total female population unmated at any given point in time.

The remaining skipper species (*Polites mystic, Hesperia sassacus, Euphyes vestris,* and *Epargyeus clarus*) were intermediate with respect to both population density and mating frequency. And in all except *P. mystic* (which had the highest population density of these four species), virgin females occurred at appreciable frequency (4–6% of the sample).

Although the suggested inverse correlation between population density and mating frequency is less well supported by data from butterflies, it is significant that *Danaus gilippus*, a mobile species, was sampled in a much dispersed, very low density state, and that it (together with *Pieris rapae*) set the record for high mating frequency. These two species, in which multiply mated females were so common, are also the only butterflies in which virgins were found.

In a summary of the North American history of the invading Eurasian skipper *T. lineola* and an analysis of its capacity to spread, I argued that the founder principle must be operating strongly in the recurrent process of colony formation.[18] Eight wild-caught females successfully launched a population in an unoccupied field in which they were released; and it seemed likely that even one displaced female, or a row of her eggs transported on a piece of hay, could start a new colony. To these points can now be added the fact that females of this species usually mate but once, with the result that a founding female will generally carry genetic information from only herself and a single male.

In species of skippers and butterflies in which females mate only about once, gene flow from deme to deme may be extremely low, being accomplished chiefly by emigrating females ovipositing in foreign areas. Since females of most species appear to be mated promptly upon emerging from the pupa, they are very likely mated by males of their own local population. By the time dispersing males reach another population, females there will probably have been serviced already. Speculations similar to these have been made with reference to the butterfly *Euphydryas editha* (Nymphalidae).[6, 20] Low female mating frequency may sometimes be correlated with extensive microgeographic variation, which nowadays can be profitably assessed at the molecular level with electrophoretic techniques. In natural populations of numerous species of skippers and butter-

flies, esterases in particular are highly variable[1-3] and provide excellent systems for just such detection and analysis of microgeographic differentiation.

Summary.—Typically, with each mating, a male lepidopteran leaves one spermatophore in the female, where it persists indefinitely. The number of spermatophores in a dissected female therefore shows how many times she has mated. Spermatophore counts in wild-caught females are invaluable in analyzing genetic polymorphism, preferential mating, and mating frequency. Among species of skippers and butterflies, mating frequency varies greatly, from little more than once to several times per female. Virgins are generally rare. Mating frequency appears to be species-specific and, especially in skippers, to be inversely correlated with population density. The low mating frequency of *Thymelicus lineola*, a colonizing skipper, must enhance the operation of the founder principle in that species. Certain assumptions, reservations, and sampling problems involved in various aspects of this work are critically discussed.

I thank the many individuals who, in various ways, have furthered this project, and specifically acknowledge the fine technical assistance lent by Cynthia G. McWhorter, Barbara L. Scott, and Rogene G. Gillmor.

* This research was supported in part by a National Science Foundation grant (GB 5935) and by Mountain Lake Biological Station of the University of Virginia (National Science Foundation grant GB 3439).

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