Another way of being anisogamous in *Drosophila* subgenus species: Giant sperm, one-to-one gamete ratio, and high zygote provisioning

(evolution of sex/paternity assurance/male-derived contribution/*Drosophila littoralis*/*Drosophila hydei*)

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ABSTRACT It is generally assumed that sexes in animals have arisen from a productivity versus provisioning conflict; males are those individuals producing gametes necessarily small, in excess, and individually bereft of all paternity assurance. A 1- to 2-cm sperm, 5–10 times as long as the male body, might therefore appear an evolutionary paradox. As a matter of fact, species of *Drosophila* of the *Drosophila* subgenus differ from those of other subgenera by producing exclusively sperm of that sort. We report counts of such giant costly sperm in *Drosophila littoralis* and *Drosophila hydei* females, indicating that they are offered in exceedingly small amounts, tending to a one-to-one gamete ratio after a single mating. As a result, most of them are successfully involved in a fertilization. Hence, the concept of “paternity assurance of individual sperm” arises. Evidence is further provided here that almost the entire sperm is incorporated into the egg during fertilization. Labeling with specific antibodies in fertilized eggs reveals intact axonemes up to late gastrulation. The question, then, is why selection has favored such an unusual strategy. Explanations related to some prefertilization functions are ruled out. It is therefore tentatively proposed that virtually every giant sperm constitutes a “direct paternal legacy to the embryo,” which, in contrast to any male-derived nuptial gift, cannot be minimized by female remating. We suggest that dramatic shortage of giant sperm with a high prospect of fusion and increased zygote provisioning is merely another way of being anisogamous.

The evolution of anisogamy (two sexes) is traditionally viewed as resulting from the conflicting demands of gamete productivity on the one hand and zygote provisioning on the other (1–8). Central to the issue is the assumption that males and females devote nearly equal amounts of resources to gamete production and that provisioning strongly affects zygote survival. Implicit in this is a negative correlation between sperm and oocyte outputs and between sperm output and sperm length (9). However, drive to produce smaller and smaller (i.e., male) gametes in increasing numbers might be countered because the zygotes produced have less provisioning and are consequently less viable and require more time to mature (10). The question is then whether drive to produce enlarged male gametes can be evolutionary stable.

The evolution of sperm of inordinate length has sporadically occurred in the arthropod phylogenetic tree: ostracods in crustaceans, *Scutigera* in millipedes, and waterbugs, ptiliid beetles, and fruitflies in insects (11, 12). Within *Drosophila*, this trend has seemingly been magnified uniquely in the subgenus *Drosophila* including *Drosophila littoralis* and *Drosophila hydei*, whose sperm length is on average 7.3 and 16.9 mm, respectively; the latter produces the largest sperm so far known among metazoans (12). In this way, *Drosophila* species provide a remarkable diversity of situations ranging from

within-ejaculate short sperm heteromorphism in the *Drosophila obscura* species group (*Sophophora* subgenus) to giant sperm found solely within the *Drosophila* subgenus. The most extreme pairwise comparison of sperm length between these taxonomic groups represents a factor of growth of 300 (12). In all *Drosophila* species described so far in this respect, sperm contain a short acrosome, a filament haploid nucleus, and a flagellum composed of two inactive mitochondrial derivatives (13, 14) flanking one axoneme along its overall length: the longer the sperm, the larger the flagellum and hence the more mitochondrial material.

The cost of such exaggerated sperm and more generally of sperm production is a matter of controversy in current thought on the evolution of sex since it happens to be substantial (15). Although definitely present in many organisms, and more especially in vertebrates, the traditional view on the universality of anisogamy has nevertheless been tempered by recent findings in nematodes that individuals may produce more oocytes than sperm and that fecundity may be limited by sperm production (16, 17). However, these organisms might appear somewhat heterodox considering they are self-fertilizing hermaphrodites and their gamete production switches from sperm to oocytes within each gonad (18, 19). If the productivity versus provisioning conflict is valid, there should exist a number of alternative solutions.

In fact, we report here empirical data based on sperm counts in females and labeling in fertilized eggs in *D. littoralis* and *D. hydei* consistently showing that the costs of making sperm happen to be considerably high in outcrossing organisms as well. Giant-sperm *Drosophila* have cracked the productivity/provisioning problem the other way round: it turns out that some, though not all, *Drosophila* lineages have evolved radically opposite evolutionary trends where male gametes combine low productivity and high zygote provisioning with exaggerated size. The issue of why there is one such sperm cell loaded with, for example in *D. hydei*, more than 3 cm of inactive mitochondrial aggregates is actually a functional as well as an evolutionary riddle. A possible clue to this is proposed in the present work suggesting that giant sperm in these species, beyond transferring paternal genetic information, may be a direct resource donated to the offspring.

