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Functional nonequivalence of sperm in *Drosophila pseudoobscura*

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ABSTRACT **We report on a form of sperm polymorphism, termed polymegaly, that occurs in species of the *Drosophila obscura* group. Individual males of species in this group characteristically produce more than one discrete length of nucleated, motile sperm. Hypotheses suggested to explain the evolutionary significance of sperm polymorphism have been either nonadaptive or adaptive, with the latter focusing on sperm competition or nutrient provisioning. These hypotheses assume all sperm types fertilize eggs; however, no data have been gathered to test this assumption. We found that two size classes of sperm are produced and transferred to females in approximately equal numbers by the male; only long sperm persist in significant numbers in female sperm storage organs. Furthermore, we used a DNA-specific dye (bisbenzimide) and sperm-specific antibodies to ask if both sperm types fertilize eggs in *Drosophila pseudoobscura*. Confocal microscopy and immunofluorescent analyses of fertilized eggs using anti-sperm polyclonal antisera demonstrated that only long sperm participate in fertilization. These data falsify those hypotheses in which all sperm types are assumed to be functionally equivalent (fertilize eggs). Any remaining or new hypotheses for the evolutionary significance of polymegaly must incorporate these findings. Several new areas of research are suggested.**

Morphologically variant sperm have been observed in a number of species including (*i*) plants, which produce pollen with varying numbers of apertures (1), (*ii*) lepidopterans (butterflies and moths), which produce nucleated (eupyrene) and nonfertilizing anucleated (apyrene) sperm (2), (*iii*) pentatomids (stinkbugs), which produce sperm containing chromosome numbers ranging between 1 and 100 (3), and (*iv*) mammals with sperm that have multiple or missing tails ("aberrant sperm"; refs. 4 and 5). Despite the wide taxonomic range in which this phenomenon occurs, sperm polymorphism has been a conundrum for evolutionary theory since it was first described in 1902 (6). Relatively few data have been gathered to address this issue (2, 7–9), even though many hypotheses have been suggested to explain its persistence (2, 4, 7–13). These hypotheses have been nonadaptive or adaptive.

The nonadaptive hypothesis suggests that aberrant sperm are simply the consequence of errors in meiosis due to chiasmata dysfunction and thus have no adaptive significance (11). Alternatively, adaptive hypotheses can be grouped into two categories, one involving sperm competition strategies and the other involving male-derived nutritive contributions. Three hypotheses based on sperm competition theory are that (*i*) not all sperm are produced to function in fertilization; nonfunctional "kamikaze" sperm of one male block important areas along the female reproductive tract and do not allow a second male's sperm to enter female sperm storage organs (12); (*ii*) different sperm morphs are capable of fertilizing eggs but do so under different situations or at different times after mating (4) and that, specifically, sperm polymorphism should be expected under alternative selective contexts, such as facultative polygamy (7, 8); and (*iii*) nonfunctional sperm, such as the apyrene sperm of lepidopterans, may serve to increase female remating latency by serving as "filler" in the female reproductive tract (2). The nutritive contribution hypothesis suggests that one sperm type may be broken down and used by the other type of sperm or incorporated into either the ovarian oocytes or somatic tissue of females as in other insect species (14, 15).

Species of the *Drosophila obscura* group are well-suited to investigating the foregoing hypotheses. All males of this species group characteristically produce, throughout their lifetime, more than one size of motile, nucleated sperm, a phenomenon known as polymegaly (refs. 16–20; Fig. 1*A*). This system differs from other species that exhibit sperm polymorphism in that all sperm types possess the correct amount of DNA and arise through seemingly identical spermatogenesis processes (21, 22). Thus, it has been assumed that all sperm morphs function in fertilization (4, 7, 8, 10, 21). This assumption has been critical to the development of several evolutionary hypotheses proposed to explain polymegaly (4, 7, 8, 10). However, earlier preliminary studies have noted that in *D. pseudoobscura* and its relatives, only one sperm type appears to be present in sperm storage organs of females (23, 24), suggesting that both morphs may not participate equally in fertilization. Our goal was to determine whether all sperm types do, in fact, fertilize eggs. We performed experiments on *D. pseudoobscura* to (*i*) determine the patterns of sperm morph production and transfer to females by males, (*ii*) determine the proportions of sperm morphs that are being stored by females and are thus available for fertilization, and (*iii*) determine directly, using sperm-specific antisera and confocal microscopy, which sperm type(s) fertilize eggs.

