Drosophila subobscura Short Sperm have no Biochemical Incompatibilities with Fertilization

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Abstract: *Drosophila obscura* group species produce two distinct sizes of nucleated sperm that differ only in head and tail lenghts. Between both sperm there is no differences in location of the acrosome and flagellum during spermiogenesis where each sperm type develops in its own bundle. Fertile sperm accumulate in the seminal vesicles. Fertilization is exclusively monospermic and in a previous study we suggested that both types of sperm are fertilization-competent on the basis of similar DNA content and storage in females also if morph variations are consistent with a fertilization-related selection for optimal sperm size. This assumption is in agreement with previous studies that demonstrated that only long sperm fertilize eggs. In this study fertilization of *Drosophila subobscura* is examined using anti-sperm surface β-N-acetylhexosaminidases and α-L-fucosidase antibodies. Beta hexosaminidases are intrinsic proteins of the sperm plasma membrane in spermomomorphic species of the melanogaster group closely related to *Drosophila melanogaster*. These enzymes had been previously identified as putative receptors for glycoconjugates of the egg surface, structurally and functionally conserved. Here their localization has been investigated in *Drosophila subobscura*. Consistent with our previous study, short and long sperm are functionally equivalent. More data are needed to clarify the consequences and adaptative significance of morph variations.

Keywords: *Drosophila*, reproduction, sperm dimorphism, gametes.

Males of all *obscura* group species produce two kinds of sperm, a long and a short morph [1]. Spermatogenesis in *Drosophila obscura* is characteristic of most Diptera, with large numbers of individual spermatids developing within a pair of cyst cells to form a spermatocyst or ``sperm bundle''. Five synchronous mitotic divisions are followed by two meiotic divisions [2, 3] and at the end, each resulting sperm bundle contains 128 spermatids. Sperm length varies between, but not within, sperm bundles [4], so the long and short sperm develop separately and both are nucleated. Across the *obscura* group, short sperm are 2-13 times shorter than long sperm and in particular, in *D. subobscura* dimorphism, long sperm (in different strains ranging from 0.199 mm [5], to 0.448 mm [6], with a nuclear lenght of 0.039 mm [7]) are almost two times as long as the short sperm (in the same strains ranging from 0.085 mm to 0.256 mm, with a nucler lenght of 0.018 mm). At individualization, the proportion of short sperm produced is 66 % [1]. Both size classes of sperm are motile, nucleated and transferred in the same percentage to females by the male. The factors determining the developmental fate of each type are unknown. The two sperm types have similar ultrastructure with only minor differences between the two sperm types in acrosome size, nucleus morphology, and the relationship between the nucleus and minor mitochondrial derivatives, cytochemical characters and similar DNA content [7]. Based on these similarities we previously concluded that both sperm morphs are potentially capable of egg penetration and fertilization. Similarly, *Anopheles gambiae* produced polymorphic nucleated sperm with a wide range of tail lenghts [8] in contrast to the sperm dimorphism found in lepidopterans in which males produce conventional larger sperm and smaller anucleated apyrene type that have non involvment in fertilization, although transferred to the female in large numbers [9].

Kinetic analysis of sperm in species of the *obscura* group at different times after copulation demonstrated that the long but not the short sperm are physiologically affected by storage [10] and that the long sperm are the principle morph for fertilization [11]. Thus, short sperm would be the first to be utilized by singly mated females or would gain immediate fertilization success in females that are storing sperm from a previous mating. Long sperm would be preferentially used when females begin to use sperm in long-term storage. Despite these evidences and a tendency of short sperm to arrive first at the sperm storage organs [12], direct measurements of sperm in eggs in several members of the *D. obscura* group (*D. pseudoobscura*, *affinis*, *athabasca*, *miranda*, *persimilis*, and *subobscura)* (see diagram at page 4 to analyze the relationships between species) suggested that the shorter type is infertile because only long sperm are ever found inside eggs [12-14]. The mechanism for the assumed fertilization incompetence is unclear. Snook and Karr [13] provided two hypotheses (functional evidences): that short sperm may have physical incompatibilies with the egg, e.g. the head of the parasperm appears to be wider than eusperm [3, 7] and that only long sperm might contain surface receptors necessary for fertilizing eggs while parasperm may be unable to enter the micropyle of the egg (biochemical incompatibilities that do not permit proper interaction with the egg). Short,

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nonfertilizing sperm have been suggested to function as cheap filler in the female reproductive tract [15] or to be a male counter adaptation to spermicide, protecting eusperm in the female reproductive tract [16, 17].

