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MALE GAMETIC STRATEGIES: SPERM SIZE, TESTES SIZE, AND THE ALLOCATION OF EJACULATE AMONG SUCCESSIVE MATES BY THE SPERM-LIMITED FLY *DROSOPHILA PACHEA* AND ITS RELATIVES

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Abstract.—The gametic strategy of males comprises the amount of energy invested per sperm, the total amount invested in sperm production, and the pattern of sperm allocation among successive reproductive bouts. All of these variables were measured for each of the four species constituting the *nannoptera* species group of the Drosophilidae. Extreme interspecific variation was identified for all variables and enigmatic male reproductive strategies, including submaximal insemination of females, partitioning of ejaculate among successive mates, and production of few large sperm, were observed. Variation among species in female remating behavior was found to occur concomitantly with male remating behavior, probably because of female fertility demands. Relationships among testes size, sperm size, sperm numbers, and mating systems in these fruit flies are examined. These relationships are not consistent with patterns identified in studies of vertebrate taxa and suggest fundamental differences between vertebrates and invertebrates with respect to these traits. Hypotheses to explain the maintenance of male ejaculate delivery patterns that are consistent with sperm competition and bet-hedging theory are examined, as are potential selection pressures responsible for sperm-size evolution.

Disruptive selection favoring both smaller, motile gametes and larger, nutrient-rich gametes ultimately led to the origin of sexes, according to the most widely accepted theory (Parker et al. 1972; Alexander and Borgia 1979). Subsequent selection on males and females has generated sex-specific reproductive strategies. Models for the evolution of such reproductive strategies have considered a complex array of coevolving traits, including maturation rate, longevity, investment in gametes, investment in offspring, and the nature of competition for mates (see e.g., Hirshfield and Tinkle 1975; Low 1978).

This study is concerned with the evolution of a specific portion of an organism's reproductive strategy, the "gametic strategy," or pattern of gamete production and usage. An organism's gametic strategy is characterized by the amount of energy invested per gamete, the total amount of energy invested in the production of gametes, and the pattern of gamete allocation among successive mates, clutches, or reproductive episodes. Although they undoubtedly evolve as a co-adapted character suite, it is convenient to consider these traits as discrete components. With respect to each component, the reproductive biology of females is

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perceived as interspecifically diverse and has thus received considerable attention. For instance, many models have examined how females should optimally resolve the trade-off between egg size and clutch size and how they should allocate their reproductive effort among successive reproductive episodes (review in Stearns 1992). Much less attention has been paid to the gametic strategy of males.

This lack of attention is partially due to the perception that male gametic strategies are relatively nondivergent. The typical male makes the minimal possible investment in individual sperm in order to maximize the number of sperm produced (Parker 1970, 1984; Cohen 1973). Vast numbers of gametes are produced, such that sperm numbers do not limit male reproductive success (Thornhill and Alcock 1983; Clutton-Brock 1991). Males are expected to saturate female sperm-storage capacity (Parker 1970) or at least to contribute a modest excess of sperm relative to the amount required by females to fertilize all potentially laid eggs (Knowlton and Greenwell 1984), and to replenish their sperm supply quickly after copulation (Trivers 1972).

The sexual difference in variation in gametic strategies is due in part to a difference in the functional significance of egg and sperm size. Egg size for each species represents the evolutionary outcome of diverse selection pressures created by competition, predation, and environmental uncertainty faced by propagules and subsequent life stages, the nature of postzygotic parental care, and an assortment of developmental and physiological constraints (reviewed in Clutton-Brock 1991). Sperm, on the contrary, are expected to be the minimum size possible while maximizing motility and survival until fertilization. In fact, species with external fertilization tend to share a primitive type of spermatozoa that is simple in morphology and approximately 50 μm long (Roldan et al. 1992), which likely reflects an optimal architectural design for motility in water.

The seemingly ubiquitous nature of male gametic strategies is also attributable to sperm competition. When ejaculates of several males occur simultaneously within the reproductive tract of females, competition tends to be numerical (Parker 1970, 1984), with males that transfer greater numbers of sperm siring more offspring (Martin et al. 1974). Parker (1982, 1984) has argued that sperm competition is nearly ubiquitous and that even a low incidence favors the transfer of large numbers of sperm by males. Because individuals have a finite amount of energy to invest in gamete production, an inverse relationship between gamete size and number is expected (Smith and Fretwell 1974; Winkler and Wallin 1987). Consequently, sperm competition also favors the production of tiny sperm.

Among organisms with internal fertilization, however, there are a great many exceptions to the traditional view of the male gametic strategy, and radically divergent male gametic strategies are observable even among closely related species. Only recently have evolutionary biologists begun to recognize and attempt to explain this variation. One reason for this historical neglect is the crucial role that the differential investment by the sexes in individual gametes has played in the development of sexual selection theory (Bateman 1948; Trivers 1972). A change in philosophy came with the recognition that sperm are delivered to females in packages (i.e., ejaculates or spermatophores) that may be costly or limiting (Dewsbury 1982). This realization stimulated research that more closely

examines the relationship between male fertility and mating success, and these studies have broadened our understanding of male gametic strategies in two ways. First, males may use their sperm or ejaculates judiciously, transferring differential amounts of material under different sociosexual situations (Baker and Bellis 1989; Bellis et al. 1990; Parker 1990*a;* Gage and Baker 1991; Birkhead and Fletcher 1992) or on the basis of the reproductive status of the female (Linley and Hinds 1975; Svard and Wiklund 1986; Parker 1990*b;* Suter 1990). Second, in some species the production rate of sperm or other ejaculatory materials may limit male reproductive success (review in Dewsbury 1982; see also Hihara 1981; Nakatsuru and Kramer 1982; Dewsbury and Sawrey 1984; Gibbons and McCarthy 1986; Dewsbury 1988; Gwynne 1990; Kirkendall 1990; Birkhead 1991; Pitnick 1993).

Another constraint on our ability to recognize and explain variation in male gametic strategies has been a lack of information concerning the functional significance of sperm morphology as it relates to motility, given the specific morphological and biochemical conditions faced by sperm within the female reproductive tract (reviewed in Roldan et al. 1992) and the nature of interaction between the sperm and the ova, both during and after fertilization (see Karr 1991). For instance, the view of sperm competition as a raffle in which all sperm are created equal as they race to reach the ova may be overly simplistic for some taxa.

In fact, recent studies of vertebrate taxa examining the first component of the male gametic strategy, sperm-size variation, suggest that all sperm are not created equal and that longer sperm may swim faster than small sperm and therefore be favored by sperm competition (Gomendio and Roldan 1991; Briskie and Montgomerie 1992). Much more variation in sperm length is observable among invertebrates than among vertebrates, however, and, as no studies examining the relationship between sperm length and mating systems have been conducted with invertebrate taxa, it remains to be seen whether sperm competition can explain this impressive level of variation.

Given the expected trade-off between sperm size and the number of sperm produced (Parker 1982), sperm competition may select for diametrically opposed traits. Studies of the second component of the male gametic strategy reveal a partial resolution to this conflict, an increase in the total amount of energy invested in sperm production with increasing sperm competition. Short (1979) was the first to propose that the number of sperm produced will be related to testes size and that males in species with promiscuously mating females will therefore have larger testes than males in species in which females generally mate with only one male per reproductive episode. The hypothesized connection between testes size and mating system has received considerable support (Short 1979; Harcourt et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986; Møller 1991), as has the implicit assumption of Short's (1979) model that testes size of different species relates closely to sperm production and transfer rates (Møller 1988*a,* 1988*b,* 1989; Pierce et al. 1990).

The third component of the male gametic strategy addresses the question: Given the availability of sperm, how should males allocate them among successive reproductive bouts in order to maximize fitness? Although males of most species produce great numbers of sperm, so many sperm are contained in an

ejaculate that successive ejaculates may contain significantly reduced numbers of sperm, and male sperm stores after copulation may remain depleted for prolonged periods (reviewed in Dewsbury 1982). Dewsbury (1982) therefore suggested that constraints posed by factors such as pregnancy initiation requirements, sperm competition, female choice, and mate-searching costs may select for male ejaculate allocation strategies different from "mate with as many females as possible." Because we have an insufficient understanding of the production and composition of nonsperm ejaculatory components, it is necessary for the purposes of this study to discriminate the allocation of sperm from the allocation of ejaculates. Doing so requires information regarding the relative number of sperm contained in successive ejaculates, the rate of sperm production or availability of sperm stores, and the sperm-storage capacity of females (wherever female sperm-storage organs of finite capacity exist, as in most invertebrates and birds; Smith 1984; Birkhead and Møller 1992*a*). Few studies have examined all of these characters (but see Pierce et al. 1990; Birkhead 1991). To understand the adaptiveness of sperm allocation patterns requires further information including female remating frequencies in nature and patterns of sperm precedence.

This study examines all three components of male gametic strategy: sperm size, total male investment in sperm production, and the pattern of ejaculate allocation among successive matings, as well as the trade-off between sperm size and the number of sperm produced. Relations between the sperm content of ejaculates, female sperm-storage capacity, and female remating interval are specifically examined. Comparisons are made among all four species of the *Drosophila nannoptera* species group (Heed 1982). Extreme variation in sperm size and number within this species group allows the influence of these traits on male and female reproductive strategies to be discerned. Relations between various components of male reproductive effort and the coevolution of male and female reproductive strategies are discussed. Additionally, alternative hypotheses to explain the maintenance of divergent male reproductive strategies are examined.

