

## *Drosophila subobscura* Short Sperm have no Biochemical Incompatibilities with Fertilization

Maria Enrica Pasini\*

Università degli Studi di Milano, Dipartimento di Scienze Biomolecolari e Biotecnologie, Via Celoria, 26-20133 Milano, Italy

**Abstract:** *Drosophila obscura* group species produce two distinct sizes of nucleated sperm that differ only in head and tail lengths. 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  $\beta$ -N-acetylhexosaminidases and  $\alpha$ -L-fucosidase antibodies. Beta hexosaminidases are intrinsic proteins of the sperm plasma membrane in spermomomorph 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 adaptive 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 length 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 nuclear length 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 poten-

tially capable of egg penetration and fertilization. Similarly, *Anopheles gambiae* produced polymorphic nucleated sperm with a wide range of tail lengths [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 involvement in fertilization, although transferred to the female in large numbers [9].

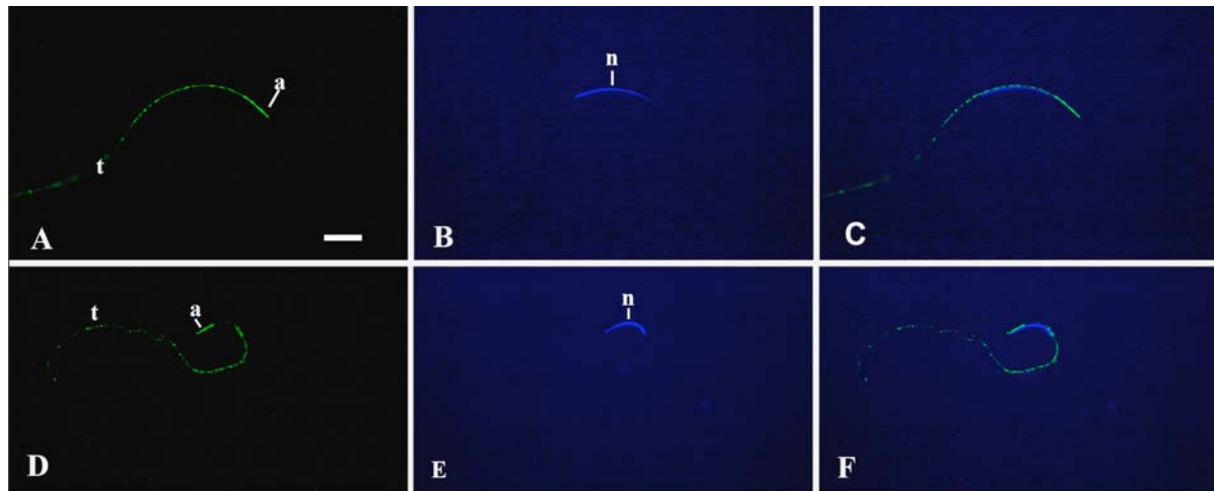
Kinetic analysis of sperm in species of the *obscura* group at different times after copulation demonstrated that the long but not the short sperm are physiologically affected by storage [10] and that the long sperm are the principle morph for fertilization [11]. Thus, short sperm would be the first 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 incompatibilities 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,

\*Address correspondence to this author at the Università degli Studi di Milano, Dipartimento di Scienze Biomolecolari e Biotecnologie, Via Celoria, 26-20133 Milano, Italy; Tel: +39-02-503.14887; Fax +39-02-503.14881; E-mail: maria.pasini@unimi.it