MATERIALS AND METHODS

D. pseudoobscura. Flies were wild-caught in Tempe in 1991 and were raised on standard cornmeal/molasses/agar medium. Virgins were separated by sex upon eclosion and were stored until reproductive maturity in 10-dram (1 dram = 4 ml) food vials containing active yeast with no more than 10 same-sex individuals per vial. Flies were 5 days old when used. All flies were kept at 22–25°C on a 12-hr light/12-hr dark photoperiod.

Sperm Length and the Number of Sperm Types. Previous investigations disagree as to whether males of *D. pseudoobscura* produce two (19, 20) or three (16, 17) distinct types of sperm. Further, these studies do not agree as to the total length of sperm types, differing in some cases by over 100 μm. Because of these difficulties, we repeated sperm head, tail, and total length measurements using Hoechst 33258 [2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride pentahydrate], a DNA-specific stain, in conjunction with glycerol. Hoechst stain allows sperm heads to be visualized, and addition of glycerol to the medium allows tails to be discerned.

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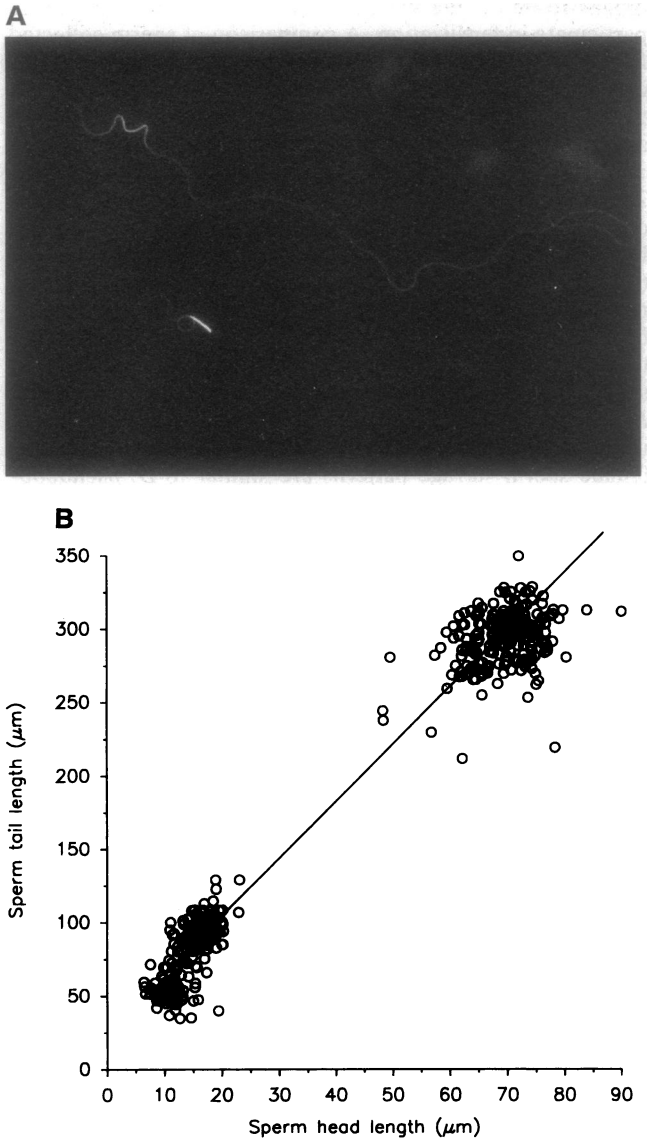

FIG. 1. (*A*) Sperm from seminal vesicles of a reproductively mature virgin *Drosophila pseudoobscura*. The disparity in "shorter" and long sperm head and tail lengths (and thus, total length) between morphs is visible by epifluorescence microscopy. (×370.) (*B*) The relationship between sperm head and tail lengths, which illustrates the accuracy of assessing shorter vs. long sperm types by either head or tail length.