MATERIAL AND METHODS

Cultures and Stock Maintenance

The collection information for *Drosophila nannoptera* Wheeler, *Drosophila acanthoptera* Wheeler, and *Drosophila wassermani* Pitnick and Heed is presented elsewhere (Pitnick et al. 1991). *Drosophila pachea* Patterson and Wheeler stocks were collected by S.P. near San Carlos, Sonora, Mexico, and never exceeded four generations in the laboratory. All four species were cultured in uncrowded conditions on banana medium with live yeast at 24° ± 1°C at an approximate 12L:12D photoperiodic cycle and an approximate 1:1 sex ratio. Medium for *D. pachea* additionally contained necrotic, autoclaved tissue of senita cactus, *Lophocereus schottii* (Englemann) Britton and Rose, the natural host of *D. pachea* (Fogleman et al. 1986). Virgin flies used in experiments were collected on the day of eclosion, anesthetized with ether to facilitate sorting of sexes, and maintained from that time in 8-dram vials containing medium, live yeast, and no

more than 10 other same-sex individuals. These vials were stored in an incubator with a 12L:12D photoperiod and a 26°C day to 22°C night temperature cycle. Body size was determined by thorax length, which has been demonstrated to be a reliable indicator of body size in *Drosophila* (Robertson and Reeve 1952; S. Pitnick and T. A. Markow, unpublished data).

Morphological Measures

For each morphological measure, reproductively mature males and females were drawn from population bottles and etherized prior to dissection in phosphate-buffered saline (PBS) on subbed glass slides. To measure sperm length, fine probes were placed on the distal tip of a testis and then gently pulled apart while maintaining even pressure. This action served to "unzip" the testicular tissue, thereby releasing the contents of the testis onto the center of the slide. Individual sperm cysts, or bundles (Lindsley and Tokuyasu 1980), could then be gently teased free and measured through the ocular micrometer of the dissecting microscope accurate to 0.006 mm. The mean length of the four longest bundles was recorded for each male, and five males were examined for each species. The number of sperm bundles present in a midtestis cross-section were counted for each male. Multiplying this value by the number of sperm per bundle (32 for each species; S. Pitnick, unpublished data) provided the index of sperm production.

To examine testicular morphology, testes were transferred from PBS into white paraffin oil on a glass slide and outstretched. The length of each long, thin, cylindrical testis (l) was recorded, as were five equidistant width measures, the mean of which (w) was used to calculate testis volume with the formula $\pi(0.5w)^2l$. Ten males were measured per species. Length of female genital chambers and oviducts were measured in PBS; seminal receptacles and spermathecal ducts were measured after transfer to white paraffin oil. The short (a) and long axis (b) of each elliptical spermatheca were measured to calculate spermatheca volume with the formula $0.75\pi a^2b$. For a description of *Drosophila* male and female reproductive organ morphology, see Patterson and Stone (1952).

To measure the percentage of the total dry body weight that is comprised by dry testes weight, testes were dissected from each male into double-distilled H_2O and then transferred to a preweighed piece of aluminum foil. All remaining tissue was placed on another piece of preweighed foil and the head and thorax were ruptured to facilitate desiccation. The samples were oven-dried at 55°C for 1 h prior to weighing on a Cahn C-31 microbalance accurate to the nearest 1.0 μg.

Dissections and Sperm Counts

Immediately after copulation, female *D. acanthoptera, D. wassermani,* and *D. pachea* were transferred by aspiration to vials containing nonoviposition medium (agar, sugar, and water). After 6 h each female was dissected. The intact reproductive tract was isolated into a drop of PBS on a glass slide with fine forceps. The sperm-storage organs, or spermathecae, were then pared free from the remainder of the tract, and the remaining tissue was discarded. Each storage organ was ruptured with fine probes, and the sperm mass was removed and gently

teased apart. The slide was then dried in an oven at 40°C prior to fixation in methanol/acetic acid (3:1) and staining in a 5×10^{-7} M solution of Hoechst 33258 as described by Sakaluk and O'Day (1984). This stain selectively binds to DNA, thereby facilitating quantification of the absolute number of sperm stored by each female through epifluorescence microscopy; no method of subsampling the sperm population was required.

Unlike females of the other species, female *D. nannoptera* store sperm in the seminal receptacle. Because it was not possible to extract all of the sperm from the receptacle, females of this species were dissected immediately after copulation. The sperm mass was easily removed intact; no female had any sperm present in the seminal receptacle by the time of dissection.

Experiment 1: Number of Sperm Transferred per Copulation

Ten mature males were randomly drawn from a single population bottle, and each was paired with a reproductively mature virgin female. Females were then processed for sperm counting as described above. This experiment was replicated twice for each species, with males in different replicates coming from different population bottles.

Experiment 2: Number of Sperm in Female Storage Organs

For each of two replicates per species, 10 females were drawn at random from a single population bottle and immediately dissected for sperm counting as described above. For *D. nannoptera,* in which dissection of sperm from the storage organ was not possible, the proportion of the seminal receptacle that was occupied by sperm was estimated. A similar estimate was made for single-mated females of this species to compare sperm loads among these two categories of females.

To examine whether sperm-storage values for female *D. pachea* in the wild are similar to those in the laboratory, a system of estimating the proportion of female sperm-storage volume that is occupied by sperm (Jefferson 1977) was modified to facilitate the estimation of female sperm loads. In the laboratory, when females were dissected for sperm counting, the spermathecae were examined through a stereoscopic microscope with a transmitted light source at 40× magnification before rupturing them. The transmitted light silhouettes the sperm inside, permitting estimation of the percentage of the total volume of each spermatheca that is occupied by sperm. Each pair of spermathecae was given a rating from zero (both spermathecae empty) to eight (both spermathecae full). Sperm were then dissected from these spermathecae, stained, and counted as described above. Thus, it was possible to correlate sperm-storage values with actual measures of number of sperm in storage (fig. 1). Subsequently, females were collected on cactus necroses in the wild, dissected, and assigned sperm-storage values.

Experiment 3: Allocation of Ejaculate among Successive Mates

Males were radiolabeled by transferring 50 early first instar larvae from culture bottles for each species to vials containing 5 g of culture medium to which 50 μCi of an L-amino acid mixture of ^{14}C (ICN Lot no. 10147) had been integrated.

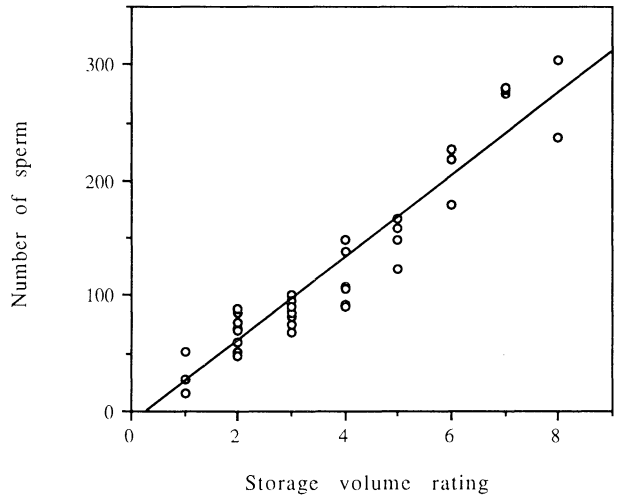

FIG. 1.—Correlation between values rating the proportion of the total volume of females' sperm-storage organs that are occupied by sperm and the number of sperm actually in storage, as determined by staining and counting, for female *Drosophila pachea* randomly collected from laboratory cultures ($y = -10.426 + 35.715x$, $r^2 = 0.902$, $N = 40$).

Thus, radioactive carbon was incorporated into protein molecules in adult flies reared from these cultures. After eclosion, labeled males were stored 10 per food vial with live yeast until reproductive maturity. Once mature, each labeled male was presented with a succession of nonlabeled virgin females over the course of a day. Matings were conducted in vials containing banana medium with two virgin females per vial. Whenever a copulation began, the noncopulating female was gently aspirated out of the vial. Immediately after the cessation of each copulation, the male was transferred by aspiration to a fresh vial containing another two virgin females, while his previous mate was processed for scintillation counting, as described for the previously published "0-hour" experiment (Pitnick et al. 1991). In this way the size of the radiolabeled ejaculate received by each female was directly quantifiable as the number of disintegrations per minute (DPM). At the end of the day, all males were similarly processed for scintillation. No live yeast was provided during the mating experiment in order to minimize nutritional intake by males.

The pattern of allocation among successive matings of the sperm component of the ejaculate was examined in *D. pachea* only. The experiment was conducted as described for ejaculate allocation, except that males from the standard culture, rather than a radiolabel culture, were used and sperm counts were done in lieu of scintillation counting. Immediately after each copulation, the female was transferred by aspiration to a vial containing nonoviposition medium to await processing for sperm counts as described above. Also, males were provided with a succession of mating opportunities on two consecutive days, rather than just one. The intent was to exhaust males' sperm supplies on the first day, so that the

second day would reveal how males allocate a single day's production of sperm among successive matings.

A field trial of this experiment was conducted to verify that behaviors observed in the laboratory, including both the number of sperm transferred and the pattern of sperm allocation, were not laboratory artifacts. A rotting senita cactus arm supporting a large population (ca. 150 individuals) of *D. pachea* was located in the desert near San Carlos, Sonora, Mexico. Shortly after sunrise, as soon as courtship behavior was observed on the cactus and before any males secured their first copulation of the day, males were collected by aspiration and placed in food vials, each containing two laboratory-reared virgin females. The mating experiment was then conducted in the shade at the base of the rotting cactus arm with protocol identical to that of the laboratory replicate.

Experiment 4: Female Remating Interval

For each species, reproductively mature virgin females were placed singly in vials containing food and oviposition substrate with two reproductively mature virgin males. Once copulation began, the noncopulating male was aspirated out of the vial. After mating the females were provided with twice-daily opportunities to remate by placing two males in each vial during 2 h in the morning and 2 h in the late afternoon. Females were transferred to vials containing fresh food and oviposition substrate daily, and each female was tested until she remated or died. For each species, two replicates were performed with 30 females per replicate.

