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FURTHER OBSERVATIONS ON POLYMEGALY IN SPECIES OF THE *DROSOPHILA AFFINIS* SUBGROUP

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Polymegaly, or the production of two or more size classes of sperms by the same male, has been known in *Drosophila* for more than a decade. More recently polymegaly (dimegaly, or two size classes) was found in the *Drosophila affinis* subgroup. Additional observations on polymegaly in six *D. affinis* subgroup species: *D. affinis, D. algonquin, D. athabasca, D. azteca, D. narragansett,* and *D. tolteca* are reported here. The two kinds of sperms differ strikingly in the nature of the head, which is elongate (threadlike) in "long" sperms, short and dense in "short" sperms. Ultraviolet fluorescence following treatment with 4′, 6-diamidino-2-phenylindole (DAPI) made it possible to recognize these distinctive heads in developing spermatids and in mature sperms. Investigations of spermatids were supplemented with observations of Feulgen-stained preparations and by electron microscopy. "Short" sperms were found to be much more numerous than "long" ones in the seminal vesicles of males and in the uteri of recently inseminated females. However, the proportions of "short" sperms were greatly reduced in the sperm storage organs of mated females. Inspection of squash preparations of newly laid eggs revealed flagella of "long" sperms but no unequivocal evidence of "short" sperms.

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INTRODUCTION

A multiplicity of sperm types among sperms of the same male has long been known in various invertebrates [*see* review of Roosen-Runge (1977, Ch. 10)]. Polymegaly, or the existence of two or more size classes of sperms (formerly called "polymorphism"), was reported in several *Drosophila* species of the *D. obscura* group by Beatty and Sidhu (1969): *D. ambigua, D. obscura, D. persimilis, D. pseudoobscura,* and *D. subobscura* [independently confirmed for *D. persimilis* and *D. pseudoobscura* by Policansky (1970)]. In all these species except *D. subobscura* there were three size classes of sperms, but only two in *D. subobscura.* Sanger and Miller (1973) demonstrated sperm dimegaly (two size classes) in six closely related species of the *D. affinis* subgroup (*D. obscura* group): *D. affinis, D. algonquin, D. athabasca* (three partially isolated semispecies), *D. azteca, D. narragansett,* and *D. tolteca.*

It has also been recognized that the different sperm size classes of polymegalous species may differ as to the nature of the head. In certain invertebrates (*e.g.,* prosobranch snails) sperms of different classes have been reported to vary as to presence and quantity of chromatin, as recognized in the terms "eupyrene," "oligopyrene," and "apyrene" of Meves (1903). Even where there was no demonstrated difference in quantity of chromatin (or DNA) it could sometimes be seen that the appearance of the sperm head differed radically from one class to another. For example, Bowen (1922) found that "large" sperm heads in pentatomid bugs, of the genus *Euschistus* are "exceedingly delicate and threadlike" though not differing from those of "smaller" sperms (two classes) in amount of chromatin. Likewise, the longest sperms of several polymegalous *Drosophila* species have been found to have a concentration of DNA well removed from the sperm tip (Policansky, 1970; Beatty and Burgoyne, 1971; Hauschteck-Jungen and Maurer, 1976). As pointed out by Policansky (1970), the fact that the "long" sperms of *D. pseudoobscura* stain differently from the "shorter" ones is evidence against the interpretation that the "shorter" sperms are fragments of the "long" ones.

Numerical relations between sperms of different classes vary. For example, in the *D. obscura* group species studied by Beatty and Sidhu (1969) sperms from the seminal vesicles of males sometimes had the "longest" class more frequent than the others (*e.g., D. pseudoobscura*), sometimes one of the shorter classes (*e.g., D. persimilis*). In *D. pseudoobscura* these authors found that recently inseminated females tended

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to preserve the proportions of the different sperm classes in the uterus (though individual females varied widely). On the other hand, incorporation of sperms into the storage organs of females was found to result in striking changes in the proportions of the size classes—*e.g.*, Beatty and Sidhu (1969) reported progressive decrease in the frequency of "short" sperms in *D. obscura* and Beatty (1972) found that only the "longest" sperms reached storage organs in *D. pseudoobscura, D. persimilis,* and *D. subobscura,* although Policansky (1970) reported sperms of all sizes reached the female's storage organs in *D. pseudoobscura.*