MATERIALS AND METHODS

Two species of the genus *Drosophila*, both of the subgenus *Drosophila*, were used:

- (i) *D. littoralis*: Paris area, 1980 (collected by J. David).
- (ii) *D. hydei*: Mt. Kenya (Kenya), altitude of 3050 m, September 1984, Meteorological Station site, at top of bamboo zone on the west side along the Naro Moru track (collected by D. Lachaise, M. L. Cariou, and M. Ashburner).

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Table 1. Number of giant sperm involved in one mating in two representatives of the *Drosophila* subgenus

Species	Sperm offered		Sperm stored				Sperm used		Sperm used/ sperm offered
	<i>n</i>	mean $\pm$ SE	<i>n</i>	Receptacle	Spermatheca	Total	<i>n</i>	mean $\pm$ SE	
<i>D. littoralis</i>	30	233.6 $\pm$ 13.9	30	54.0 $\pm$ 2.7	115.7 $\pm$ 3.9	169.7 $\pm$ 5.2	18	133.8 $\pm$ 5.2	0.57
<i>D. hydei</i>	30	83.4 $\pm$ 3.3	20	67.0 $\pm$ 1.8	3.1 $\pm$ 0.8	70.1 $\pm$ 1.7	18	67.1 $\pm$ 2.0	0.80

n, Number of individuals tested.

Both strains have been maintained in mass culture since collection. Considering late sexual maturity (20, 21), virgin 7-day males and females were allowed to mate once and then separated. *Sperm transferred*. Thirty females for each species were dissected in PBS immediately after mating, and the contents of their uterine cavity were transferred onto a 12-mm diameter coverslip. This preparation was air dried, fixed in ethanol (4 min at 20°C), air dried again, and then incubated in a 4',6-diamidino-2-phenylindole solution (2 μ g/ml in PBS) for 20 min at 20°C. The coverslip was then rinsed in PBS, mounted, and observed on an epifluorescence microscope. Sperm heads were counted exhaustively owing to their particular filiform shape. *Sperm stored*. For each species 30 females from another series were dissected 3 hr postmating and the same method was used on female storage organs. 4',6-Diamidino-2-phenylindole-labeled sperm were counted through the wall of both the unpaired ventral receptacle and the paired spermatheca. *Sperm used*. Eighteen inseminated females per species were individually placed in oviposition boxes, and the hatched eggs were recorded daily, giving an estimation of the number of sperm used.

For confocal microscopic detection of the axoneme, embryos were prepared according to Karr (22). A monoclonal antibody, TAX49 (23), directed against axonemal tubulin from *Paramecium*, the specificity of which is similar to that of the polyclonal antibody TAPp (24), was used as primary antibody. This was followed by a fluorescein isothiocyanate-coupled anti-mouse antibody as secondary antibody. Embryos were then mounted in a drop of aqua-poly/mount (Polysciences, Warrington, PA) between two hairs and examined under a Bio-Rad MRC600 confocal microscope.

RESULTS

Table 1 reports exhaustive counts of sperm offered, stored, and used after one mating in *D. littoralis* and *D. hydei*. It appears that in both species the number of sperm involved in one mating is strikingly reduced.

The number of sperm a male transfers to a female is unusually small in both species—on average <250 in *D. littoralis* and <100 in *D. hydei*. For comparison, in *Drosophila melanogaster* for instance, the amount of sperm offered after one mating reaches 2000–4000 (25)—that is, 8–16 and 20–40 times as much while sperm are 4 and 8.5 times shorter, respectively (12).

Sperm stored represent a proportion of 0.73 and 0.84 of sperm offered in *D. littoralis* and *D. hydei*, respectively. Sperm storage organs in *Drosophila* females include an unpaired tubular ventral receptacle and paired fungus-shaped spermatheca. The former appears to yield significantly more sperm in *D. hydei* than the latter and vice versa in *D. littoralis*. Consistent with this, the receptacular volume is greater in *D. hydei* than in *D. littoralis*, and the spermathecal volume is extremely reduced in the former while highly developed in the latter. The reason for such a difference in storage remains unclear and, therefore, the discussion will henceforth refer to total stored sperm.