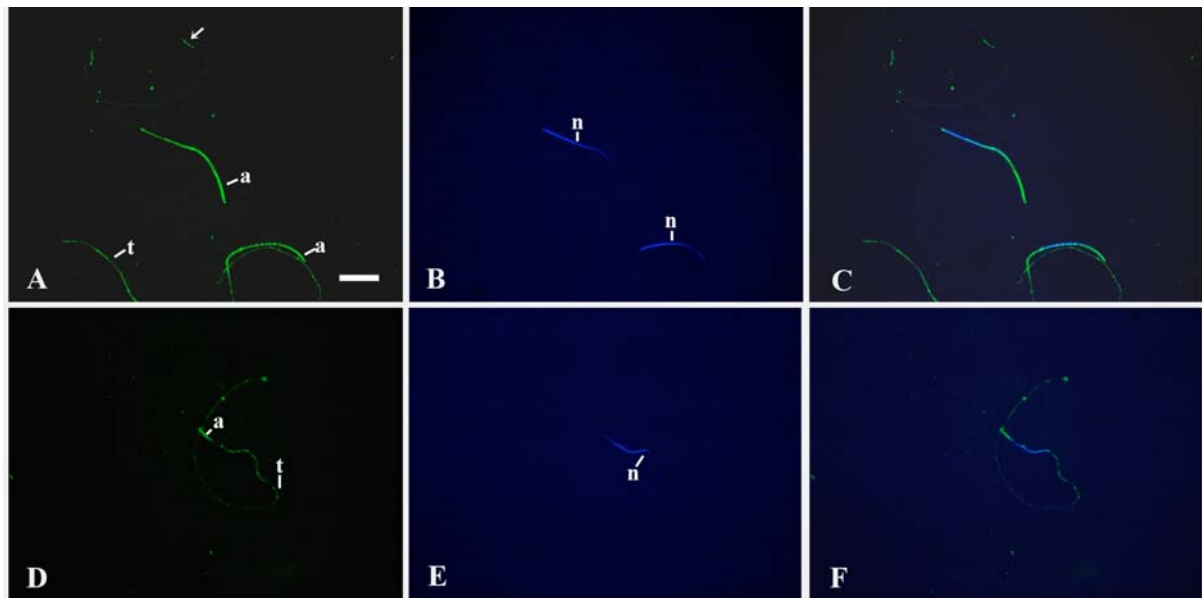
nonfertilizing sperm have been suggested to function as cheap filler in the female reproductive tract [15] or to be a male counter adaptation to spermicide, protecting eusperm in the female reproductive tract [16, 17].

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 specificity 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 amphibi-

ans [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, participate in the degradation of glycoproteins, glycolipids and glycosaminoglycans. HEXA is a heterodimer of subunits  $\alpha$  (encoded by the gene *HEXA*) and  $\beta$  (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  $\mu$ m.



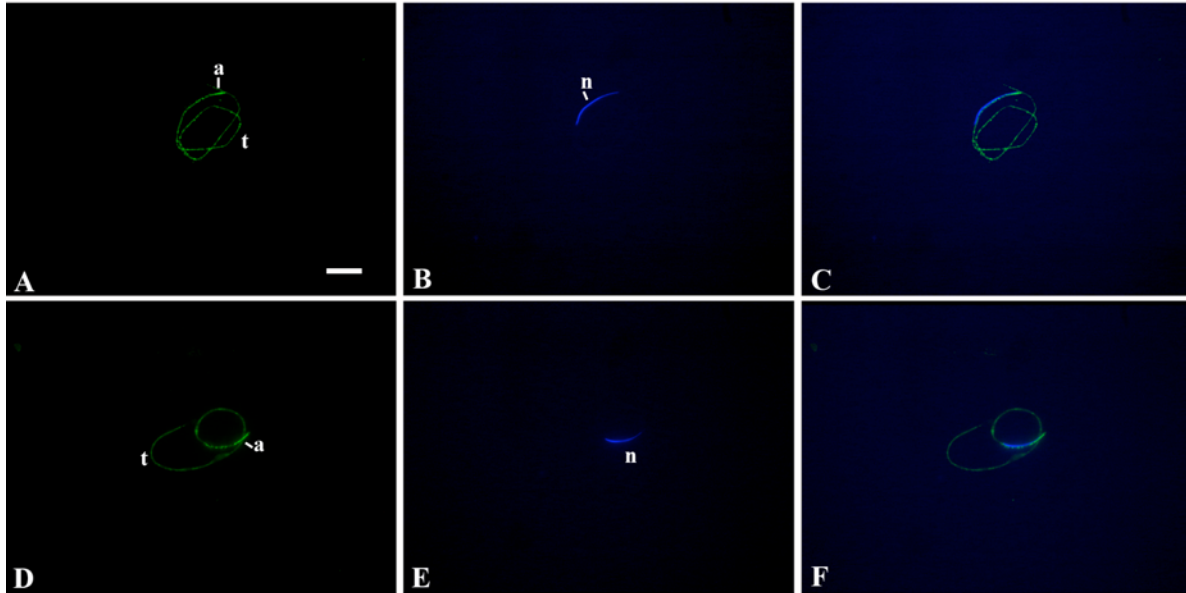
**Fig. (2).** *D. subobscura* sperm labeled with rabbit anti-*Hexo2* followed by goat antirabbit-Alexa Fluor 488 (green), and Hoechst 33342 for nuclei counterstaining (blue). (A-C) Immunolabeling of long sperm. A strong signal over the acrosome is evident. Labeling of the nucleus and of the tail were 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  $\mu$ m.