To obtain sperm, virgin males were ether-anesthetized, and the seminal vesicles (where mature sperm are located) were removed. Sperm samples from each male were dissected in phosphate buffer solution (PBS) on a gelatin/chrome alum-coated microscope slide and spread evenly; a drop of glycerol was added to the PBS before drying. Sperm were then fixed and stained following a described protocol (25). Photographs of 80–100 sperm per male were taken from these samples, which were viewed using a Nikon Diaphot microscope equipped with epifluorescence illumination (see Fig. 1*A*). Sperm heads and tails were measured from these photographs using a Summagraphics digitizer and SIGMASCAN (Jandel, Corte Madera, CA). Regression analysis was performed on sperm heads and tails to determine if sperm type could be accurately predicted from sperm head length. This has previously been shown to be the case (26).

Sperm Production and Transfer by Males and Sperm Storage by Females. Sperm samples from males or females were dissected in PBS on a gelatin/chrome alum-coated microscope slide, spread evenly, and dried. Sperm were then fixed and stained according to a described protocol using Hoescht 33258 stain, which causes sperm heads to fluoresce (25). As stated, sperm head length has been shown to be highly predictive of total sperm length (26). After staining, sperm samples were counted using an epifluorescent microscope.

To establish the ratio of sperm morphs produced by males, seminal vesicles of virgin reproductively mature males were dissected. For the remaining samples (sperm transfer to females and sperm storage by females), reproductively mature virgin females were mated to reproductively mature virgin males. To document the ratio of sperm morphs transferred to females, the female reproductive tract was removed, and the uterus was dissected immediately postcopulation. To determine the proportions of sperm morphs stored by females, the female reproductive tract was removed at specified intervals postcopulation, and the uterus and sperm storage organs (ventral receptacle and paired spermathecae; ref. 27) were isolated on different slides. Once each tissue was separated from the others by scissoring with fine probes, sperm were evacuated by placing pressure on the tissue. Tissues were then dissected to make sure all sperm were removed. Since 25,000 sperm are transferred in a single copulation (R.R.S., unpublished data) and even more sperm are produced than what is transferred, determining the proportions of sperm types in seminal vesicles and the uterus is time consuming. Thus, proportions of sperm morphs in the seminal vesicles were quantified by subsampling in which the sample was distributed on a microscope slide etched with a 5-mm^2 grid, and two areas on the grid, an exterior (1 mm × 1 mm) and an interior (3 mm × 3 mm), were counted. The proportion of sperm morphs transferred was determined by performing three different random counts of at least 500 sperm (total of 6000 sperm per sample) in each area. Females also retain many sperm in the uterus until oviposition begins. A similar subsampling protocol was followed to determine the proportion of sperm types in the uterus, except that only one count of at least 500 sperm (total of 1000 sperm per sample) in each area was performed. For time points after mating and oviposition, total enumeration in the uterus was possible. The much lower numbers of sperm in the storage organs (minimum = 0, maximum = 2345) allowed complete counts without subsampling. For each time point examined, three replications of three to five females each were performed. All statistics, except testing for an area effect, were performed on arcsine-transformed proportion data. Statistics for determining an area effect were performed on the actual number of sperm counted; exterior and interior areas were compared for a specific sperm type. Area-effect analyses were performed separately for the two sperm morphs. Only those individuals with sperm in a particular tissue were included in analyses, since proportion data were used. All statistical analyses were performed using the STATISTIX (version 3.5; Analytical Software) package.

Antibody Production/Egg Collections. To determine the sperm type(s) present in fertilized eggs, we directly measured sperm found in embryos using a sperm-specific polyclonal antibody and indirect immunofluorescence. In *Drosophila* the entire sperm enters the egg and remains intact during and after fertilization, allowing for sperm measurement within the embryo (28). Precise estimates of sperm length in fertilized eggs can then be made from three-dimensional reconstructions of confocal images.