The ratio of sperm stored to sperm offered is 0.73 and 0.84, while the ratio of sperm used to sperm stored is 0.79 and 0.96 in *D. littoralis* and *D. hydei*, respectively.

Finally, the numbers of sperm used and sperm offered after one mating are very close to one another (compared to nonrelated *D. melanogaster* and *a fortiori* nonrelated clades), indicating that wastage is limited and gradual. As a result, the ratio of sperm used to sperm offered (that is, chance of fusion for each giant sperm) after one mating reaches exceedingly high values—0.57 in *D. littoralis* and 0.80 in *D. hydei*, respectively.

Of interest is the comparison of these data with some available from the literature involving *D. melanogaster*, various other nonrelated insects (*Papilio zelicaon* and *Schistocerca gregaria*), and mammals (Fig. 1). Paternity assurance of individual sperm—that is, the ratio of sperm used to sperm offered—increases from mammals to insects as a positive function of sperm length. Of primary importance is that while insignificant (i.e., 10^{-9} – 10^{-6}) in the former phylum, this value becomes close to 1 in species producing exceedingly long sperm.

We used specific antibodies to label giant sperm axoneme within just fertilized eggs and early embryos of the two species (Fig. 2). Evidence is thereby provided for the incorporation of this complete or almost complete arthropod sperm into the egg. In both species, the axoneme is detectable during the first developmental stages. The giant sperm tail is tightly wound into a ball at the anterior end of the embryo up to early ventral furrow formation (i.e., early gastrulation). In late gastrulation (early germ-band retraction), the axoneme has still not been disorganized, though it is found at the level of the medial gut (yolk region) containing the metabolic wastes.

DISCUSSION

Sperm gigantism is an evolutionary trend that has convergently appeared within various arthropod lineages. In an attempt to understand why selection has not countered such an exaggerated trait, we have made counts of giant sperm offered, stored, and used and have labeled sperm within

FIG. 1. Paternity assurance of individual sperm (ratio of sperm used to sperm offered, logarithmic scale) as a positive function of sperm length (logarithmic scale) after a single mating. ♦, Sheep (26, 27); ●, primates (26, 28): chimpanzee, human, orangutan, and gorilla; ▲, rabbit (26, 29); ■, insects: *Papilio zelicaon* (14, 30), *Schistocerca gregaria* (C. Bressac, unpublished data; ref. 31), *D. melanogaster* (12, 25), *D. littoralis* (12), and *D. hydei* (12). The dashed line indicates the value 1.

fertilized eggs. Evidence is provided here, from direct and exhaustive counts of such giant sperm offered after one mating in two species of the *Drosophila* subgenus, that exaggerated sperm length goes together with extremely limited numbers. Unlike the nematode *Caenorhabditis elegans*, which combines sperm shortage and relatively cheap sperm (19), these *Drosophila* thus combine sperm shortage with highly costly sperm. Contrary to the common expectation that giant sperm act solely as copulatory plugs (32), it is shown here that the overwhelming majority of them are actually involved in a fertilization. As a result, there is a nearly equivalent amount of sperm and egg usage. This is in contrast to most species where relatively tiny male gametes are in large excess, and hence it appears as an alternative to traditional anisogamy.

A limitation, however, could be that females of the species studied here inevitably remate daily, and the egg output may then go up 2- to 3-fold (20, 21). Unlike *D. melanogaster* (25), however, the males favored in multiple matings in *D. littoralis* and *D. hydei* are the first two to mate instead of the last (20). As a result, in spite of remating, the giant sperm offered in such a system with "reversed predominance" (that is referring to *D. melanogaster*) have, individually, a high prospect of fusion.

Although a similar prospect of fusion for both sexes may explain the high cost of sperm, the question nevertheless remains as to why exaggerated length has been selected for. Central to the debate is the fate of giant sperm. Females contribute to the process in that they have similarly giant unpaired tubular storage organs. Interestingly, a similar

positive correlation between sperm length and the length of female sperm storage tubules was found in passerine birds (33). Few prefertilization functions can satisfactorily account for exaggerated length of both male gametes loaded with respiratorily inactive mitochondrial derivatives and female storage organs. One possibility is better retention and higher survival of the former within the latter in obligatorily multiple-mating systems, but this would only make sense if it resulted in considerably delayed fertilization, a result that is inconsistent with experimental data.