It is generally accepted that initial sperm-egg binding is mediated by sperm surface carbohydrate-binding proteins such as lectins, glycosyltransferases and glycosidases, that have a high affinity and specficity for complex glycoconjugates in the extracellular coat of eggs. Glycosidases of the sperm membrane are involved during fertilization in the primary carbohydrate receptor-based gamete recognition mechanism in mollusks [18], in ascidians [19], in amphibians [20] and in mammals [21, 22]. Enzymes specifically responsible for carbohydrate hydrolysis have been classified in families of glycosyl hydrolases (GH) [23]. Beta-*N*-acetylhexosaminidases (EC 3.2.1.52) (hereafter referred to as *N*acetylglucosaminidases (NAGs)), enzymes that hydrolyze non-reducing terminal GlcNAc or GalNAc residues of oligosaccharides and their conjugates, inserted by GlcNAc-transferase I, belong to family 20 (GH20) [23]. In mammals two lysosomal isoforms, HEXA and HEXB, partecipate in the degradation of glycoproteins, glycolipids and glycosaminoglycans. HEXA is a heterodimer of subunits α (encoded by the gene *HEXA*) and β (encoded by the gene *HEXB*),

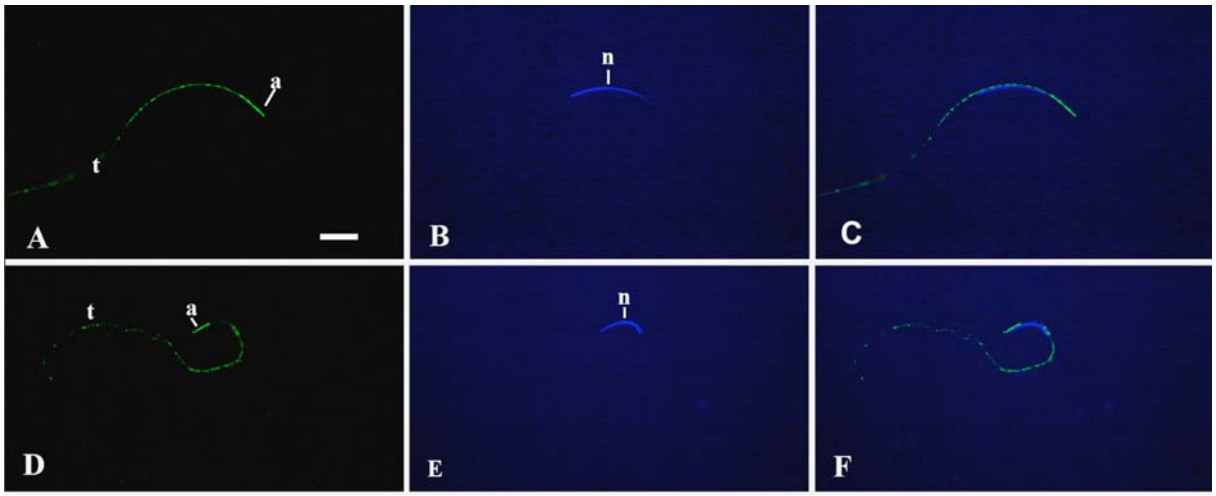


Fig. (1). Immunolocalization of *Hexo1* product on *D. subobscura* spermatozoa. Primary antiserum binding was evidenced with Alexa Fluor 488-conjugated secondary antiserum (green) and nuclei were counterstained with Hoechst 33342 (blue). (**A-C**) Immunolabeling of long sperm. The plasma membrane over the acrosome is fluorescent. Labeling of the nucleus was slightly weaker and absent from the tail. In C, panels **A**, **B** have been merged. (**D-F**) Immunolabeling of short sperm. The labeling pattern is similar to the one observed in the long sperm. a, acrosome; n, nucleus; t, tail. Bar, 5 μm.