whereas HEXB is a homodimer of  $\beta$  subunits. Sperm surface glycosidases, an  $\alpha$ -L-fucosidase and two  $\beta$ -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].

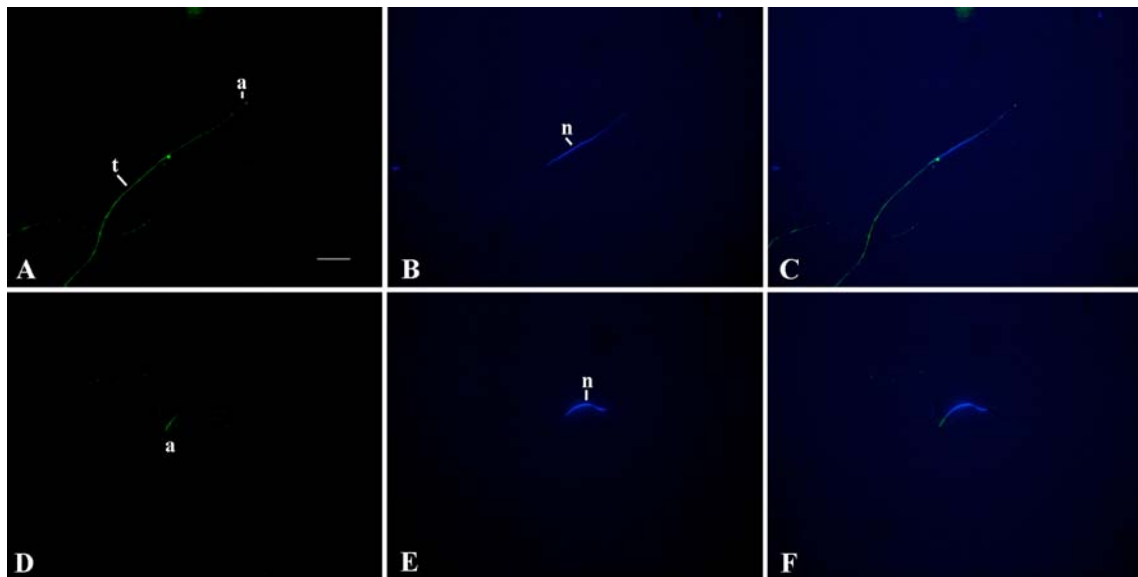
In *D. melanogaster* spermatozoa HEXA is an heterodimer with an  $\alpha\beta_2$  structure and HEXB has a  $\beta_1\beta_2$  structure. The  $\alpha$ ,  $\beta_1$  and  $\beta_2$  subunits are encoded by the homologous NAG

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  $\alpha$ -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  $\alpha$ -L-