The lack of any clear function related to sperm activity or storage in females suggests instead some postfertilization role (34). What, then, could be the fate and function of *Drosophila* sperm within the developing embryo? The hypothesis, raised for *D. melanogaster*, that the flagellum could play a mechanical role in positioning the male pronucleus in anticipation of karyogamy (22) is ruled out since it could not explain why the sperm length varies by a factor of 300 within the genus (12). The role of giant axonemes as a major supply of tubulin and cytoskeletal components during early development is also not supported by our results, since this structure is still substantially intact when in the medial gut; it is probably eliminated with metabolic wastes at hatching. In mitochondrial derivatives, autonomous transcription seems to continue in spite of paracrystalline differentiation (35). This conclusion has, however, remained controversial. If valid, these highly modified organelles might therefore represent a device for transferring to the zygote, in addition to some energetic reserves, considerable inputs of paternal transcripts associated with some metabolic function.

In the controversy on resource extraction from each parent, it is assumed that male gametes act as "parasites" of female ooplasm, which provide all nutrients, allowing complete development of embryos (2, 5, 36, 37). Incorporation of huge sperm in eggs can be seen as an unusual direct paternal contribution. Such a male's investment results in a selective advantage if and only if paternity assurance of individual sperm is high and there is no predominance of the last male to mate, two conditions that are consistently satisfied in these insect species.

In various arthropods and in some vertebrates, males transfer nutrients to females by seminal fluids (38, 39) or by overnumbered sperm (40). In some *Drosophila*, seminal secretions were shown to benefit the male's mate and hence indirectly his offspring (38, 39). Some species make large ejaculatory contributions, while males of others, like *D. hydei*, pass no nutritive substances to females (39). However, this should be tempered by our findings that nuptial gift (incorporation of male-derived substances by females) is advantageously replaced by direct donation to eggs in the form of exaggerated sperm (incorporation of male-derived substances by eggs). In contrast to the former situation, the latter will not be minimized by female remating. In fact, it can be seen as a mechanism for minimizing cuckoldry. In terms of sexual selection, this may explain why gradually enhanced sperm length has been selected for in spite of increasing cost. Table 2 summarizes the four alternatives of the possible

FIG. 2. Indirect immunofluorescence of *D. littoralis* giant sperm axoneme decorated with TAX49 in a fertilized egg. (A) Anterior end of embryo in dorsoventral view in early gastrulation: early ventral furrow formation. (B) Embryo in subdorsal view in late gastrulation: early germ-band retraction. From early to late gastrulation, the sperm tail is drawn away from the anterior end to the level of the medial gut (yolk region). ax, Labeled axoneme; mp, micropylar pole; cf, cephalic furrow; vf, ventral furrow; ih, invagination of head; ps, parasegments; gb, germ band. (Bars = 50 μ m.)

Table 2. Paternal contributions to offspring success resulting from the environment-derived or male-derived resource versus indirect or direct donation alternatives

Donation	Paternal contribution	
	Environment-derived resource	Male-derived resource
To females	Nuptial gift (prey, seed)	Cryptic nuptial gift (seminal fluid)
To offspring	Paternal care (prey)	Direct donation to embryo (giant sperm)

paternal energetic allocation to offspring, including our interpretation of giant sperm as direct donation to embryos. Thus, our data support the assumption (10) that the "provisioning effect" is most likely one of the key factors in the evolution of anisogamy.

If the solution to this productivity/provisioning problem is again the stable coexistence of two morphs for gamete size (males and females), evolution has undeniably resulted in a diversity of anisogamous systems, some of which have a nearly even prospect of fusion for male and female gametes after one single mating. The present empirical data do confirm the expectation that drive to produce longer and longer male gametes should be accompanied by decreasing number. Consistent with this is increased provisioning to the zygote. Thus, producing males does not necessarily result from producing increasing amounts of depauperate gametes.

Whether or not such resource investment to eggs by both sexes may result in or from a parental conflict is an outstanding question. Whatever the answer, it may further create opportunities at the level of the cytoplasmic conflict (41–44). In mammals, the oocyte and sperm contribute to eggs 10^6 – 10^8 and 100 mtDNA molecules, respectively (45), resulting in a serious competitive disadvantage for paternal ones. Although cytoplasmic inheritance in animals was thought to be strictly maternal, there is recent evidence for some paternal cytoplasmic contribution in mussels, mice, and *Drosophila* (46, 47). It may be suggested that exaggerated male gametes may restore, at least locally at the anterior end of the fertilized eggs, a ratio of cytoplasmic information more favorable to paternal ones; this might temporarily counter the effects of cytoplasmic matriarchy.

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