To examine whether the time intervals measured in this experiment are applicable throughout the reproductive life of females or whether they are relevant to a female's first remating only, a replicate of this experiment was continued for 10 d in *D. pachea* instead of stopping with the first remating. Also, to test the validity of conducting this experiment in vials, another experiment, in large naturalistic chambers, was conducted to measure female remating interval in *D. pachea*. Two days prior to beginning the experiment, 100 virgin females were marked with a small drop of one of 10 different colors of Testors model paint on the prothorax. At first light on the day of the experiment, 10 females, one of each color, were aspirated into each chamber containing an artificial cactus necrosis (Pitnick 1992), which provides refuge and oviposition substrate. Each chamber also received 15 reproductively mature males (10 nonvirgins and five virgins) and was then scanned for copulating pairs approximately every 20 min for 36 h. After 12 h of light, the ceiling lights in the laboratory were extinguished one bank at a time, over the course of 0.5 h, to simulate dusk. For the next 12 h, only a single red bulb was kept on in the laboratory to provide working light, and the scanning of chambers was performed with a medical penlight, which only mildly agitated the flies. Identity of the mating female, location within the chamber, and the time were recorded for all copulations. This experiment was replicated twice with six chambers per replicate.

The 38 × 20 × 15-cm Plexiglas chambers and their contents are described in detail elsewhere (Pitnick 1992). Each chamber contained an approximately 30-cm-long section of an arm of senita cactus with a "rot pocket" of oviposition

substrate. The aggregation, courtship, aggression, and oviposition behaviors of flies within these chambers are qualitatively similar to the behavior of *D. pachea* on naturally occurring senita necroses (S. Pitnick, personal observation).

RESULTS

Reproductive Organ Morphology and Sperm Production

Extensive variation in sperm and testes size exists in the *nannoptera* species group (table 1). The distribution of sperm sizes is bimodal in this clade, with *Drosophila wassermani* and *Drosophila acanthoptera* males producing relatively short sperm and *Drosophila nannoptera* and *Drosophila pachea* producing much longer sperm. Total investment in sperm production also varied among species, as determined by testes volume ($F = 38.86$, df = 3,36, $P < .0001$) and the percentage of the total dry body weight that is comprised by the dry testes weight ($F = 40.26$, df = 3,36, $P < .0001$). Significant differences in both of these characters were due to the greater investment in testicular tissue by *D. pachea* relative to the other species (table 1).

It was not possible to directly measure daily sperm production rates. However, an index of sperm production (the number of sperm bundles present in midtestis cross-sections multiplied by the number of sperm per bundle) varied greatly among species (table 1). This index of sperm production clearly indicates that the species making shorter sperm are producing many more gametes at any given time than those species making larger sperm ($F = 32.17$, df = 3,16, $P < .0001$).

Interspecific variation in the location of sperm storage within females was identified: *D. nannoptera* females store sperm in the seminal receptacle only, while females of the remaining three species use only the spermathecae for sperm storage, confirming an earlier report on some of these species (Russell et al. 1978). The volume of the spermathecae differed significantly among all species pairs ($F = 63.14$, df = 3,67, $P < .0001$; Tukey's Studentized range test at $P = .05$), with female *D. pachea* exhibiting the largest spermathecal volume. The length of seminal receptacles also varied among species and exhibited a positive relationship with sperm length (fig. 2*a*; $F = 254.99$, df = 1,30, $P < .0001$, $r^2 = 0.895$). As sperm length increased, the ratio of seminal receptacle length to sperm length decreased in a significant curvilinear fashion (fig. 2*b*; $F = 6.00$, df = 1,30, $P = .020$, $r^2 = 0.167$). With the exception of the sperm-storage organs, dimensions of female reproductive tracts, including the genital chamber, common and lateral oviducts, and the spermathecal duct, did not differ substantially among species (table 2).

Experiment 1: Number of Sperm Transferred per Copulation

Great variation exists among the *nannoptera* group species for the number of sperm transferred per typical copulation ($F = 329.32$, df = 3,76, $P < .0001$). *Drosophila acanthoptera* males transferred 1,023 ± 48 sperm per copulation.

TABLE 1

MALE BODY SIZE, SPERM SIZE AND NUMBER, AND TESTES LENGTH, VOLUME, AND RELATIVE WEIGHT FOR THE FOUR *NANNOPTERA* SPECIES GROUP MEMBERS

Species	Thorax Length* (mm)	Sperm Length (mm)	Number of Cysts	Number of Sperm	Testis Length (mm)	Testis Volume (μm^3)	Testes Dry Weight (%)
Drosophila wassermani	1.11 ± .01 (a)	4.52 ± .03	76.4 ± 1.44 (a)	2,445	7.76 ± .16 (a)	44 ± 2 (a)	3.62 ± .53 (a)
Drosophila acanthoptera	1.10 ± .01 (a)	5.83 ± .09	101.6 ± 12.81 (a)	2,845	8.20 ± .15 (a)	48 ± 2 (a)	4.53 ± .27 (a)
Drosophila nannoptera	1.03 ± .00 (b)	15.74 ± .30	28.4 ± 2.11 (b)	682	19.81 ± .57 (b)	54 ± 2 (a)	4.02 ± .20 (a)
Drosophila pachea	1.04 ± .00 (b)	16.53 ± .29	25.8 ± 1.24 (b)	826	22.75 ± .39 (c)	82 ± 4 (b)	8.58 ± .50 (b)

NOTE.—Differences between species were examined with a Tukey's Studentized range test at $P = .05$. Subset membership by this test is indicated by the letter underneath the mean (± SE) value for that variable.

* Thorax length measures from ejaculate allocation experiment. Mean thorax length of males in other experiments did not differ significantly ($P = .05$).

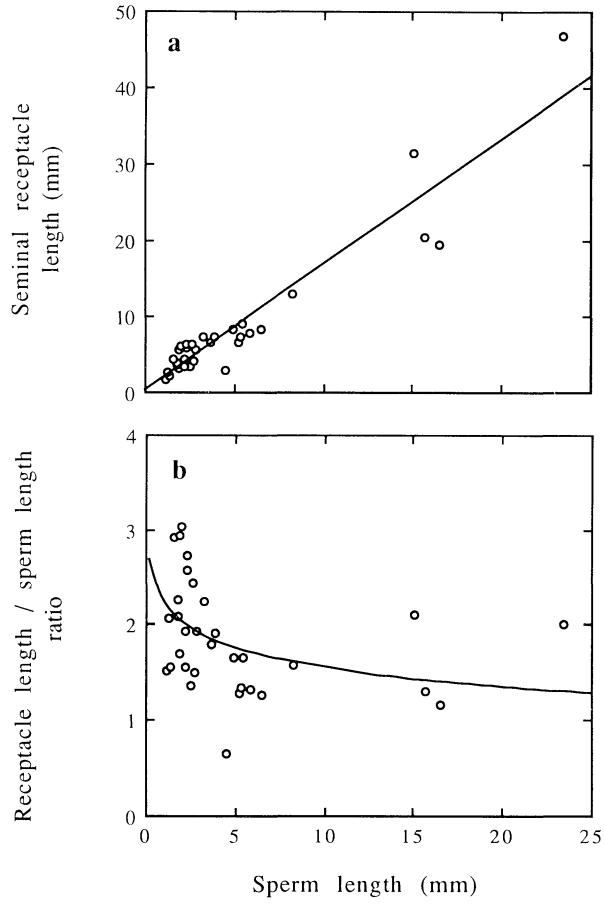

FIG. 2.—*a*, The relationship between sperm length and female seminal receptacle length (the line is described by the equation $y = 0.186 + 1.659x$); *b*, the relationship between sperm length and the ratio of female seminal receptacle length to sperm length (the line is described by the equation $y = -0.676 \times \log(x) + 2.223$). Data are from Hihara and Kurokawa (1987) (21 species), the present study (four species), and S. Pitnick (unpublished data) (seven species).

This value far exceeds the male sperm transfer numbers of its relatives. *Drosophila pachea* males, for example, transferred just 44 ± 6 sperm per copulation (fig. 3).

Experiment 2: Number of Sperm in Female Storage Organs

Female *D. pachea* randomly drawn from the population were found to contain fewer sperm than female *D. acanthoptera* and female *D. wassermani* (Tukey's Studentized range test, $P = .05$). Evidence also suggests that the sperm-storage capacity of female *D. pachea* is much less than that of its relatives. The maximum number of sperm observed to be stored by females is presented in table 2; the females in these cases appeared to have completely full spermathecae. In the

TABLE 2

FEMALE BODY SIZE, LENGTHS OF REGIONS COMPRISED BY THE FEMALE REPRODUCTIVE TRACT, AND ESTIMATED SPERM-STORAGE CAPACITY

Species	Thorax Length* (mm)	Genital Chamber (mm)	Common Oviduct (mm)	Lateral Oviduct (mm)	Seminal Receptacle (mm)	Spermathecal Duct (mm)	Sperm-Storage Organ(s)	Spermatheca Volume (μm^3)	Maximum No. of Sperm Stored
Drosophila wassermani	1.16 ± .02	.31 ± .01	.51 ± .02	.07 ± .01	3.00 ± .05	.62 ± .03	Spermathecae	22.1 ± .4	1,240
Drosophila acanthoptera	1.21 ± .01	.33 ± .01	.57 ± .02	.09 ± .01	7.70 ± .43	.27 ± .01	Spermathecae	28.0 ± 1.1	1,456
Drosophila nannoptera	1.08 ± .01	.38 ± .02	.52 ± .04	.07 ± .01	20.39 ± .68	.26 ± .01	Seminal receptacle	.8 ± .1	. . .
Drosophila pachea	1.12 ± .01	.30 ± .01	.53 ± .02	.06 ± .01	19.36 ± .96	.36 ± .03	Spermathecae	35.9 ± 2.1	304

NOTE.—Data are means ± SE; $N = 5$.

* Thorax length measures from ejaculate allocation experiment. Mean thorax length of females in other experiments did not differ significantly ($P = .05$).