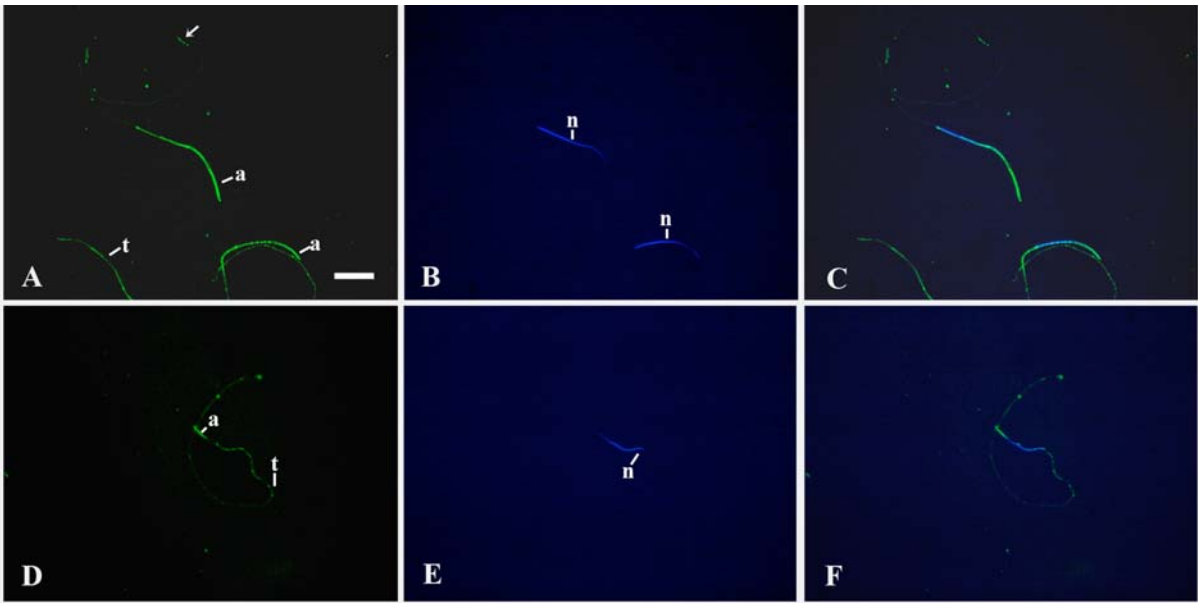


Fig. (2). *D. subobscura* sperm labeled with rabbit anti-*Hexo2* followed by goat antirabbit-Alexa Fluor 488 (green), and Hoechst 33342 for nuclei counterstaining (blue). (**A-C**) Immunolabeling of long sperm. A strong signal over the acrosome is evident. Labeling of the nucleus and of the tail were slightly weaker. In **C**, panels **A**, **B** have been merged. (**D-F**) Immunolabeling of short sperm. The labeling pattern is similar to the one observed in the long sperm. a, acrosome; n, nucleus; t, tail; arrow, tail end piece. Bar, 5 μm.

whereas HEXB is a homodimer of β subunits. Sperm surface glycosidases, an α-L-fucosidase and two β-*N*acetylhexosaminidases isoforms, traditionally referred to as *N*-acetylglucosaminidases (NAGs), named HEXA and HEXB, have been identified in the *melanogaster* group as receptors for glycoconjugates of the egg surface [24, 25].

In *D. melanogaster* spermatozoa HEXA is an heterodimer with an $\alpha\beta_2$ structure and HEXB has a $\beta_1\beta_2$ structure. The α, β_1 and β_2 subunits are encoded by the homologous *NAG* genes *fdl*, *Hexo1* and *Hexo2*, respectively [24].

The presence in *D. subobscura* of the products of the *NAG* genes and of the *Fuca* gene, that codes for an α-L-fucosidase has been demonstrated with polyclonal antibodies. Three peptide segments for each sequence were selected as immunogens for the production of specific antibodies. Similar gene structures were observed between pairs of *Drosophila* species [25, 26]. Immunofluorescence labeling of *D. subobscura* whole spermatozoa showed that α-L-