To collect fertilized eggs, reproductively mature virgin females were singly mated to virgin males. Two different "treatments" of eggs were collected: (*i*) those oviposited at 24–25 hr, and (*ii*) those oviposited some time between 24 and 72 hr after mating. For eggs oviposited at 24–25 hr, 100 females were placed on nonoviposition medium (agar/sugar/water) until 24 hr postcopulation. Females were then placed

on molasses/agar plates containing a yeast paste to initiate oviposition. Eggs were harvested within 1 hr of female placement on the molasses/agar/yeast plates. For eggs collected 24–72 hr postcopulation, ≈200 singly mated females were kept on standard medium until 24 hr after mating. Females were then placed on molasses/agar/yeast plates with egg collections made randomly; no collection was made if eggs had been oviposited for longer than 2 hr since the last collection. Egg fixation and immunofluorescence were performed by standard methods (28) using a mouse polyclonal antisera diluted 1:500. The antisera was developed against *D. melanogaster* sperm by standard methods (29).

Confocal Microscope Imaging/Epifluorescent Microscopy. Confocal images (512 × 512 pixels) of antibody-labeled eggs were collected at 2-μm intervals using a Zeiss confocal laser scanning microscope and associated computer and software. The image was generated from 40 images (total of 80 μm in the z axis) using the "overlay" function of the on-board computer. The total length of the sperm was measured using the measurement function of the on-board computer software.

Because long sperm are easily discernible from shorter sperm (≈4 times the length of the shorter class), we were also able to easily determine which sperm morph was present in fertilized eggs by visual inspection of embryos using a conventional epifluorescence microscope.

RESULTS

Sperm Length. We found evidence for only two morphs. It is possible that there are two size classes within the smaller morph, but it would be difficult to accurately distinguish between them given the high degree of overlap between these potentially different size classes (Fig. 1*B*). Further, since other published distributions of sperm length (16, 20) indicate a high degree of overlap between the "short" and "medium" class, but no overlap between these classes and the long sperm type, we chose to base our analysis on only two sperm types. We therefore pooled short and medium sperm to create a shorter size class, which, regardless of type, does not overlap the long sperm morphs (refs. 16 and 20; Fig. 1*B*). Values of sperm head, tail, and total length are presented in Table 1. The total length of the long sperm morph is ≈4 times the length of the shorter sperm class. Additionally, we confirmed that sperm head length is highly predictive of sperm tail length (and thus, total sperm length; Fig. 1B; regression analysis, $F = 12772.45$, df = 1427, $P < 0.00001$, $r^2 = 0.968$; the line is described by the equation, tail = 25.15 + 3.83 head).

Sperm Production, Transfer, and Storage. For all experiments, there were no significant differences between subsampled counts performed in the exterior and interior areas (Kruskal–Wallis tests, not significant) or between the three counts in each area themselves (Kruskal–Wallis tests, not significant). There were no significant differences between any replications for any hour (Kruskal–Wallis tests, not significant) in any experiment. Thus, data presented are combined from replications.

We examined the proportion of each sperm morph produced and transferred by males and compared this to the proportions found in the female uterus and the sperm storage organs to determine whether both morphs are available to participate in fertilization. A comparison of sperm collected from males prior to copulation with sperm found in the uterus immediately postcopulation revealed that sperm morphs are transferred to females in approximately the same proportion as produced by males (all data presented as mean proportion ± SE; 0.44 ± 0.02 shorter sperm, 0.56 ± 0.02 long sperm; Fig. 2). However, at 6 hr after mating, the long sperm morph is overrepresented in female storage organs (0.74 ± 0.03), and by 48 hr after mating essentially only long sperm are present (0.97 ± 0.004; Fig. 2). In other words, while shorter sperm represent 44% of the ejaculate, by the time oviposition begins, 24–36 hr postcopulation (R.R.S., unpublished data), they represent only 14.4%, and shortly thereafter only 3%, of sperm in female storage organs (Fig. 2). Preliminary data on *Drosophila persimilis*, *D. pseudoobscura*'s sister species, indicate that both male sperm production and transfer and female sperm storage patterns are similar to *D. pseudoobscura* (R.R.S., unpublished data).

Table 1. Sperm head, tail, and total lengths (mean ± SE)

Type	n (sperm)	Mean length ± SE, μm		
		Head	Tail	Total
Shorter	5 (215)	14.2 ± 0.23	77.9 ± 1.9	92.1 ± 1.7
Long	5 (214)	56.6 ± 0.39	292.9 ± 1.3	362.5 ± 1.5

n, number of males.