The origin and function of the different sperm classes in polymegalous *Drosophila* species remain a mystery. The different classes are clearly not the result of segregation at meiosis, since bundles of spermatids (each contained within a cyst) have *not* been found to contain a mixture of sperms of different sizes (Beatty and Sidhu, 1969; Policansky, 1970). Neither is polymegaly attributable to differences in number of mitotic (or meiotic) divisions in the development of a sperm cyst (*e.g.*, fewer divisions in a "long" one), since the number of spermatids per bundle appears to be the same (the characteristic species number) in cysts of the different kinds (Beatty and Burgoyne, 1971). It seems probable that sperms of different size classes differ in their ability to accomplish fertilization of eggs. Beatty (1972) expressed doubt that any sperms other than the "long" ones of *D. pseudoobscura* can participate in fertilization, since, in that species, only "long" sperms were found in the female's ventral receptacle. The possible function of non-fertilizing sperms in polymegalous species has been the subject of speculation—*e.g.*, the non-eupyrene sperms of prosobranch gastropods, *etc.* may perform a mechanical function, or may provide nutrition for the eupyrene sperms [*see* review by Baccetti and Afzelius (1976, Ch. 14)]. However, in no polymegalous *Drosophila* species has it yet been demonstrated that one or more of the size classes is actually incapable of fertilizing eggs and/or performs some function other than fertilization.

Our observations on developing spermatids and mature sperms of the polymegalous *D. affinis* subgroup species provide additional evidence on the nature of polymegaly in *Drosophila.* In general, our results are consistent with those reported for other *Drosophila* species—though major questions (*i.e.*, the origin of polymegaly and the functions of the different kinds) remain unanswered.

MATERIALS AND METHODS

The strains used in this study were: *D. affinis* Lincoln, Nebraska, and an XO strain; *D. algonquin* Honeyoye Falls, New York; *D. athabasca* Netcong, New Jersey (semispecies "eastern A"), Lincoln, Nebraska (semi-species "eastern B"), and Deer Isle, Maine (semispecies "western-northern"); *D. azteca* Chilpancingo, Mexico; *D. narragansett* Bloomington, Indiana, and an XO strain; and *D. tolteca* Coroico, Bolivia. Of these ten strains, six were the same as ones used by Sanger and Miller (1973), the exceptions being the strain of *D. algonquin* and those of all three *D. athabasca* semi-species. Stocks were maintained on standard *Drosophila* medium (agar–molasses–cornmeal–Tegosept) in a room kept at 18.5 ± 2.5 C. Except where stated otherwise, all species were investigated according to a given procedure. However, due to occasional difficulties of culture and to differences between species as to favorableness or appropriateness for certain observations, parts of this investigation were restricted to only one or several of the species.

To observe stages of spermiogenesis within testes, our procedure was patterned after that of Peacock and Miklos (1973). The testes of 7-day-old adult males were dissected out in 0.7% NaCl and fixed in a fresh mixture of one part glacial acetic acid and three parts absolute alcohol. The testes were stored in a freezer at about -7 C from 3 to 10 hr. They were then placed in 5N HCl at room temperature for 5 min, stained for 30 min in the Feulgen reagent, and each mounted in 45% acetic acid on a slide under a coverslip. The weight of the coverslip usually ruptured the testicular envelope causing spermatid cysts and other contents to spill out into the surrounding liquid; if this did not happen, a little pressure was applied to cause breakage and dispersal. Observations of these non-permanent preparations were made with phase contrast illumination using a Zeiss photomicroscope. Although some photographs were taken, the outlines of spermatid bundles and locations of Feulgen-positive material in the "long" ones were recorded by tracing on paper using a Zeiss drawing attachment on the microscope. The lengths of bundles, whether "long" or "short," and the lengths of "long" spermatid heads were then determined with a Tacro No. 4720 rotameter applied to the drawings.

Mature sperms from seminal vesicles were prepared for observation in the way described by Sanger and Miller (1973) —*i.e.*, by dispersal of sperms in Bodenstein's solution, air-drying, washing to remove salt, and final air-drying. In addition, efforts were made to stain the sperm heads. Although it was found that the "short" sperms of these species had heads that stained well by the Feulgen technique (or by Giemsa stain or acetic orcein), the "long" sperm heads remained generally invisible after the staining procedure. On the other hand, it was found possible to observe the heads of both "short" and "long" sperms by ultraviolet fluorescence after treatment with 4′, 6-diamidino-2-phenylindole (DAPI). DAPI was applied in a concentration of 2 μg/ml of distilled water, the preparations sealed by nail polish, and observation done with a Zeiss reflected light, fluorescence microscope with transmitted phase. As described by Williamson and Fennell (1975), the

complex of DNA and DAPI fluoresced in ultraviolet with a brilliant light-blue coloration. We found that not only the DNA of the sperm heads glowed brightly but sperm flagella were revealed throughout most of their length by the dim fluorescence of mitochondrial DNA. In *D. athabasca,* DAPI fluorescence was also observed in bundles of spermatids from the testes, revealing essentially the same nuclear structure visible after Feulgen treatment.