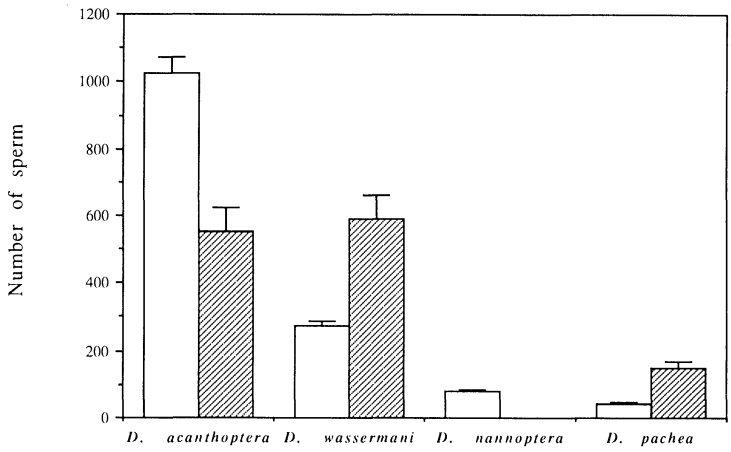

FIG. 3.—Number of sperm transferred per copulation by typical males of each species (*open bars;* mean ± SE) and number of sperm contained within sperm-storage organs of typical females in the population (*hatched bars;* mean ± SE). No sperm-storage data were obtained for *Drosophila nannoptera*. Each bar represents combined data from two replicates; $N = 20$. As ANOVA tests treating "replicate" as the class variable and "sperm number" as the dependent variable produced nonsignificant results ($P > .05$) for each species, replicates were combined for statistical analyses.

case of *D. pachea,* it was possible to be more quantitative since the method of rating the proportion of the spermathecae that is occupied by sperm (fig. 1) produces an independent storage maximum estimate of 264.5 sperm, which can be compared with the maximum observed value of 304 for this species. Additional data for *D. pachea* revealed that wild females and laboratory females had very similar numbers of sperm in storage (table 3), suggesting that the laboratory measures are not artifacts.

We compared the typical number of sperm possessed by females with the typical number received per copulation within species. For all three species examined, these values differed significantly (*D. acanthoptera:* $F = 29.28$, df = 1,38, $P < .0001$; *D. wassermani:* $F = 20.62$, df = 1,38, $P < .0001$; *D. pachea:* $F = 34.00$, df = 1,38, $P < .0001$; fig. 3). However, the direction of the inequality varied among species. *Drosophila acanthoptera* females received almost twice as many sperm per copulation (1,023 ± 48 sperm) as females were typically found to have in storage (552 ± 73 sperm). The pattern of sperm transfer to and possession of sperm by females was very different for *D. wassermani* and *D. pachea*. In these species, females contained many more sperm than were transferred in a typical copulation (fig. 3). In other words, males submaximally inseminate females. The greater number of sperm in storage than is received in a typical copulation must be a consequence of overlapping ejaculates from successive matings by females. These data suggest that female *D. pachea* contained, on the average, sperm from 3.48 males, whereas *D. wassermani* females contained sperm from 2.16 males. For *D. nannoptera* females, examination of the propor-

TABLE 3

SPERM-STORAGE RATING AND STORAGE ESTIMATES FOR FEMALE *DROSOPHILA PACHEA* FROM A REMATING EXPERIMENT, LABORATORY CULTURES, AND A FIELD COLLECTION

Population	Storage Rating	Storage Estimate
Remating experiment ($N = 30$)*	5.48 ± .34	208.92
Laboratory cultures ($N = 20$)	4.25 ± .48	162.03†
Field collected ($N = 214$)‡	4.88 ± .34	185.44

* Females dissected after 10 d of twice-daily opportunities to remate. See text for description of experiment.

† Actual sperm count = 152.95.

‡ Data for all dissected females collected from eight populations in January 1991.

tion of the seminal receptacle that is occupied by sperm revealed that typical females from the population contain significantly more sperm (rating = 2.33 ± 0.28 of 4) than do singly mated females (rating = 0.83 ± 0.32; unpaired *t*-test, $t = 3.494$, df = 22, $P < .01$). It can, therefore, be concluded that *D. nannoptera* males also submaximally inseminate females.

Experiment 3: Allocation of Sperm among Successive Mates

Because of the labor intensiveness of examining the allocation of sperm among successive mates, we instead quantified allocation of the entire ejaculate for each species using radiolabeled males. This protocol was justified in two ways. First, males of these species do not produce seminal materials that are later incorporated into female soma and oocytes (Pitnick et al. 1991), as has been reported for some *Drosophila* species (see, e.g., Markow and Ankney 1984), and the correlation between the total amount of sperm transferred in the first mating (number of sperm × sperm length; see above, *Experiment 1*) and ejaculate size (radiolabel) of first matings was found to be quite high (one-tailed Pearson $r = 0.837$, $.025 < P < .01$). Second, in *D. pachea,* these measures were found to be highly congruent across the entire series of matings (see below). We therefore conclude that ejaculate allocation patterns measured here reliably indicate patterns of sperm allocation for each of the species.

Results of the radiolabel experiment revealed extreme interspecific variation in how males partition ejaculate among successive partners (fig. 4). Males varied with respect to three discrete characters: ejaculate size, determined as the proportion of a male's premating radioactivity level (DPM) transferred to the female in a single copulation, the number of matings achieved in 1 d by each male under the condition of ad lib. receptive virgin females, and total ejaculate transferred, which was the sum of all individual ejaculates transferred throughout the day, calculated as the percentage of each male's premating, or virgin, radioactivity level that was passed to females.

The species varied in the amount of time and material invested by males in

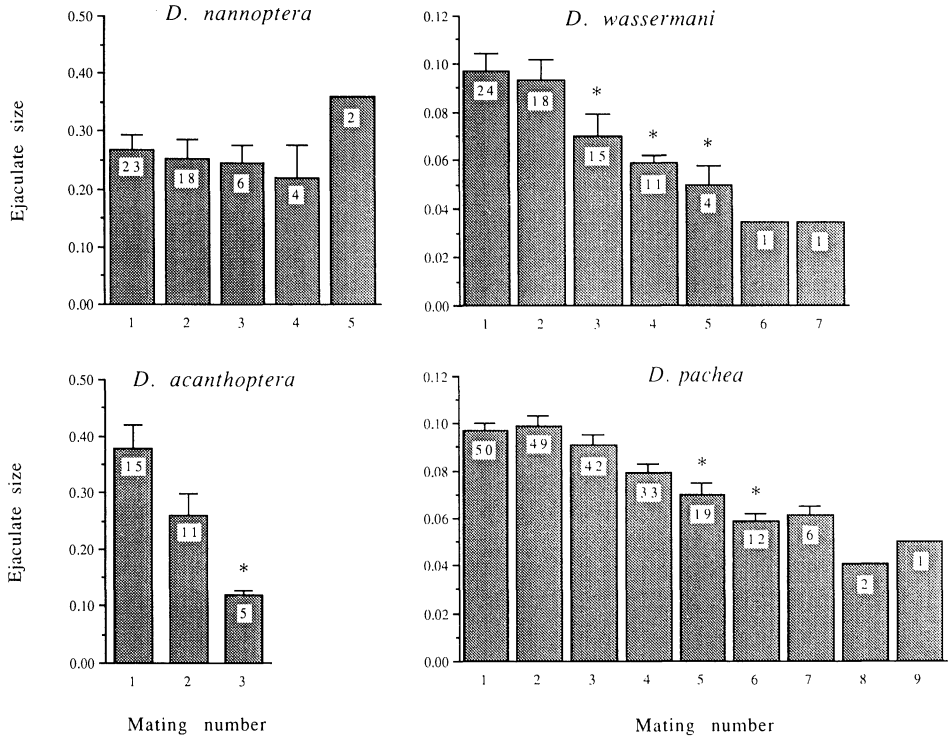

FIG. 4.—Size of ejaculate transferred (mean ± SE) in successive copulations with virgin females by radioactive males of each species, measured as the proportion of each male's premating radioactivity level (DPM) transferred to each female. Numbers within *bars* indicate sample size for each mating number category. Each *asterisk* denotes a significant difference in ejaculate size ($P = .05$) by repeated measures ANOVA between females in that mating number category relative to females in mating number category 1 for each species. Note that vertical axes scales differ among species.

each mating (table 4). Duration of copulation was significantly different among all species ($F = 327.98$, df = 3, 106, $P < .0001$). *Drosophila acanthoptera* have the most prolonged copulation of any *Drosophila* species studied (137 min); it lasts more than 17 times longer than that in *D. nannoptera*. Ejaculate size also varied among species ($F = 60.33$, df = 3, 107, $P < .0001$). *Drosophila pachea* and *D. wassermani* males transferred ejaculates of identical size, while *D. nannoptera* and *D. acanthoptera* males passed 2.8 and 3.9 times more material in each copulation, respectively (table 4).

Although less variable than measures of investment per copulation, the ability of males to mate repeatedly also showed interspecific variability ($F = 15.11$, df = 3, 108, $P < .0001$). This difference was primarily due to a significantly greater number of matings achieved by *D. pachea* males relative to males of the other three species (table 4).

Total ejaculate transferred over the course of the day also varied among species ($F = 16.57$, df = 3, 106, $P < .0001$). *Drosophila nannoptera* and *D. acanthoptera*

TABLE 4

COMPONENTS OF MALE REPRODUCTIVE STRATEGY MEASURED IN A RADIOACTIVITY EXPERIMENT FOR THE FOUR *NANNOPTERA* SPECIES GROUP MEMBERS

Species	Copulation Duration (min)	Ejaculate Size (DPM)	Total Ejaculate Transferred (DPM)	No. of Mates per Day
Drosophila wassermani (N = 24)	14.17 ± .62 (c)	.097 ± .003 (c)	.25 ± .03 (b)	3.12 ± .33 (a)
Drosophila acanthoptera (N = 15)	137.13 ± 8.10 (b)	.377 ± .042 (b)	.61 ± .07 (a)	2.07 ± .21 (a)
Drosophila nannoptera (N = 23)	7.73 ± .59 (a)	.269 ± .025 (a)	.60 ± .07 (a)	2.30 ± .25 (a)
Drosophila pachea (N = 49)	39.54 ± 1.46 (d)	.097 ± .007 (c)	.38 ± .02 (c)	4.32 ± .24 (b)

NOTE.—Ejaculate size and total ejaculate transferred per day are measured in disintegrations per minute (DPM) contained in ejaculate(s), standardized as a proportion of individual male premating whole-body DPM. Values for copulation duration and ejaculate size are for the first mating only in each male's mating series. Differences between species were examined with a Tukey's Studentized range test at $P = .05$. Subset membership by this test is indicated by the letter following the mean (± SE) value for that variable.

males transferred equivalent total amounts of ejaculate, and either transferred significantly more material than *D. wassermani* and *D. pachea* males. The latter two species also differed significantly from each other, with *D. wassermani* males transferring the smaller quantity (table 4).