Sperm that have not migrated to the storage organs are pushed from the uterus during oviposition. Sperm found in the uterus after oviposition were primarily the long morph (Fig. 2 and Table 2). These sperm either leaked out of sperm storage organs (30) or remained due to incomplete and differential evacuation.

While primarily long sperm are stored by females, a small number of shorter sperm are observed in storage (Table 2), suggesting that they may also function in fertilization, especially at the onset of oviposition (Fig. 2).

Confocal Imaging/Epifluorescent Microscopy. Three-dimensional reconstruction of fertilized eggs collected 24–72 hr postcopulation indicated that all sperm lengths in eggs were >320 μm (n = 12; Fig. 3). Additionally, we scored sperm length by visual inspection of fertilized eggs using an epifluorescent microscope. This was possible because of the disparity in sperm lengths. The sperm in Fig. 3 is marked at the length expected for the shorter sperm morph. While we initially examined 325 eggs collected 24–72 hr after mating, we specifically scored a group of eggs (n = 32) that were the first to be oviposited by a group of females. In no case was a fertilized egg observed to contain a short sperm.

DISCUSSION

Despite the production and transfer of two morphologically variant sperm types, both of which contain DNA, only long sperm are seen to participate in fertilization in *D. pseudoobscura*. Most previous speculations on the adaptive significance of polymegaly (7, 8, 10) and sperm polymorphism in general (4) were predicated on the assumption that all sperm function in fertilization. Knowledge that only one sperm morph functions in fertilization (at least for *D. pseudoobscura* and probably *D. persimilis*) can now be integrated into evolutionary hypotheses on the significance of polymegaly. These hypotheses, discussed earlier, must suggest why shorter sperm are produced and transferred to females when they do not function as fertilizing gametes.

The nonadaptive hypothesis, which implies that shorter sperm are the consequences of errors in meiosis due to chiasmata dysfunction (11), does not apply to *Drosophila*. In the *D. obscura* group, sperm develop in bundles of 128 sperm with each bundle containing only one sperm type (16, 17). Each bundle is derived from a single stem cell at the beginning of its development. Because the fate of a bundle is determined at its inception before spermiogenesis begins, errors in meiosis can not explain polymegaly. Further, crossing-over does not occur in *Drosophila* males, eliminating chiasmata dysfunction as an explanation for polymegaly. Additionally, the fact that males of *D. pseudoobscura* produce nearly