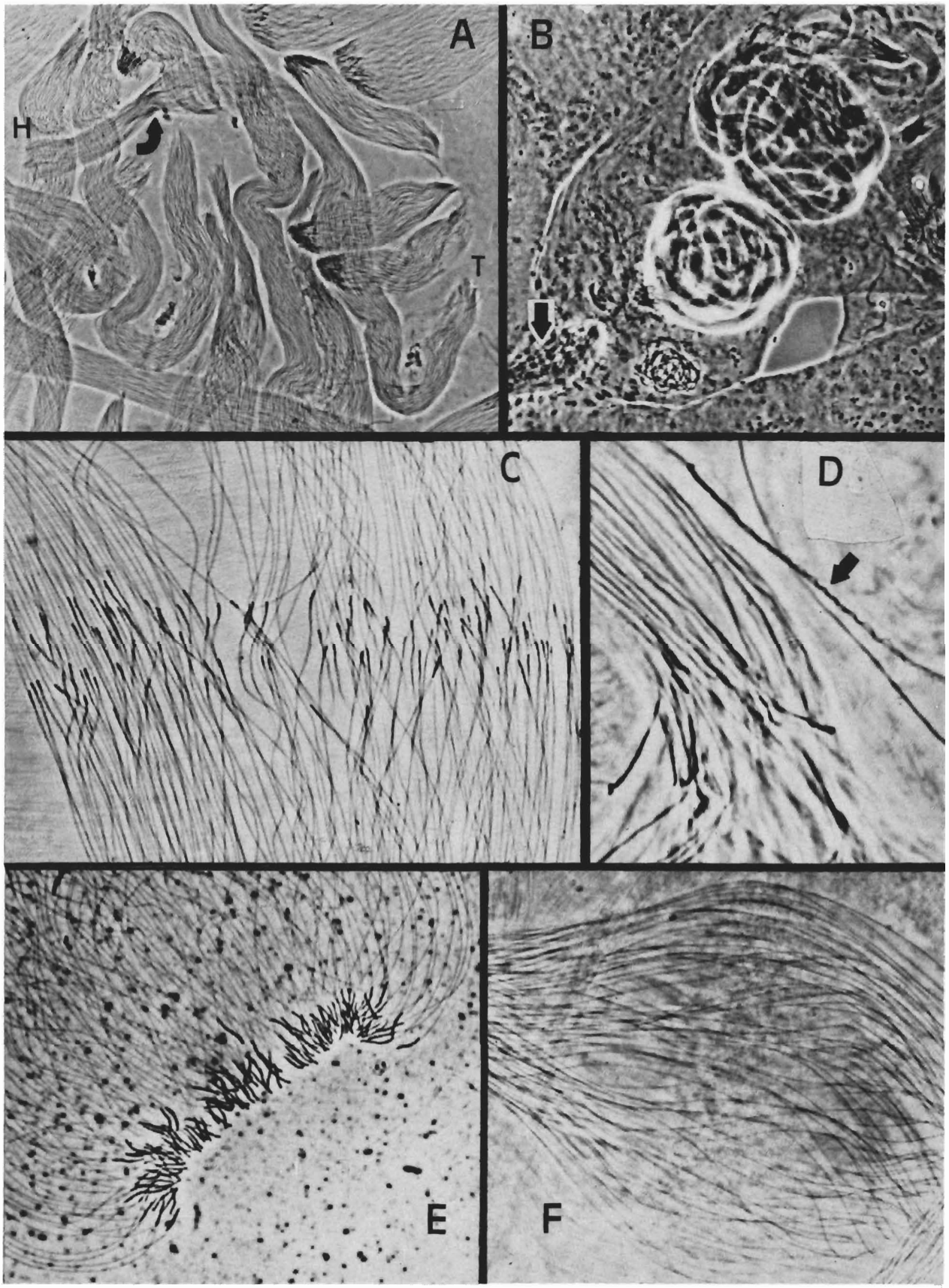
Sections of adult male testes of the *D. athabasca* semispecies "eastern B" and "western-northern" were examined, thick sections by light microscopy and thin ones by transmission electron microscopy. These observations were part of another project (to be reported later) to look for signs of impaired fertility in hybrid males between these semispecies. Testes were dissected in glutaraldehyde (2% in 0.1M phosphate buffer) and allowed to fix for 2 hr. After fixation the testes were rinsed with phosphate buffer five times at 1-hr intervals and then post-fixed for 1 hr in 1% osmium tetroxide in 0.1M phosphate buffer. The testes were next dehydrated in an alcohol series. After two 5-min periods in propylene oxide they were left overnight in a mixture of propylene oxide and Epon. The testes were then cut into four or five segments of approximately equal length, embedded in Epon, and subjected to polymerization at 60 C. Both thick and thin sections were made with a Porter-Blum ultra-microtome MT-1. Thick sections were stained in 1% methylene blue and examined at about 1000X with a Zeiss photomicroscope. Thin sections were double stained with 2% uranyl acetate and lead citrate and examined by means of a Philips 201 transmission electron microscope.