Total ejaculate transferred is clearly determined by ejaculate size and mating frequency, modified by some function depicting the pattern of ejaculate allocation among successive matings by males. This function varies among species (fig. 4). Repeated-measures ANOVA revealed that the size of ejaculates passed by *D. nannoptera* males to their mates did not diminish across four successive copulations. The same pattern was exhibited by *D. pachea* males, which transferred significantly less material only after four copulations in succession. *Drosophila acanthoptera* and *D. wassermani* males, in contrast, transferred significantly less ejaculate by the third copulation (ANOVA of contrast variables comparing the first and third copulations: *D. nannoptera:* $F = 0.94$, df = 1,5, $P = .38$; *D. acanthoptera:* $F = 7.91$, df = 1,4, $P < .05$; *D. wassermani:* $F = 6.73$, df = 1,14, $P < .05$; *D. pachea:* $F = 0.88$, df = 1,40, $P = .36$).

We further examined these ejaculate allocation patterns using repeatability statistics to verify that comparisons made above, on the basis of mean values, were indicative of allocation patterns by individual males of each species. The repeatability statistic quantifies stability of a phenotypic trait within individuals relative to differences in that trait among individuals (Falconer 1989). In this case, it is the intraclass correlation coefficient calculated from a one-way ANOVA with individual males as the independent variable and ejaculate size as the dependent variable (Lessels and Boag 1987). The higher the repeatability measure, the more similar the repeated measurements of a given individual are relative to differences among individuals. The repeatability statistics presented in table 5 confirm the ejaculate allocation patterns as illustrated in figure 3. Considering the first three

TABLE 5

REPEATABILITY OF EJACULATE SIZE AMONG THREE SUCCESSIVE MATINGS BY RADIOLABELED MALES

Species	*F* Value	df	Repeatability
Drosophila nannoptera	5.18**	5,12	.582
Drosophila acanthoptera	.90	4,10	−.033
Drosophila wassermani	1.57	14,30	.160
Drosophila pachea	2.44***	41,83	.324

** $P < .01$.
*** $P < .001$.

matings of each male, *D. nannoptera* and *D. pachea* males were highly consistent in the amount of ejaculate transferred in each mating, relative to *D. acanthoptera* and *D. wassermani* males. In the case of *D. acanthoptera,* within-male variation in size of successive ejaculates exceeded among-male variation.

To examine the congruence between ejaculate and sperm allocation, an experiment was conducted in the laboratory and in the wild with *D. pachea* to examine how males allocate specifically the sperm component of their ejaculate among successive matings. In the laboratory, males were tested across two successive days. Laboratory males given unlimited access to virgin females mated more times (5.44 ± 0.22 mates) on the first day, relative to the second day (4.08 ± 0.18 mates) and to males in the wild (3.50 ± 0.34 mates; fig. 5). Additionally, the pattern of sperm allocation was similar on the first day to the pattern of ejaculate allocation (see fig. 4, *D. pachea*). Repeated-measures ANOVA revealed that the number of sperm transferred by males on the first day in the laboratory did not decline significantly across seven successive copulations (fig. 5).

This pattern was substantially different from the sperm allocation pattern for the second day and for the field replicate, where male mating success and the patterns of ejaculate allocation were nearly identical, with males transferring significantly fewer sperm by the second copulation and sperm quantity gradually declining with each successive mating (fig. 5). These similarities were expected, since most reproductively active males in the wild are expected to be nonvirgins.

As with the ejaculate allocation data, the consistency of sperm transfer by males to different mates, relative to variation among males, was examined by repeatability statistics of the first three copulations in each trial (table 6). Males exhibited relatively high repeatability in sperm transfer on the first day in the laboratory and when considering the combined data from both days in the laboratory or the repeatability among six copulations per male. However, there was very low repeatability when considering only the second day in the laboratory and for males in the field.

Experiment 4: Female Remating Interval

Female *D. pachea* and *D. nannoptera* both remated very rapidly, after only 0.68 ± 0.05 d and 0.68 ± 0.04 d, respectively (fig. 6). In both species, more than 66% of females remated on the first afternoon of the experiment, and more than

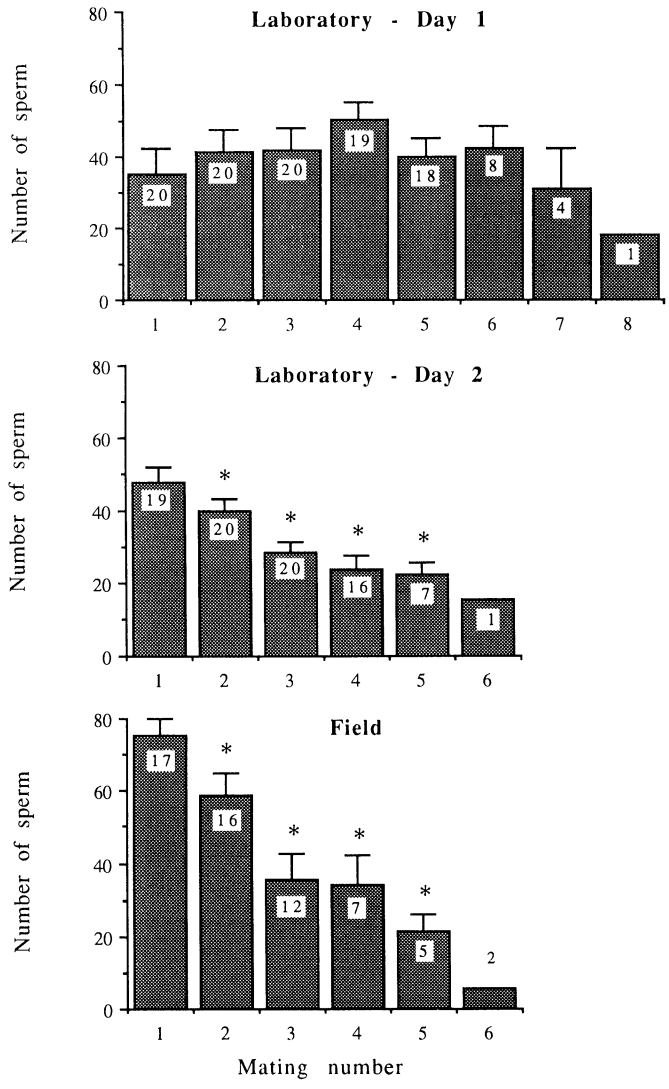

FIG. 5.—Number of sperm transferred (mean ± SE) in successive copulations with virgin females by *Drosophila pachea* males on two consecutive days in the laboratory and by males in the field. Numbers within *bars* indicate sample size for each mating number category. Each *asterisk* denotes a significant difference ($P = .05$) by repeated-measures ANOVA between females in that mating number category relative to females in mating number category 1, with respect to the number of sperm received.

TABLE 6

REPEATABILITY OF NUMBER OF SPERM TRANSFERRED AMONG THREE SUCCESSIVE MATINGS BY LABORATORY-REARED *DROSOPHILA PACHEA* MALES ON TWO CONSECUTIVE DAYS AND BY WILD MALES IN THE FIELD

Experiment	*F*-value	df	Repeatability
Laboratory, day 1	6.89****	19,38	.662
Laboratory, day 2	1.66	19,39	.180
Days 1 and 2 combined	4.86****	19,97	.563
Field	.74	13,24	−.094

**** $P < .0001$.

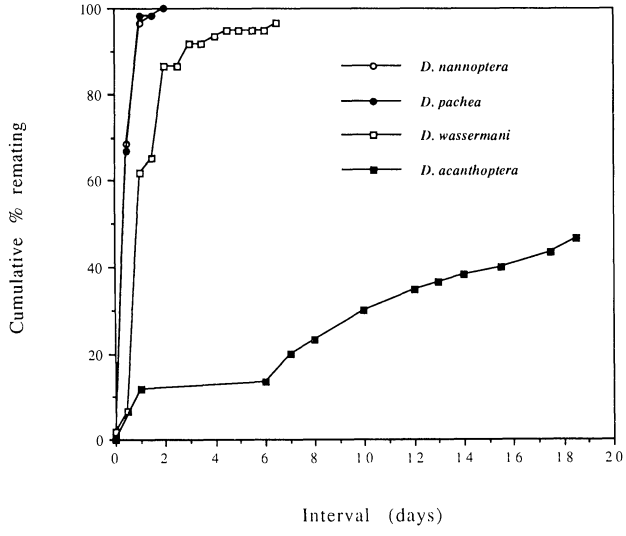

FIG. 6.—Cumulative percentage of females remating across days after a loss of virginity on day 0 for each of the *nannoptera* group species. Each *line* represents combined data from two replicates ($N = 60$). As ANOVA tests treating replicate as the class variable and "remating interval" as the dependent variable produced nonsignificant results ($P > .05$) for each species, replicates were combined for statistical analyses.