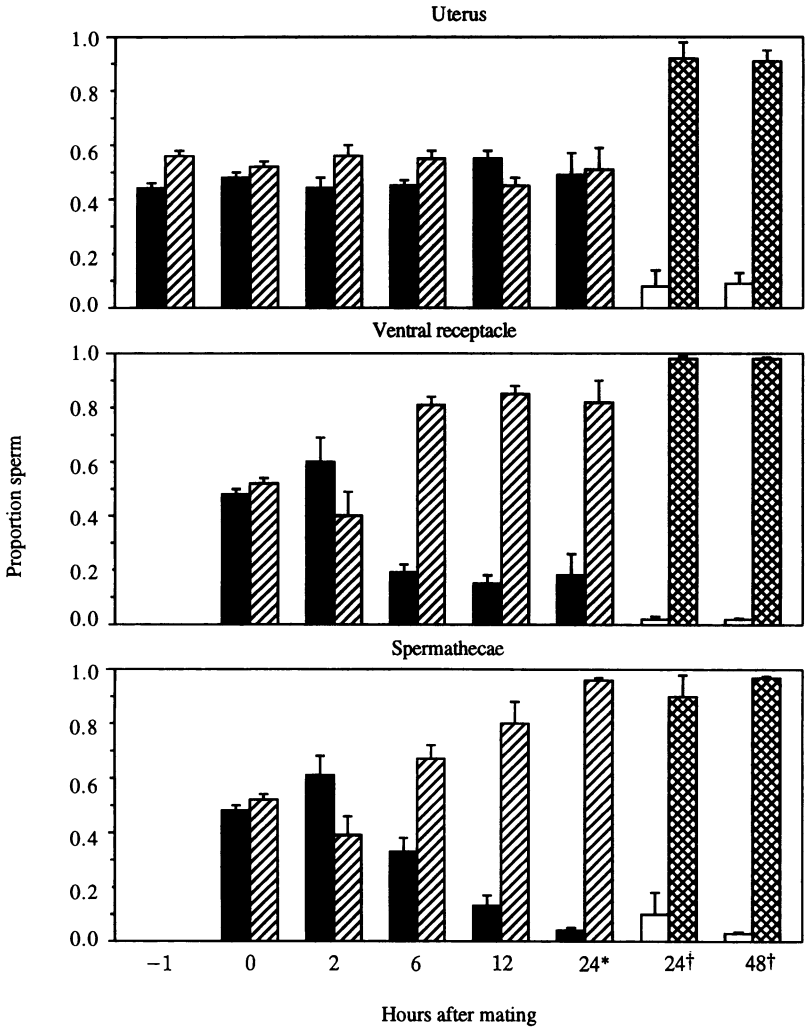

FIG. 2. Proportion (+SE) of sperm present in the uterus, ventral receptacle, and spermathecae at various times after mating. Values at −1 hr represent sperm in male seminal vesicles; values at 0 hr represent sperm in uterus immediately after copulation. Females do not have sperm present in sperm storage organs at 0 hr after mating. This is presented in the figure to indicate what was originally transferred to females and, thus, is available for movement into storage. Sample sizes are 12 for −1 hr, 11 for 0 hr, 15 for 2-hr uterus, 13 for 2-hr ventral receptacle, 11 for 2-hr spermathecae, 15 for 6 hr, 13 for 12 hr, 8 for 24* hr, 4 for 24† hr, 11 for 48† hr uterus, and 15 for 48† hr both sperm storage organs. The unequal sample sizes associated with 2 hr postcopulation are due to some females not yet having sperm in storage. The unequal sample sizes associated with 48 hr postcopulation are due to some females not having sperm in the uterus. 24*, females sampled at 24 hr after mating that had not oviposited; 24† and 48†, females sampled at 24 and 48 hr, respectively, after mating that had oviposited. Filled bars, shorter morphs in nonovipositing females; hatched bars, long morphs in nonovipositing females; open bars, shorter morphs in ovipositing females; cross-hatched bars, long morphs in ovipositing females.

identical proportions of each morph argues against developmental accidents for the origin of shorter sperm.

The sperm competition hypothesis suggested that shorter and long sperm morphs are used to fertilize eggs under alternative situations (4, 7, 8) can also be rejected. Additionally, in a study looking at motility differences between sperm types in several members of the *D. obscura* group, it was suggested that shorter sperm morphs must be utilized first in fertilization because their motility does not change in storage (10). However, the critical prediction of this hypothesis was not met in the present study as eggs oviposited first had only long sperm present.

The remaining sperm competition hypotheses, such as those involving kamikaze sperm (2, 12) or increased remating interval (2), and provisioning hypotheses involving incorporation of sperm-derived materials in females/offspring (2) are not falsified by these data.

Why might only long sperm fertilize eggs? Perhaps only long sperm contain surface receptors necessary for sperm entry into the micropyle. While no one has found evidence for sperm receptors in insects, this may be a system where differences in functionality of the sperm types could prove a useful research tool in testing for insect sperm receptors. Physiologically, only the long morph could conceivably form the species-specific stereotypical three-dimensional sperm structure, such as seen in *D. melanogaster*, which may serve a role in the positioning of the male pronucleus prior to karyogamy (28). Evolutionarily, the persistence of the sperm

Table 2. Number of sperm morphs present in male or female reproductive tracts at various times postcopulation