Mated females of the *D. affinis* subgroup receive sperms into a relatively wide uterus, from which they are subsequently incorporated into the sperm storage organs: a slender, blind, transparent ventral receptacle, and a pair of dorsal mushroom-shaped, dark-brown spermathecae. Although sperms may readily be seen in these organs in fresh preparations of mated female reproductive tracts, it is difficult to distinguish "long" and "short" sperms and determine their numbers by flagellum lengths alone–especially in the storage organs. However, DAPI fluorescence made distinguishing the two kinds of sperms by their heads possible, facilitating recognizing and counting "long" and "short" sperms in these locations–although adequate extraction and dispersal were still important. Observations of the two kinds of sperms in the uterus were done for *D. affinis, D. algonquin, D. athabasca,* and *D. narragansett.* Inspection of sperm storage organs for these sperms was restricted to *D. affinis, D. athabasca,* and *D. narragansett,* with actual counts being made only in *D. affinis,* in which the relative shortness of "long" sperms appeared to aid their dispersal. Females to be dissected were aged 7 days and then paired with males for 30 hr, after which each female's reproductive tract was dissected in Bodenstein's solution. In most cases, uterus, seminal receptacle, and spermathecae were put on separate slides in DAPI solution. Air was bubbled from a clean pipette in the uterus preparations to help spread the sperms, after which a coverslip was added, the slide sealed with nail polish, and then observed with a Zeiss fluorescence microscope. Pressure was applied on the coverslip in an effort to break the sperm storage organs and release the sperms, after which these slides were also sealed and examined. To promote counting the two kinds of sperms in *D. affinis,* uterus and sperm storage organ slides were set up in Bodenstein's solution, an effort was made to rupture the organs with dissecting needles and disperse the sperms, and the slide was then air-dried. DAPI was then applied to each air-dried slide, a coverslip applied and sealed, and the slide examined by fluorescence microscopy. This procedure was found better than putting the female's organs directly in DAPI, since the sperms dispersed themselves less readily in DAPI than in Bodenstein's solution.

Recently laid eggs of mated females were inspected for sperms using the squash technique of Patterson (1945). Eggs were collected from *Drosophila* medium where mated females had deposited them. Such eggs were put on a glass slide, to which they soon stuck as the adhering medium dried. Each egg was then gently stroked with a needle, causing the chorion to break and the egg (enclosed by its vitelline membrane) to emerge from the chorion. Four or five dechorionated eggs were arranged in a row on a slide, a small drop of Bodenstein's solution or DAPI added, a coverslip carefully put on the preparation and pressure applied to crush each egg to a thin film, and the slide sealed with nail polish. Each squash was examined microscopically for sperms using phase contrast illumination (supplemented with ultraviolet where DAPI had been applied).

RESULTS AND DISCUSSION

Figure 1A shows several Feulgen-stained spermatid bundles from an adult testis of *D. affinis.* A single "long" bundle appears in its entirety along with several "short" ones. Although the strands in a "short" bundle have a distinctly terminal Feulgen-positive head, the Feulgen-positive material of a "long" bundle is concentrated in a clearly subterminal zone, though much closer to one end than to the other. A similar difference was seen in all our species–in agreement with that already reported for *D. pseudoobscura* by Policansky (1970). Close examination of the Feulgen-positive zone of a "long" bundle showed that the stained material of each strand actually tapers off to a narrow thread towards the near end of the bundle, and, in favorable cases, could be seen attached laterally as an undulating ribbon (Fig. 1C, D). Although the difference of appearance between this stained structure and the head of a "short" spermatid (Fig. 1E) was striking, it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seemed appropriate, following the examples of others (Beatty and Burgoyne, 1971), to regard the region from juncture of Feulgen-positive material and flagellum to the near end of each "long" strand as the head of the strand, though it was never possible to trace Feulgen staining all the way to the end—partly due to the presence of a non-staining acrosome at the tip of the strand (demonstrable by using both phase contrast and UV fluorescence with DAPI, as described below). The fact that Feulgen-positive material could occasionally be seen as if laterally attached by an undulating membrane (Fig. 1D) made these heads sometimes seem "crenellated"—in contrast to the observation of Beatty and Burgoyne (1971) that, in *D. pseudoobscura,* "the flagellum but not the head is minutely crenellated."