96% had remated by the end of the next morning. In the case of *D. pachea*, females maintained their brief remating interval over a prolonged period, mating on the average with 14.67 ± 0.71 (range: 5–22) males during a 10-d period. *Drosophila wassermani* females took substantially longer, 1.51 ± 0.14 d on average, to remate. Only 6.7% of these females remated on the first day of the experiment, with 61.7% of the females having remated by the end of the next morning. After 3 d, 13.3% of *D. wassermani* females still had not remated. The remating behavior of *D. acanthoptera* females was in sharp contrast to that of the other three species. Only 46.7% of these females ever remated, despite the fact that

females never remating lived an average of 25.0 ± 0.8 d. Of the females that did remate, the mean remating interval was 8.84 ± 1.10 d.

To examine the validity of the one-female–two-males protocol used to determine female remating interval in laboratory vials, we also measured the remating interval of *D. pachea* females at higher population densities but in large chambers in which females were able to avoid persistent courtship of males. Of females initially mating in the morning or the afternoon, the mean (± SE) remating interval was 0.34 ± 0.05 d (range = 0.008–1.004 d, $N = 47$) in the first replicate and 0.70 ± 0.04 d (range = 0.117–0.983 d, $N = 45$) in the second replicate. The number of matings achieved by each female throughout the day was 2.21 ± 0.18 (range = 1–5, $N = 33$) in the first replicate and 1.28 ± 0.08 (range = 1–2, $N = 29$) in the second replicate. These data lead us to conclude that the experiment in vials produced conservative estimates of the rapidity of remating in this species.

Within the experimental chambers, the only oviposition substrate was the necrotic tissue within the rot pockets. Initiation of oviposition could be conservatively estimated as the time at which each female was first seen within a rot pocket ($N = 52$; first replicate only). Once a female entered the rot pocket, she typically remained within for long periods of time and often remated there (71.2% of all subsequent rematings). The relationship between estimated time of first oviposition and the mating status of females is illustrated in figure 7. Many females (42.3%) remated at least once before ovipositing and therefore before using any sperm, and some females (9.6%) remate multiply before beginning to oviposit.

DISCUSSION

The gametic strategy of males is determined by the size of sperm, the total amount of energy allotted to sperm production, and the pattern of sperm allocation among successive matings. As the expression of any of these traits will likely influence expression of the others, they are expected to evolve as a functional character suite. Moreover, many traits not examined in this study that affect male energy budgets, such as the composition of the nonsperm portion of the ejaculate, may also influence gametic strategy evolution. Knowledge of relations among each of the components comprising male gametic strategies, interspecific variation in such strategies, and the selection pressures responsible for evolutionary divergence in such strategies is scarce, particularly for invertebrates. This study reveals that male gametic strategies can vary greatly, even among closely related species, and provides an opportunity to examine patterns among characters and to evaluate various hypotheses for the evolution of such traits.

Sperm Size and Number

Males of most species make small sperm, but within all higher-level taxa considerable variation in sperm length is observable. For example, sperm length in mammals ranges from 33.5 μm in the hippopotamus, *Hippopotamus amphibius,* to 356.4 μm in the honey opossum, *Tarsipes spenserae* (Cummins and Woodall 1985); in birds from about 60 μm to 300 μm (Birkhead and Møller 1992a); and in

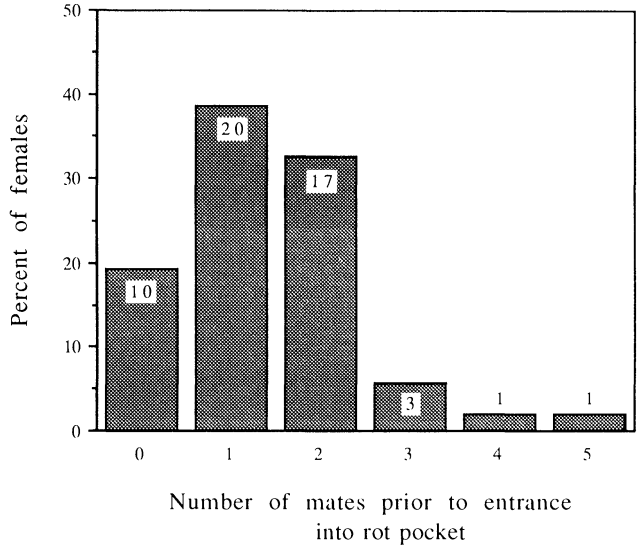

FIG. 7.—The mating status of female *Drosophila pachea* with respect to the initiation of oviposition, operationally defined as entering the rot pocket of the experimental chamber.

fish from aflagellate, amoeboid sperm in the Mormyridae, or elephant fish, to 272 μm in the Australian lungfish, *Neoceratodus fosteri* (Jamieson 1991). For unknown reasons, sperm length is far more variable among invertebrates than among vertebrates, as illustrated by the Drosophilidae, in which sperm length ranges from 56 μm in *Drosophila pseudoobscura* (short sperm morph; Joly et al. 1989) to approximately 24,000 μm in *Drosophila hydei* (S. Pitnick and T. A. Markow, unpublished data). Further, while the production of relatively giant sperm is uncommon, it is taxonomically widespread (see review in Sivinski 1984), with the length variation primarily attributable to variation in length of the sperm tail (i.e., flagellum or principal piece).

Although extreme interspecific variation in sperm length has been reported for *Drosophila* (Joly et al. 1991), the present study indicates that even very closely related species can be highly variable with respect to this trait; sperm length differs dramatically among the *nannoptera* group species. *Drosophila pachea* produces one of the longest sperm in nature; each cell is approximately seven times the total body length of the male. In contrast, the sperm of its sister species (Heed 1982), *Drosophila wassermani,* are only about one-fourth as long.

Spermatozoa of different species can vary in many distinct morphological ways (see, e.g., Jamieson 1987, 1991). Although sperm length is a somewhat imprecise morphological character, it is easy to quantify and is likely the most reliable universal indicator of the energetic cost of the gamete, which is crucial to analyses of reproductive strategy evolution. Within species, any mutation for an increment change in gamete size is expected to have an inverse effect on the number of gametes produced by that individual. Intraspecific studies of sperm production

relative to male body size lend support to the notion that significant costs may be incurred in the production of individual sperm in some species (Berrigan and Locke 1991; S. Pitnick and T. A. Markow, unpublished data). However, this sperm size and number trade-off may not be observable in interspecific comparisons as species may vary in the total amount of energy invested in sperm production (see, e.g., Gomendio and Roldan 1993). Still, among closely related species it is reasonable to expect the macroevolutionary pattern to reflect the microevolutionary gamete size and number trade-off.

This expectation is met among members of the *nannoptera* species group. A significant negative correlation was found between sperm size and an index of the number of sperm produced. This index is probably conservative, as longer sperm likely require more time to mature. Therefore, males of species with larger sperm produce relatively few of them, despite their making a greater total investment in gamete production (see below).

In those species in which ejaculates contain few sperm, the cost to males of producing relatively few, long sperm may be mitigated by adaptations of females or of the sperm, which enhance the efficiency of sperm use. For example, in the self-fertilizing nematode, *Caenorhabditis elegans,* fewer sperm than eggs are produced. When fertilization occurs, excess sperm are often carried or pushed by the fertilized zygote into the uterus. These sperm then actively migrate back to the spermathecae where they regain their position on the organ wall to await another oocyte. In consequence, nearly every sperm is used to produce a zygote (Ward and Carrel 1979).

Unfortunately, there are insufficient data to test this "increased efficiency with decreased sperm numbers" hypothesis with the *nannoptera* group species. However, available data on other species of *Drosophila* suggest that no such relationship exists. Male *Drosophila melanogaster* typically transfer 4,000–6,000 sperm per copulation (sperm length = 1,700 μm), of which approximately 1,100 are stored by the female. Although efficiency of sperm use varies depending on how rapidly females lay their eggs, most copulations yield 300–400 progeny, permitting an estimate of maximum efficiency of use of about two sperm per zygote (Gilbert 1981). By comparison, female *D. hydei* receive and store about 126 sperm per copulation. Single copulations yield, on the average, 57 progeny, providing an estimated efficiency of use of 2.2 sperm per zygote (S. Pitnick and T. A. Markow, unpublished data). This apparent lack of improved use efficiency with reduced sperm numbers may be due to weak selection on females. Because females can remate and thereby replenish their supply of sperm, unlike the self-fertilizing nematodes, sperm are more limiting from the perspective of males than of females. The remating frequency of female *D. melanogaster* (3–5 d; Pitnick 1991), *D. hydei* (several times daily; Markow 1985), and females of the *nannoptera* group species (figs. 6, 7) support this contention.

Testes Size

Despite their phylogenetic proximity, members of the *nannoptera* species group vary significantly in the total amount of energy invested by males in gamete production, measured by testes volume and testes weight (in terms of their per-

centage contribution to total dry body weight). Species also differ in total ejaculate transferred per day, an additional indicator of energetic investment by males in gamete production.

Comparative analyses of vertebrate taxa have demonstrated a positive correlation between testes size and female remating frequency, which is indicative of sperm competition levels (reviewed in Pierce et al. 1990). To our knowledge, no similar studies have been conducted with invertebrates. One cross-species study of butterflies (Svard and Wiklund 1989) has shown a positive association between degree of polyandry and both the mass and production rates of ejaculates, although no examination of testes size or the sperm content of ejaculates was made. In the present study, the highest relative investment in testicular tissue was found in *D. pachea,* which also produced the longest sperm. The only insect species we know of to make a greater relative investment in testes (7%–13% of total dry body weight) is *D. hydei,* which also makes much longer sperm (S. Pitnick and T. A. Markow, unpublished data).

Results of the present study show that the relation between testes size and mating systems in the *nannoptera* species group is not consistent with patterns found in studies with vertebrates. Comparative studies involving birds, primates, and mammals, respectively (Møller 1988*a,* 1988*b,* 1989), indicate a positive relationship between testes size and ejaculate quality, measured as either ejaculate size or the number of sperm per ejaculate. In this study, a positive relationship was found between testes volume and to a lesser extent the relative dry weight of testes and female remating frequency. However, the most critical prediction of the sperm competition hypothesis, a positive relation between testes size and ejaculate quality, was not met in this study. In fact, the opposite relationship was generally true. It appears that large testes have evolved in these *Drosophila* because they are necessary for the manufacture of large sperm (Hatsumi and Wakahama 1986; S. Pitnick and T. A. Markow, unpublished data). It would be informative to conduct a larger and broader comparative study of relative testes size, sperm size and numbers, and mating systems in invertebrates to know whether the present study is indicative of fundamental differences in spermatogenesis between vertebrates and invertebrates.