Time, hr	Seminal vesicle		Uterus		Ventral receptacle		Spermathecae	
	Shorter	Long	Shorter	Long	Shorter	Long	Shorter	Long
−1	232.6 ± 5.6	293.7 ± 6.0						
0			261.4 ± 5.6	285.6 ± 6.5				
2			243.4 ± 17.1	308.0 ± 16.3	44.5 ± 22.8	32.7 ± 12.6	19.1 ± 6.3	10.5 ± 5.2
6			238.3 ± 10.5	290.5 ± 9.4	91.4 ± 21.8	361.7 ± 50.3	211.4 ± 55.1	492.7 ± 154.9
12			279.2 ± 16.5	241.2 ± 16.4	53.8 ± 13.9	348.0 ± 62.5	69.4 ± 16.3	726.8 ± 191.6
24*			531.4 ± 99.1	380.0 ± 69.6	154.5 ± 118.2	328.0 ± 79.9	60.0 ± 16.8	1371.9 ± 190.1
24†			9.3 ± 8.9	18.5 ± 8.8	6.8 ± 3.5	177.7 ± 29.9	42.3 ± 27.1	1155.3 ± 320.7
48†			0.8 ± 0.3	19.1 ± 7.4	1.9 ± 0.4	107.1 ± 20.0	47.9 ± 8.0	1618.3 ± 119.5

The values given are the mean (±SE) number of sperm present in tissues at various times postcopulation. Mean (±SE) number of eggs oviposited at 24 and 48 hr, respectively, = 17.3 ± 7.8 and 58.0 ± 5.0.
*Females sampled at 24 hr postcopulation that had not oviposited.
†Females sampled at 24 and 48 hr postcopulation that had oviposited.

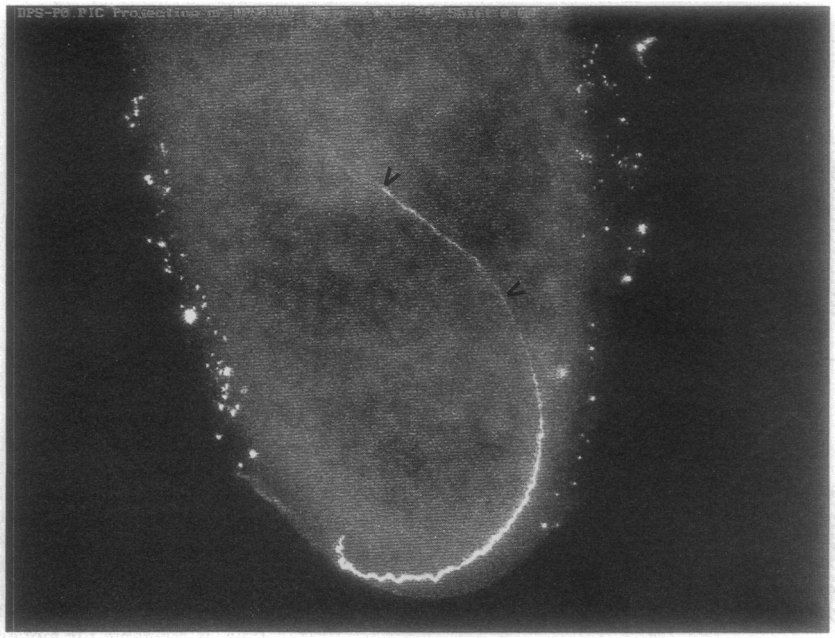

FIG. 3. Three-dimensional reconstruction of sperm in a fertilized *D. pseudoobscura* egg. The sperm is seen as the thin bright line extending into the egg from the anterior end (anterior is down). Carets mark 95 μm along the sperm, the length expected for the shorter sperm morph.

tail after fertilization, as shown in *D. melanogaster*, suggests that material delivered by the sperm may represent essential components for successful embryogenesis (28). The benefit of having long sperm fertilize eggs may be that long sperm could contain greater amounts of these essential components. None of these hypotheses, however, explain the evolutionary persistence of shorter types.

Molecular genetic studies, similar to those performed in *D. melanogaster* using enhancer trap methodologies (31), may prove useful in determining the developmental basis of polymegaly. Because sperm of different sizes and functional capacities are produced in a common testis and presumably arise from a single spermatogonial stem cell division that produces a bundle of 128 spermatids of a single sperm type (16, 17), polymegaly may result from molecular differences arising during stem cell divisions. If so, a molecular genetic study of *D. pseudoobscura* and other members of the *obscura* group may provide valuable information on the genes controlling sperm function or size. Of particular interest would be the potential for identifying genes controlling morphological parameters such as sperm length and nuclear shape.

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