Table I presents the results of measurements of "long" and "short" spermatid bundles [each interpretable as a cyst, after Bairati (1967)] and of the "long" head regions. Lengths determined for the "long" and "short" bundles agree roughly with those reported for mature sperms by Sanger and Miller (1973), the ranges of measurements overlapping in all cases. Lengths of "long" spermatid bundles are more or less related to size of testis, which varies in these species in the order (shortest to longest): *D. affinis, D. algonquin* and *D. narragansett* (about the same), *D. athabasca, D. tolteca,* and *D. azteca.* Although an early impression was that "long" heads were similar in length to "short" strands and that "short" bundles might possibly arise from the breakage of "long" ones and the metamorphosis of their head regions into "short" sperms, no evidence was found to support this idea (Chang and Miller, 1977). It may be seen that the lengths of "long" heads were, in most cases, much greater than those of "short" bundles, usually more than twice as great—but with two exceptions: *D. affinis* and *D. narragansett,* in each of which both XY and XO males had "long" head regions shorter than "short" bundles. It is an interesting and possibly significant relationship (though without explanation) that the two exceptional species have fertile XO males while most of the others are known to require the Y chromosome for male fertility. [Voelker and Kojima (1971) investigated all these *D. affinis* subgroup species for XO male fertility except "western-northern" *D. athabasca* and *D. azteca*).] On the other hand, although Hess and Meyer (1968) reported that XO *D. melanogaster* males have significantly shorter sperms than XY males of that species, our measurements of "long" and "short" spermatid bundles of XY and XO *D. affinis* and *D. narragansett* failed to show such a consistent relationship—as had already been reported for mature sperms of XY and XO males of these species (Sanger and Miller, 1973).

An effort was made to recognize developmental stages in "long" and "short" spermatid bundles of the *D. affinis* subgroup species. After Feulgen staining of whole testes it was possible to observe, even with a stereoscopic microscope (no more than 100X magnification), that a testis has two regions differing in staining response. These are a small, apical (anterior) region, where spermatogonial cells are abundant and primary spermatocytes may be found, and a large, basal (posterior) region filled with sperm cysts. The majority of "long" bundles were found to have their heads oriented towards the basal end of the testis and their tails extending towards the apical end, occupying altogether about 90% of the testis length. Large numbers of "short" bundles were found near the basal end of the testis. The "short" spermatids varied as to their nuclei, some having a more or less slender nucleus interpreted as less mature, others having a condensed, rodlike nucleus regarded as more mature and probably characteristic of the individualized spermatids. The heads of "long" bundles were difficult to judge and, moreover, the "long" bundles were very hard to observe throughout their length due to their close association with each other (coiling and looping back and forth) and with the "short" cysts. Because of the fragility of the testis preparations, it was necessary to proceed with caution in applying pressure to make the bundles separate from each other, and ordinarily only one or a few of the "long" ones could be viewed in their entirety. It

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FIGURE 1. A–F. Feulgen-stained preparations of adult testes of *D. affinis* subgroup species males, observed by phase contrast illumination. **A.** "Long" and "short" spermatid bundles (cysts) of an adult *D. affinis* testis. One "long" bundle appears in its entirety, its head end labeled "H," its tail (flagellar) end labeled "T," and its Feulgen-positive zone indicated by an arrow. Several "short" bundles are included, their strongly Feulgen-positive heads oriented to the left. *Ca.* X286. **B.** Terminal testicular region of adult testis of "eastern B" *D. athabasca.* The arrow (lower left) points to the duct to the seminal vesicle. The two large masses near the center are coiled "long" bundles. Disintegrating spermatids can be seen below, and several "short" bundles are barely visible at the upper right. *Ca.* X286. **C.** Feulgen-positive zone of a "long" bundle of "eastern B" *D. athabasca* at increased magnification (*Ca.* X630). The heads are oriented downward out of the picture. **D.** Still greater enlargement of "long" bundle strands of "eastern B" *D. athabasca.* The strand indicated by an arrow illustrates its laterally situated Feulgen-positive thread tapering off to the right. *Ca.* X148. **E.** Feulgen-positive heads of "short" bundle of *D. athabasca* "eastern B." *Ca.* X630. **F.** Cystic bulge found in a "long" bundle of *D. athabasca* "eastern B." *Ca.* X594.

TABLE I. Lengths (mm) of "long" and "short" spermatid bundles (cysts) from testes of young adult *D. affinis* subgroup species males. Each mean is given with its standard error and the observed range of measurements (extremes).