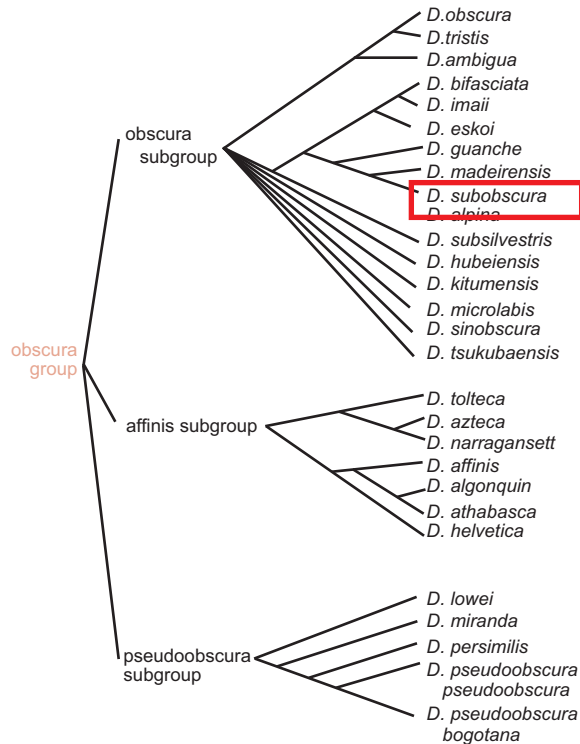


**Fig. (3).** Immunolocalization of *fdl* product on *D. subobscura* sperm plasma membrane. Primary antiserum binding was evidenced with Alexa Fluor 488-conjugated secondary antiserum (green) and nuclei were counterstained with Hoechst 33342 (blue). (A-C) Immunolabeling of long sperm. The plasma membrane 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  $\mu$ m.



**Fig. (4).** *D. subobscura* sperm labeled with rabbit anti- $\alpha$ -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  $\mu$ m.

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*.



Russo C. *et al.* Mol Biol Evol 1995; 12(3): 391-404.

## MATERIALS AND METHODS

### Flies Used

*D. subobscura* provided by Snook RR were maintained on standard cornmeal/sugar/agar food with yeast at  $22 \pm 1$  °C, 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  $\text{NH}_4\text{Cl}$  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  $\mu\text{g}/\text{mL}$  primary antiserum in 1% BSA-PBS for 1 h, and, following PBS washing, for 1 h in 5  $\mu\text{g}/\text{mL}$  Alexa Fluor 488-goat antirabbit/antimouse antiserum (Molecular Probes, Eugene, OR) supplemented with 3

$\mu\text{g}/\text{mL}$  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).