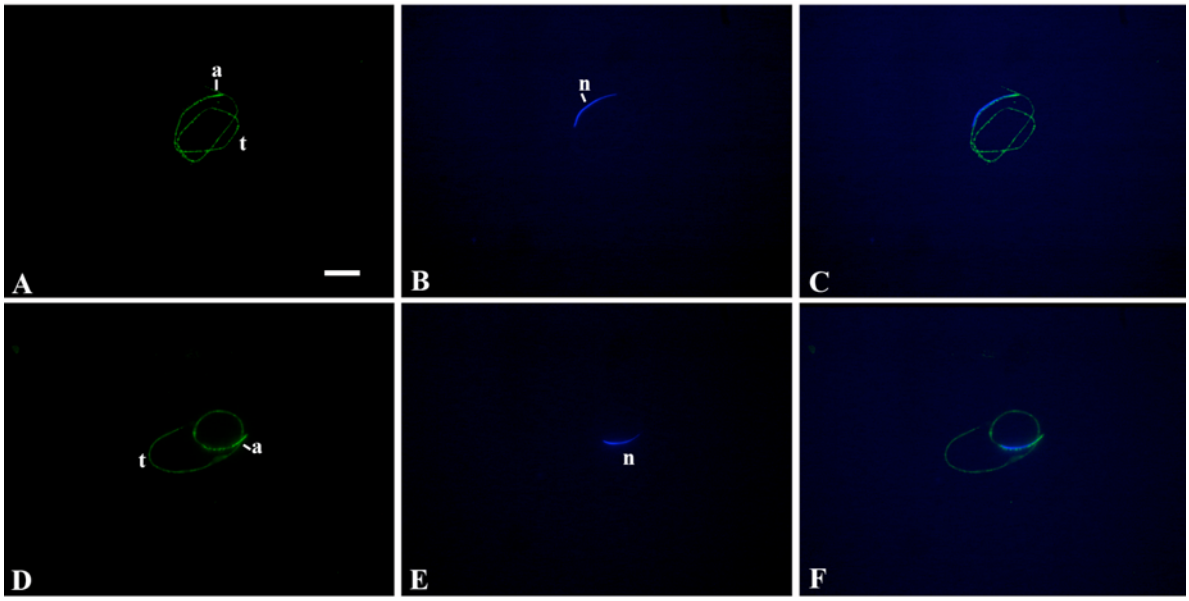


Fig. (3). Immunolocalization of *fdl* product on *D. subobscura* sperm plasma membrane. Primary antiserum binding was evidenced with Alexa Fluor 488-conjugated secondary antiserum (green) and nuclei were counterstained with Hoechst 33342 (blue). (**A-C**) Immunolabeling of long sperm. The plasma membrane over the acrosome and the tail is fluorescent, whereas it is negative over the nucleus. In C, panels **A**, **B** have been merged. (**D-F**) Immunolabeling of short sperm. The labeling pattern is similar to the one observed in the long sperm. a, acrosome; n, nucleus; t, tail. Bar, 5 μm.

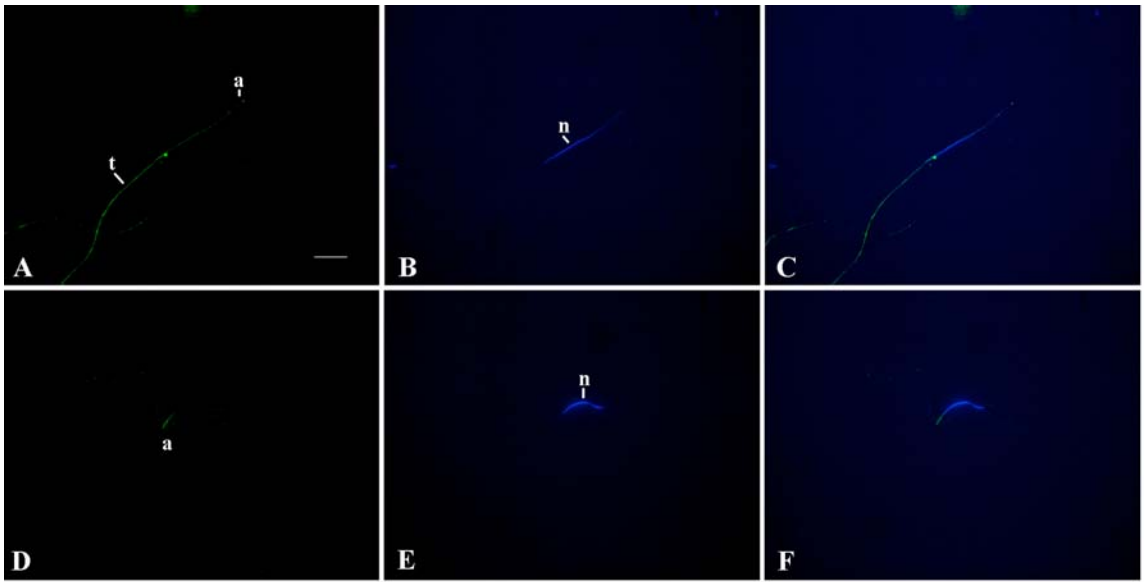
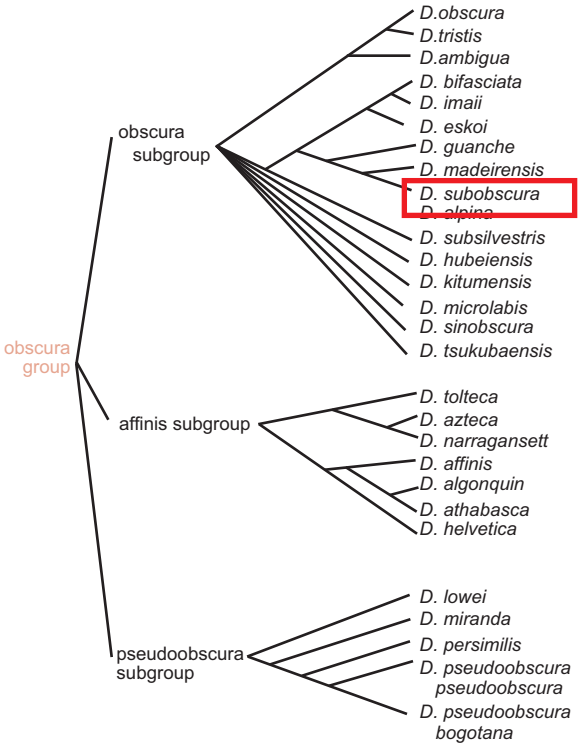