	"Long" Bundles			"Short" Bundles	
	Entire bundle $\bar{x}$	Head region $\bar{x}$	n	Entire bundle $\bar{x}$	n
D. affinis					
XY strain	0.47 ± 0.005 (0.39–0.54)	0.07 ± 0.002 (0.05–0.10)	45	0.11 ± 0.002 (0.06–0.19)	91
XO strain	0.46 ± 0.005 (0.39–0.64)	0.08 ± 0.002 (0.06–0.14)	58	0.11 ± 0.001 (0.06–0.15)	122
D. algonquin	0.96 ± 0.010 (0.82–1.10)	0.26 ± 0.005 (0.18–0.33)	37	0.12 ±0.10 (0.09–0.17)	7
D. athabasca					
"eastern A"	1.69 ± 0.068 (1.40–1.94)	0.48 ± 0.010 (0.45–0.51)	6	0.13 ± 0.003 (0.09–0.19)	71
"eastern B"	1.48 ± 0.030 (1.18–1.73)	0.34 ± 0.007 (0.24–0.39)	25	0.13 ± 0.001 (0.10–0.17)	72
"western-northern"	1.48 ± 0.015 (1.42–1.56)	0.42 ± 0.004 (0.41–0.45)	8	0.15 ± 0.008 (0.10–0.17)	18
D. azteca	2.09 ± 0.021 (1.78–2.31)	0.45 ± 0.014 (0.33–0.54)	21	0.20 ± 0.004 (0.13–0.24)	35
D. narragansett					
XY strain	0.87 ± 0.009 (0.87–0.97)	0.06 ± 0.004 (0.04–0.09)	14	0.21 ± 0.019 (0.16–0.23)	3
XO strain	0.97 ± 0.010 (0.90–1.05)	0.09 ± 0.003 (0.07–0.12)	22	0.17 ± 0.003 (0.14–0.19)	13
D. tolteca	1.94 ± 0.058 (1.72–2.33)	0.37 ± 0.022 (0.33–0.53)	9	0.12 ± 0.005 (0.09–0.16)	16

was sometimes possible to recognize, in a "long" cyst, a cystic bulge (Fig. 1F) like those reported in sperm cysts of *D. melanogaster* by Tokuyasu, Peacock, and Hardy (1972a). According to these authors, passage of the cystic bulge from head to flagellar end of a cyst signals progress of individualization, *i.e.*, termination of the syncytial state of the spermatid bundle. Coiling and discarded wastes associated with "long" cysts in *D. affinis* subgroup species could also be seen, as reported in *D. melanogaster* by Tokuyasu, Peacock, and Hardy (1972b). In "short" cysts, although no clear cystic bulge or coiling could be observed, occasionally a waste bag could be seen at the tip of the cyst. It was ordinarily very difficult to count the two kinds of cysts in the testes of our species, but the impression was general that the "short" cysts were much more numerous than the "long" ones. In one especially clear preparation from a *D. athabasca* "eastern A" male it was possible to count 64 "short" cysts and 23 "long" ones. Thus, the *D. affinis* subgroup species appear to resemble certain other *D. obscura* group species in having more "short" sperms than "long" ones—as, for example, *D. persimilis* in the report by Beatty and Sidhu (1969).

Sections of testes of *D. athabasca* "eastern B" and "western-northern" revealed cross-sections of sperm cysts at varying stages of development, though it was not possible to distinguish "long" and "short" bundles. As in other *Drosophila* species [*e.g.*, *D. melanogaster* (*see* Tokuyasu, Peacock, and Hardy (1972a)], preindividualized cysts show a syncytial structure while individualized cysts have well-separated strands. Spermatids per cyst were counted in thick sections with the Zeiss photomicroscope at 1000X. Numbers per cyst averaged about 124; specifically, 109 cysts of *D. athabasca* "eastern B" averaged 123.6 ± 0.7 (range: 106–128), while the mean for *D. athabasca* "western-northern" was 124.8 ± 0.5 (range: 114–128). There was no suggestion of a bimodal distribution that might mean a difference in number between "long" and

"short" cysts. Moreover, a few counts of spermatid nuclei in favorably spread Feulgen-stained spermatid bundles showed no striking difference between the two kinds: in particular, the bundle of "long" spermatids of Figure 1C had 117 nuclei, and that of "short" spermatids of Figure 1E had 107. One may conclude from these observations that, in the *D. affinis* subgroup species, from an initial spermatogonium there are derived additional cells by five mitotic and two meiotic divisions, resulting in $2^7 = 128$ spermatids—the observed deviations being attributable to occasional loss of spermatids and to errors in counting. Kurokawa and Hihara (1976) pointed out that the number of first spermatocytes per cyst is "generally consistent within taxa" in *Drosophila* and that the number previously reported for the *D. obscura* group [*e.g., D. pseudoobscura* by Dobzhansky (1934)] is 32; hence, it is not surprising that the number of spermatids per cyst in these *D. affinis* subgroup species tends toward 128 (*i.e.,* the number expected from 32 primary spermatocytes after two meiotic divisions).