## REFERENCES

- [1] Snook RR. Is the production of multiple sperm types adaptive? *Evolution* 1997; 51: 797-80.
- [2] Fuller MT. In: Bate M, Martinez Arias A, Eds. *The Development of Drosophila melanogaster*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press 1993; vol. 1: pp. 71-148.
- [3] Takamori H, Kurokawa H. Ultrastructure of the long and short sperm of *Drosophila bifasciata* (Diptera: Drosophilidae). *Zool Sci* 1986; 3: 847-58.
- [4] Beatty RA, Burgoyne PS. Size classes of the head and flagellum of *Drosophila* spermatozoa. *Cytogenetics* 1971; 10: 177-89.
- [5] Joly D, Lachaise D. Polymorphism in the sperm heteromorphic species of the *Drosophila obscura* group. *J Insect Physiol* 1994; 40: 933-38.
- [6] Snook RR, Markow TA, Karr TL. Functional Nonequivalence of Sperm in *Drosophila pseudoobscura*. *Proc Natl Acad Sci USA* 1994; 91 (23): 11222-26.
- [7] Pasini ME, Redi CA, Caviglia O, Perotti ME. Ultrastructural and cytochemical analysis of sperm dimorphism in *Drosophila subobscura*. *Tissue Cell* 1996; 28: 165-75.
- [8] Klownen MJ, Chambers GM. Production of polymorphic sperm by anopheline mosquitoes and their fate within the female genital tract. *J Insect Physiol* 2004; 50(12): 1163-70.
- [9] FriedlŠnder M, Seth RK, Reynolds SE. Eupyrene and Apyrene Sperm: Dichotomous Spermatogenesis in Lepidoptera. *Adv Insect Physiol* 2005; 32: 206-308.
- [10] Bressac C, Joly D, Devaux J, Serres C, Feneux D, Lachaise D. Comparative kinetics of short and long sperm in sperm dimorphic *Drosophila* species. *Cell Mot Cytoskel* 1991; 19(4): 269-74.
- [11] Bressac C, Hauschteck-Jungen E. *Drosophila subobscura* females preferentially select long sperm for storage and use. *J Insect Physiol* 1996; 42: 323-28.
- [12] Snook RR, Markow TA, Karr TL. Functional nonequivalence of sperm in *Drosophila pseudoobscura*. *Proc Natl Acad Sci USA* 1994; 91: 11225-29.
- [13] Snook RR, Karr TL. Only long sperm are fertilization-competent in six sperm-heteromorphic *Drosophila* species. *Curr Biol* 1998; 8: 291-94.
- [14] Snook RR, Markow TA. Mating system evolution in sperm heteromorphic *Drosophila*. *J Insect Physiol* 2001; 47(9): 957-64.
- [15] Silberglied RE, Shepherd JG, Dickinson JL. Eunuchs: the role of apyrene sperm in Lepidoptera. *Am Nat* 1984; 123: 255-65.
- [16] Swallow JG, Wilkinson GS. The long and short of sperm polymorphisms in insects. *Biol Rev* 2002; 77(2): 153-82.
- [17] Holman L, Freckleton RP, Snook RR. What use is an infertile sperm? A comparative study of sperm-heteromorphic *Drosophila*. *Evolution* 2008; 62: 374-38.
- [18] Focarelli R, La Sala GB, Balasini M, Rosati F. Carbohydrate-mediated sperm-egg interaction and species specificity: a clue from the *Unio elongatulus* model. *Cells Tiss Organs* 2001; 168: 76-81.
- [19] Matsumoto M, Hirata J, Hirohashi N, Hoshi M. Sperm-egg binding mediated by sperm alpha-L-fucosidase in the ascidian, *Halocynthia roretzi*. *Zool Sci* 2002; 19(1): 43-8.
- [20] Martinez ML, Martelotto L, Cabada MO. Purification and biological characterization of N-acetyl- Dglucosaminidase from *Bufo arenarum* Spermatozoa. *Mol Reprod Dev* 2000; 57(2): 194-203.
- [21] Perez Martinez SL, Menendez Helman RJ, Zitta KS, Brandelli A, Miranda PV. Characterization of human sperm Nacetylglucosaminidase. *Int J Androl* 2007; 31: 315-24.

- [22] Nixon B, Aitken RJ, McLaughlin EA. New insights into the molecular mechanisms of sperm-egg interaction. *Cell Mol Life Sci* 2007; 64(14): 1805-23.
- [23] Henrissat B, Bairoch A. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* 1993; 293: 781–88.
- [24] Cattaneo F, Pasini ME, Intra J, Matsumoto M, Briani F, Hoshi M, Perotti ME. Identification and expression of *Drosophila melanogaster* genes encoding-hexosaminidases of the sperm plasma membrane. *Glycobiology* 2006; 16(9): 786-800.
- [25] Intra J, Cenni F, Pavesi G, Pasini ME, Perotti ME. Interspecific analysis of the glycosidases of the sperm plasma membrane in *Drosophila*. *Mol Reprod Dev* 2009; 76: 85-100.
- [26] Pasini ME, Pavesi G, Intra J. Expression study of -L-fucosidase gene in *Drosophila*. *Gene* 2008; 420: 23-33.

---

Received: September 16, 2009

Revised: November 07, 2009

Accepted: February 08, 2010

© Maria Enrica Pasini; Licensee *Bentham Open*.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.