Sperm Allocation Patterns and Female Remating

Anecdotal evidence suggests that various sperm allocation strategies occur in invertebrates. For instance, a previous study (Markow 1985) revealed that male *D. hydei* gain relatively few offspring per copulation but will copulate with up to 10 females in a single morning. The number of progeny produced remains the same over six successive copulations, suggesting that males of this species partition their ejaculate among multiple matings. Svard (1985) and Svard and Wiklund (1986, 1991) found that males of the swallowtail butterfly *Papilio machaon* L. provide more sperm and more accessory gland secretions to initial versus subsequent mates and attributed this variation to males transferring more material to virgin than nonvirgin females. Finally, males of some species only partially fill the sperm-storage organs of their mates. This phenomenon has been observed in the ant *Atta sexdens* (Kerr 1961), the honey bee *Apis mellifera* (Kerr et al. 1962),

the fruit fly *Drosophila euronotus* (Stalker 1976), and several species of odonates (Waage 1984; Siva-Jothy 1988).

Among members of the *nannoptera* species group, the pattern of sperm use by males varies greatly. For example, despite transferring 62% less ejaculate over the course of a day than a typical *Drosophila acanthoptera* male, an average *D. pachea* male mates with about twice as many females. Only *D. acanthoptera* males employ the expected male strategy of completely filling the sperm-storage capacity of their mates. *Drosophila nannoptera, D. wassermani,* and *D. pachea* males fill only a fraction of each female's sperm-storage organ(s) (fig. 3). In fact, comparison of values for the total number of sperm transferred by males in 1 d (172 ± 20 sperm for wild males, 226 ± 30 sperm in the laboratory) with the measure of female sperm-storage capacity (304 sperm) strongly suggests that male *D. pachea* are incapable of totally filling even a single female's sperm-storage organs. It seems, then, that males of this species have compromised the number of sperm in each ejaculate to achieve increased potential for multiple mating.

Several lines of evidence indicate that *D. pachea* males are not simply transferring all of their mature sperm in each copulation and undergoing spermatogenesis between copulations to replenish sperm supplies. First, although virgin males had many more mature sperm available on day 1 than they did 1 d later after mating with several females, the number of sperm transferred in the first copulation of each day did not differ significantly from each other. Second, it seems unlikely that the number of sperm transferred in the second copulation of day 1, for instance, could have been produced during the average 7.4-min-long interval between copulations or during the copulation itself. Finally, although the actual numbers of sperm have not been quantified, males' seminal vesicles, vas deferens, and ejaculatory ducts always contain large numbers of sperm immediately after single matings (S. Pitnick, personal observation). Thus, the extent of submaximal insemination is not determined by a male's supply of mature spermatozoa but by partitioning of their sperm among successive matings.

Of the variables measured in this study, ejaculate allocation patterns are associated with sperm size and female remating behavior. *Drosophila pachea* and *D. nannoptera,* the species that produce relatively long sperm and are the most sperm limited, exhibit the greatest degree of ejaculate partitioning. A study of *D. hydei,* which also produces very long sperm (S. Pitnick and T. A. Markow, unpublished data), reveals a similar pattern of ejaculate allocation (Markow 1985), thereby strengthening this association. The relation between these characters is not clear, however, for *D. wassermani* males produce the shortest sperm of the four species, and they also submaximally inseminate females while *D. acanthoptera* males do not.

Female remating interval showed a strong positive relationship with the number of sperm received per copulation, suggesting that female remating is driven by fertility requirements. *Drosophila acanthoptera* females typically receive from a single copulation a sperm supply that is adequate for the fertilization of all of the eggs they will lay throughout their lives, and so they typically do not remate, while *D. pachea* and *D. nannoptera* females must remate frequently to ensure a sperm supply adequate for fertilization. In a laboratory study, *D. pachea* females

laid on average 34.6 ± 1.4 eggs per day (range: 15–48) across 6 d (Pitnick 1993). Even if females of this species use sperm efficiently, the supply of 44 sperm received in an average copulation in the laboratory in the present study will quickly be exhausted. This proposed relationship between sperm supply and fefemale remating is supported by studies of *D. melanogaster* showing that female remating behavior appears to be inhibited until the supply of stored sperm falls below a critical threshold (Gromko et al. 1984; Letsinger and Gromko 1985; Gromko and Markow 1992).

Why Partition Sperm among Mates?

Previous comparative studies have all demonstrated a positive relationship between the size of ejaculates and the degree of female polyandry (Møller 1988*a*, 1988*b*, 1989; Svard and Wiklund 1989). The implicit assumption of these studies was that female remating frequencies evolved independently from ejaculate size. Consequential variation in the intensity of sperm competition favored different patterns of sperm production and transfer. Where sperm competition is the most intense, males transferring the greatest number of sperm to their mates are at an advantage due strictly to the relative numerical representation of their gametes within the population of sperm competing for fertilization (see, e.g., Dickinson 1986).

The sperm-partitioning behavior exhibited by *D. pachea, D. nannoptera,* and, to a lesser extent, *D. wassermani* is therefore puzzling as it is inconsistent with this "numerical response to sperm competition" theory. In fact, the opposite relation between degree of polyandry and ejaculate size was found. *Drosophila acanthoptera* males transfer the largest ejaculate, containing the most sperm, and females of that species exhibit the lowest level of remating. Likewise, male *D. pachea* produce the smallest ejaculate while females exhibit a very brief remating interval and typically contain the ejaculates of multiple males.

Two alternative hypotheses that are consistent with our findings address the maintenance of sperm-partitioning behavior. The "sperm conservation" hypothesis proposes that, in the face of intense sperm competition, males should conserve their gametes by judiciously transferring to mates only the number of sperm that they are likely to use prior to remating. Advantages of employing such a strategy would be greatest in species such as *D. pachea,* where sperm numbers can be limiting (Pitnick 1993). The "bet-hedging" hypothesis proposes that in species with limited quantities of sperm, a strategy of partitioning gametes may be favored over one of maximally inseminating females because it is less risky.

With regard to the first hypothesis, results of the female remating experiment in chambers suggest that females do not use all of the sperm from a mating prior to remating, as many females remated prior to initiating oviposition (fig. 7). In vials, females were observed to typically, but not always, lay some eggs between copulations. Further, comparison of female sperm-storage data and sperm-transfer data from the field as well as the laboratory clearly indicates that sperm from several matings tend to accumulate within females (fig. 3). Sperm competition theory therefore predicts that males should exhibit a sperm conservation strategy only when there is extremely high last-male sperm precedence (e.g. displacement of first male's sperm). If the sperm of multiple males mix inside the

female reproductive tract and thereby compete directly for fertilizations, then males transferring relatively large quantities of sperm in each ejaculate will be favored via the numerical response already discussed.

Sperm competition mechanisms and the patterns of sperm precedence are not known for the *nannoptera* group species. However, as noted above, *D. pachea, D. acanthoptera,* and *D. wassermani* females store sperm only in their spermathecae. It has been suggested that such spheroid structures promote sperm mixing (Walker 1980) rather than displacement, defined here as the movement of another male's sperm to a position within the female that is disadvantageous with respect to fertilization. Our study demonstrates that sperm from multiple males do cooccupy the spermathecae and, in each species, all of the sperm within each spermatheca are intertwined into a single round mass, suggesting that no sperm displacement occurs. The potential for sperm displacement to occur is higher in *D. nannoptera,* since females of this species store sperm in the seminal receptacle, which is a long, thin tubule. The fact that *D. nannoptera* and *D. pachea* exhibit nearly identical patterns of female remating and ejaculate allocation, despite this variation in female sperm-storage morphology, further weakens the hypothesis that male ejaculate delivery patterns are adaptations to sperm competition. Finally, in *D. hydei,* in which males produce relatively few, long sperm and exhibit a pattern of sperm allocation similar to that of *D. pachea* and *D. nannoptera,* it has been demonstrated that sperm of multiple males do mix randomly within female storage organs (Markow 1985).

The second hypothesis is based on bet-hedging theory, or the occurrence of selection that favors phenotypes with low variances in reproductive success over alternatives with higher variances and potentially higher mean fitnesses (Gillespie 1974; Slatkin 1974). In this case, as sperm become more limiting, selection may favor males that partition their sperm among several females, thereby reducing variance in the number of sperm used in fertilization, over males that more rapidly deplete their sperm supply by providing many sperm to only one or two females. This could occur despite a greater mean fitness of the latter strategy (see "diversified bet-hedging" in Seger and Brockmann 1987).

It is possible to speculate on mechanisms by which sperm-partitioning behavior would reduce variance in male reproductive success when sperm are limiting in these species. For example, the ephemeral nature of necrotic cactus tissue requires frequent dispersal. As the probability of any individual successfully colonizing a newly forming necrosis composed of suitable oviposition substrate may be low, the optimal strategy for males may be to maximize the probability of some gametes reaching new populations by inseminating as many females as possible instead of risking all gametes in one dispersing female, despite its greater potential fitness payoff.

Even if ecological conditions conducive to sperm partitioning do exist, this behavior can only be maintained if there is a sufficiently high probability of males acquiring multiple mates each day. Relevant data are only available for *D. pachea*. Natural populations of this species exhibit operational sex ratios (Emlen and Oring 1977) that vary from slightly male biased to extremely female biased (Pitnick 1993). When considered with the high remating propensity of females, it

would appear that any male transferring all of his limited gametes to a single female would incur potentially huge costs with regard to mating success. In contrast, because *D. acanthoptera* females remate at a low frequency, the operational sex ratio of populations in this species is expected to be extremely male biased. As a result, males of this species should be subject to strong intrasexual competition for mates and hence have a low probability of acquiring multiple mates.