Observations of thin sections of testes by electron microscopy revealed some sections through cysts at the level of the nucleus and some at the level of the flagellum. Two kinds of nuclei were found, condensed and uncondensed, but it is not known whether these represent "long" and "short" cyst nuclei or nuclei of one or both classes at early and late stages. Each section at the nuclear level showed the nucleus accompanied by an axonema and one mitochondrial derivative. Thus, as Hauschteck-Jungen and Maurer (1976) reported in *D. subobscura*, the nucleus extends alongside axonema and mitochondrial derivative. Sections through the flagellum showed, on the other hand, an axonema accompanied by two mitochondrial derivatives (Fig. 2B). The axonema was found to have a typical "9 + 2" organization of flagellar microtubules, as was reported by Kiefer (1973) for *D. melanogaster.* In *D. melanogaster* the minor mitochondrial derivative reduces to an extremely small size after individualization, while the major one contains a paracrystalline body that is not much different in size from that of the preindividualization state. In *D. athabasca,* on the other hand, two mitochondrial derivatives were seen after individualization, each containing a paracrystalline body. Moreover, these were of about the same size. As reported for several other *Drosophila* species by Sidhu (1974), evidence was also derived that mature sperm flagella, of both the "long" and "short" classes, consist of three fiberlike elements capable of dissociating (Fig. 2C).

Observation of DAPI-stained mature sperms by ultraviolet fluorescence revealed the distinctive heads of "long" and "short" sperms very clearly (Fig. 2A)—and of spermatids in cysts of *D. athabasca.* Sperm heads of the two kinds were visible in all species in the reproductive tracts of mated females without any difference of appearance with respect to that seen in the seminal vesicles of males. DAPI-treated *D. affinis* subgroup species sperm heads observed by ultraviolet fluorescence look rather like those of *D. subobscura* illustrated by Hauschteck-Jungen and Maurer (1976), who used a different fluorescing substance, BAO. To look for the two kinds of sperms in female reproductive tracts and determine their relative numbers, mated *D. affinis* females were dissected. In one female that had cohabited with a male for 17 hr but had not begun to lay eggs, the uterus was found to contain 3,051 "short" sperms and 1,305 "long" sperms, while the ventral receptacle had two "short" sperms and 69 "long" sperms, and the spermathecae had 55 "short" sperms and 176 "long" sperms. In another one that had been kept with a male the same length of time, although no count was made of sperms in the uterus, it was found that the ventral receptacle had no "short" sperm but 117 "long" sperms, and the spermathecae had six "short" sperms and 200 "long" ones. Nine females were dissected after egg laying had started. As expected, the uterus contained no sperm. In the ventral receptacles of these females were a total of eight "short" sperms (all but three having no "short" sperm in this organ) and 767 "long" sperms. Counts of sperms in the spermathecae were made on just two egg-laying females; one had no "short" sperm but 45 "long" ones, while the other had one "short" sperm and 36 "long" ones in the spermathecae. These data surely indicate a decrease in proportions of "short" sperms in the sperm storage organs with respect to those in the uterus—even taking into account that it was difficult to extract all sperms from these organs (especially the spermathecae), since it would be expected that the relatively small "short" sperms would disperse more readily than the "long" ones and, hence, be more likely to be available for counting. Our observations agree with those of Beatty and Sidhu (1969), who reported a progressive decrease in proportions of "short" sperms in the female's storage organs in *D. obscura.* Nevertheless, *some* "short" sperms were detected in both ventral receptacle and spermathecae.

Freshly laid egg squash preparations from mated females revealed sperm flagella (Fig. 2D) but not sperm heads. Hence, it was not possible to identify "short" and "long" sperms by their heads using ultraviolet fluorescence after DAPI treatment. However, some of the observed flagella were clearly within the range of lengths of "long" sperms (and outside that of "short" sperms), though others were intermediate, as short as, or even shorter than "short" sperms. In cases in which the flagellum in an egg squash was within the length range of "short" sperms, it was not possible to be sure that the flagellum of a "long" sperm had not broken—either as a natural phenomenon related to its disintegration or due to the pressure exerted on the coverslip to make the squash. The *D. algonquin* flagellum of Figure 2D (not shown in its entirety) measured 0.8 mm, which is a little shorter than previously reported for "long" sperms of that species (Sanger and Miller, 1973) but too long to be within the range of "short" sperms. Thus, it appears that in these species "long" sperms *are* capable of penetrating the egg, but we have no