Fig. (4). *D. subobscura* sperm labeled with rabbit anti-α-L-fucosidase followed by secondary antiserum-Alexa Fluor 488 (green) and Hoechst 33342 for nuclei counterstaining (blue). (**A-C**) Immunolabeling of long sperm. The plasma membrane over the acrosome and the tail is fluorescent, whereas it is negative over the nucleus. In **C**, panels **A**, **B** have been merged. (**D-F**) Immunolabeling of short sperm. The labeling pattern on the acrosome is similar to the one observed in the long sperm. a, acrosome; n, nucleus; t, tail. Bar, 5 μm.

fucosidase, HEXA and HEXB are localized on the plasma membrane overlying the acrosome and the tail of both long and short spermatozoa (Figs. **1**-**4**). Their localization over the acrosome support their participation in sperm-egg interactions. Here new insight is gained from immunocytochemistry that demonstrated that in both the long and short sperm of *D. subobscura* enzymes with a role of egg receptors at fertilization are present on the plasma membrane. The results confirm the existence of two types of fertile spermatozoa in *D. subobscura*.



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MATERIALS AND METHODS

Flies Used

D. subobscura provided by Snook RR were maintained on standard cornmeal/sugar/agar food with yeast at 22 ± 1 ºC, and a 12:12-hour light:dark photoperiodic cycle. Adult males and females were separated at eclosion and used 6 days later.

Immunofluorescence

Antibodies were elicited against synthetic peptides encompassing two/three different regions for each of the polypeptides encoded by *Hexo1* (CG1318), *Hexo2* (CG1787), *fused lobe (fdl)* (CG8824) and *Fuca* (CG6128) genes, as previously described [24, 26]. Mature spermatozoa from the seminal vesicles were thoroughly washed in PBS (pH 7.2), fixed for 10 min with 2% paraformaldehyde in PBS at room temperature, blocked with 0.2 M NH_4Cl for 30 min, washed in PBS, and blocked again with 10% normal goat serum in PBS supplemented with 1% BSA for 30 min. They were then incubated in 40 µg/mL primary antiserum in 1% BSA–PBS for 1 h, and, following PBS washing, for 1 h in 5 µg/mL Alexa Fluor 488-goat antirabbit/antimouse antiserum (Molecular Probes, Eugene, OR) supplemented with 3 µg/mL Hoechst 33342. Microscopic analysis was carried out with a Leica DMRB microscope equipped with a 100X oil immersion objective, the CCD-camera indicated above and manufacturer's filters for the fluorescent dyes (for Hoechst 33342, the filter set BP340–380, RKP 400, and LP 430; for Alexa Fluor 488, the fluorescein filter set BP488, BP 450–490, RKP 510, and BP 525/20).

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