Why Make Such Long Sperm?

The question, Why do males produce so many tiny sperm? was addressed in a model exploring why species do not revert back toward isogamy with the advent of internal fertilization and hence the loss of the selective advantage of small male gametes (Parker 1982). Parker's mathematical argument can be summarized as follows. Any increase in the size of sperm would probably represent a negligible increase in provisioning to the zygote but would significantly reduce the total number of sperm that could be produced. A decrease in sperm number would prove detrimental to male fitness, given that sperm competition is common and competition for fertilizations tends to be numerical. Therefore, any mutation affecting an increase in sperm size, which has the pleiotropic effect of reducing sperm quantity, would be selected against. The model also predicts that in the complete absence of sperm competition increases in sperm quality at the cost of sperm quantity will be favored.

While Parker's model provides an explanation for the maintenance of the typical male gamete production strategy, it requires expansion, for, as this study clearly illustrates, males of some species produce relatively few long sperm, despite the presence of sperm competition. Four selection pressures have been suggested to contribute to evolution of sperm-size variation: sperm competition, provisioning of gametes as a paternal investment in offspring, selection posed by the environment of the female reproductive tract, and selection to prevent hybridization. As the limited amount of evidence for the third selective pressure was the subject of a recent review (Roldan et al. 1992), we will not address it here except to the extent that it pertains to the *nannoptera* group species.

Increased sperm length may represent a nonnumerical adaptation to sperm competition. This was convincingly demonstrated in a recent study of sperm-size variation in primates and rodents (Gomendio and Roldan 1991). Comparative investigations revealed positive relationships between sperm length and maximum sperm velocity and between sperm length and levels of female promiscuity, generating the conclusion that the greater length of sperm observed in polyandrous versus monandrous species of mammals was the outcome of sperm competition selecting for longer, faster sperm. A similar comparative study of birds found no relationship between the length of sperm and mating systems (Briskie and Montgomerie 1992).

Studies of birds have, however, identified a negative correlation between the length of sperm and the number of females' sperm-storage tubules (SSTs). This relationship has been interpreted as the consequence of selection favoring longer sperm, because of a motility advantage, as they compete for access to limited storage sites in females (Briskie and Montgomerie 1992, 1993). Alternatively,

evolution of SST numbers may be driven by sperm numbers, and males producing longer sperm may transfer fewer sperm per mating. This explanation is supported by the observations that SST number is positively correlated with the number of sperm per ejaculate and that females of species with large numbers of SSTs tend to copulate less frequently than females with fewer SSTs (Birkhead and Møller 1992*b*).

It is unlikely that this simple motility advantage to longer sperm in the race to fertilize ova or reach a storage site can explain sperm-length variation in invertebrates, at least not variation of the magnitude described here. With all of the insects producing very long sperm, it has been recognized that the sperm are much longer than the distance they need to travel within the female (Afzelius et al. 1976; Mazzini 1976; Taylor et al. 1982). This is certainly the case with the *Drosophila* species examined here (table 2), for whether sperm are traveling from the point of insemination to storage (distance = genital chamber + spermathecal duct) or from storage to the point of fertilization (distance = spermathecal duct + common oviduct), they need traverse no distance greater than about 1 mm. It is noteworthy that dimensions of the female reproductive tracts of these species are relatively invariable, suggesting that the gross variation in sperm lengths is unlikely a response to selection posed by female reproductive morphology.

Alternative mechanisms of sperm competition that may confer an advantage on longer sperm are the ability of sperm to prevent access to female storage organs by sperm of other males or to resist displacement from or within female storage organs. For example, the comparative studies of birds described above have also identified a positive correlation between sperm length and the length of SSTs (Briskie and Montgomerie 1992, 1993; Birkhead and Møller 1992*b*), which is similar to the relationship between sperm length and seminal receptacle length in *Drosophila* (fig. 2*a*) and between sperm length and spermathecae length in featherwing beetles (Dybas and Dybas 1981). With the featherwing beetles (*Bambara* spp.) there is nearly a one-to-one correspondence across species between the length of spermathecae and sperm length. The first male to mate with a female fills her spermatheca to capacity, thereby excluding sperm of potential competitors from access to her single large egg (Dybas and Dybas 1981). This mechanism seems unlikely to apply to birds and *Drosophila,* however, as with birds many SSTs remain empty or capable of storing additional sperm even after several inseminations (Briskie and Montgomerie 1993), and, with *Drosophila* species producing long sperm, sperm from multiple males are clearly not excluded from storage within females (fig. 3; Markow 1985). The ability of sperm to resist displacement may therefore provide a more likely mechanism of sperm competition in these taxa.

In both *Drosophila* and birds the ratio of female sperm-storage organ length to sperm length varies among species from one to three (fig. 2*b;* Briskie and Montgomerie 1993). Variation in this ratio may correspond to patterns of sperm precedence. When sperm and SSTs are nearly equal in length, all sperm may orient as a single layer and thus have an equal opportunity to exit the organ, such that the probability of paternity should be determined by the relative proportion of

each male's sperm that is present. When SSTs are much longer than sperm, the sperm may become stratified inside, thereby creating a "last in, first out" pattern of sperm precedence (Birkhead and Hunter 1990). Briskie and Montgomerie (1993) argue that a conflict of interest between the sexes may arise over the control of sperm-use patterns, resulting in an evolutionary arms race with ever-increasing lengths of sperm and sperm-storage organs. The arms race hypothesis generates the prediction that species with relatively long sperm, that is, those subject to strong selection, should also exhibit the highest congruence between sperm and SST, which is weakly supported by data of *Drosophila* (fig. 2*b*). Empirical studies of sperm precedence patterns relative to lengths of sperm and storage organ and of the configuration of sperm inside these organs are required before the applicability of this scenario can be assessed for either birds or *Drosophila*.

In contrast to these explanations from sperm competition theory, observed variation in sperm size may represent differential provisioning of gametes by males as a form of parental investment. We define parental investment broadly, as sperm may contribute a diversity of resources to the zygote including limiting nutrients or cytoskeletal components, mitochondrial DNA, and structural information critical to the organization of subsequent development. A role for nonnuclear sperm components in events subsequent to fertilization was first attributed to long sperm by Perotti (1973) and Afzelius et al. (1976) and was later addressed in detail by Sivinski (1984). These authors focused on the proteinaceous mitochondrial derivatives that are located adjacent to the axoneme for its entire length and compose 50%–70% of the total sperm volume of these species, as their function is unknown (Perotti 1973; Afzelius et al. 1976). These derivatives do, however, possess characteristics consistent with a paternal investment hypothesis: they do not possess the biochemical activity of mitochondria, are structurally stable during the life span of the sperm, and are metabolically inert (Sivinski 1984). They may therefore provide a nutritive resource to the zygote, or they may function in cytoplasmic inheritance. In the spermatid of *D. melanogaster*, RNA synthesis in the mitochondrial derivative is known to continue long after detectable RNA synthesis by the nucleus has ceased (Curgy and Anderson 1972; Gould-Somero and Holland 1974), thereby implying the availability of DNA for transcription in the derivative. Moreover, recent studies employing high-resolution techniques suggest that heteroplasmy may not be uncommon (Kondo et al. 1990; Gyllensten et al. 1991).

The possibility of additional postfertilization functions of sperm components was raised by a recent study of the behavior, position, and persistence of the sperm tail during embryonic development. Using sperm-specific antibodies, Karr (1991) conducted a detailed analysis of the position and fate of the 1,750-μm-long sperm tail of *D. melanogaster* during and after fertilization. It was discovered that the sperm tail coils and folds into a stereotypical structure inside the oocyte that is repeatable among embryos. The sperm tail remains intact through stages of gastrulation and undergoes reproducible changes in morphology and position throughout this developmental period. During cellular blastoderm formation the sperm tail is sequestered into the anterior yolk area, where it continues to persist

well into embryonic development. These findings are consistent with the hypothesis that sperm provide a contribution to the developing embryo, either in terms of resources or structure that guides cytoplasmic organization.

Given the numerous ways in which parents can provide nutriment to their offspring (Clutton-Brock 1991), including the production of nutritional ejaculatory secretions that are subsequently incorporated into eggs by females (reviews in Leopold 1976; Boggs 1990), the question of why males might evolve a strategy of sperm provisioning needs to be addressed. Indirect investments by the male, through substances absorbed by the female, may increase future fecundity. However, these substances may provide for ova that are subsequently fertilized by another male's sperm (Sivinski 1984; Markow 1988). Indirect investments may also be usurped by the female, incorporated into her own somatic tissue rather than the enhancement of egg production. Further, a shortened time between resource transfer and deposition into zygotes minimizes the probability of a female's dying before producing the investing male's offspring (Sivinski 1984).

Finally, it has been suggested that variation among closely related species in sperm morphology, and more specifically sperm length, may serve as a mechanism of reproductive isolation (Afzelius et al. 1976; Dybas and Dybas 1981). In the case of featherwing beetles belonging to the genus *Bambara,* the highly complementary form of sperm and spermathecae within species and the divergent nature of these structures among species were interpreted as a prezygotic, mechanical barrier to preserve reproductive isolation. The investigations by Karr (1991) of the three-dimensional shape assumed by sperm inside of eggs also suggest a possible role of sperm morphology in providing a mechanism of postzygotic reproductive isolation, as the shapes may be species specific (T. L. Karr, personal communication).

A full understanding of the origin and maintenance of variation in sperm length, in addition to other components of the male gametic strategy, awaits investigations of physiological and phylogenetic constraints on sperm production, detailed examinations of sperm use by females, and studies of functional aspects of sperm morphology, including mechanisms of sperm precedence, maintenance of sperm viability within females, and sperm/egg interactions during fertilization and throughout early development. The results of this study, which demonstrate monumental variation in sperm morphology and use in closely related *Drosophila* species, suggest that male gametic strategy merits greater attention in both theory and empirical research.

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