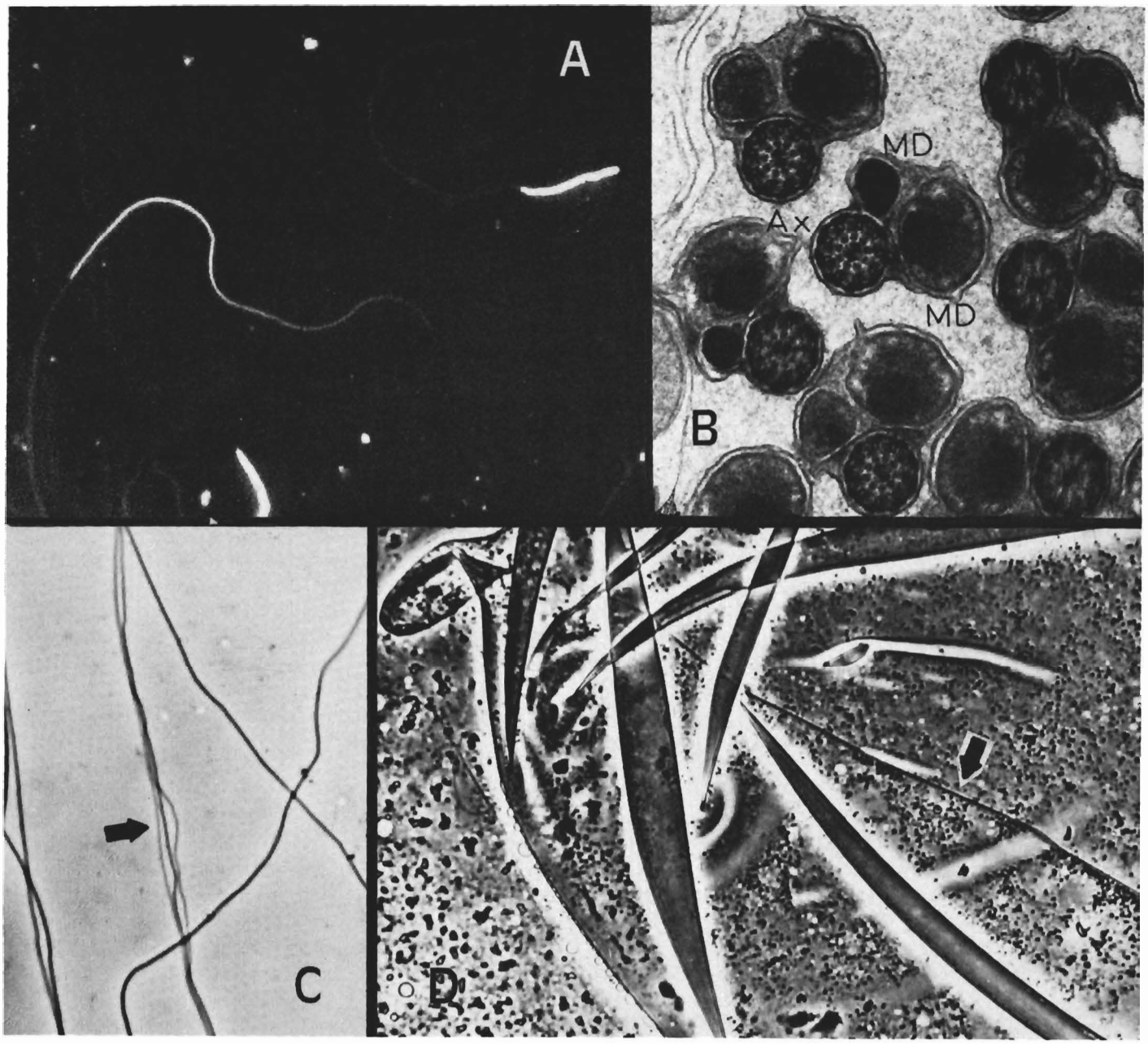

FIGURE 2. **A.** Air-dried mature sperms (seminal vesicle) of *D. affinis* treated with DAPI and observed by ultraviolet light. An entire "short" sperm appears at upper right, including its brilliantly fluorescing head and its faintly fluorescing (hardly visible) flagellum. A "long" sperm head appears on the left, diminishing in fluorescence from its head-flagellum juncture towards its tip (center); a part of the faintly fluorescing flagellum can be seen at lower left. *Ca.* X1,891. **B.** Transmission electron microscope photomicrograph of several spermatid flagella of an individualized cyst in a section of testis of "eastern A" *D. athabasca.* Each flagellum includes an axoneme (Ax) and two unequal mitochondrial derivatives (MD) containing dense paracrystalline material. *Ca.* X44,571. **C.** Air-dried mature "long" sperm flagella (seminal vesicle) of "western-northern" *D. athabasca* observed by phase contrast illumination. The arrow points to a dissociation of a strand into three fibers. *Ca.* X1,411. **D.** A squash preparation of a recently laid egg of *D. algonquin* observed by phase contrast illumination. The micropyle appears at upper left. An arrow points to a "long" sperm flagellum within the egg, extending out of the picture to the lower right. *Ca.* X733.

unequivocal evidence that "short" sperms are capable of doing so.

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