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TESTICULAR SPERMATOZOA AND MALE GERM CELLS EVOLUTION IN DINA LINEATA (HIRUDINEA: ERPOBDELLIDAE) STUDIED BY SCANNING ELECTRON MICROSCOPY

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RESUM

L'espermatozoide testicular i l'evolució de les cèl·lules germinals masculines de Dina lineata (Hirudinea: Erpobdellidae). **Estudi al microscopi electrònic d'escandallatge**

A *Dina lineata,* hirudini erpobdèl·lid amb fecundació traumàtica hipodèrmica, cada espermatogònia primitiva, lliure en els sacs testiculars, entra en un procés de set divisions mitòtiques anticlinals cariocinètiques i no citocinètiques per formar poliplasts amb 2, 4, 8, 16, 32, 64 espermatogònies i, finalment, 128 espermatòcits primaris. Les cèl·lules, en un mateix poliplast, evolucionen sincrònicament lligades per un pont a una massa citoplasmàtica central comuna anomenada citòfor. Una darrera divisió, meiòtica, porta a un poliplast amb 512 espermàtides.

Per un complex procés morfogenètic de diferenciació cel·lular, cada espermàtida, arrodonida i d'uns 1,3 μm de diàmetre, esdevé un espermatozoide filamentós i helicoïdal d'uns 48 μm de longitud per 0,3 μm de diàmetre. L'ordre cronològic de diferenciació de les diverses regions del gàmeta és el següent: elongació del flagel, elongació i espiralització del nucli, elongació de la regió mitocondrial i elongació i espiralització, primer de l'acrosoma posterior i, després, de l'acrosoma anterior.

A l'espermatozoide:

1) L'acrosoma posterior (7 μm) és una doble hèlix dextrògira formada per dues fibres, una d'estreta i una altra d'ampla, que descriuen, cadascuna, set voltes completes.

2) L'acrosoma anterior (6,5 μm) és un eix a l'entorn del qual una mateixa expansió cintiforme descriu, en sentit dextrògir, set voltes completes.

3) La regió nuclear (5,5 μm) és també una doble hèlix dextrògira integrada per dues fibres. A la base presenten el mateix gruix, però, a mesura que se n'allunyen, l'alteren a voltes alternes.

4) Les regions mitocondrial (8,5 μm) i flagel·lar (20 μm) són externament llises.

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ABSTRACT

This paper studies the male germ cells evolution in the leech *Dina lineata* by scanning electron microscopy.

A primitive spermatogonium is an oval cell measuring 1,6 per 6 μm. Seven not-finished consecutive anticlinal mitotic divisions lead to the formation of isogenic groups with 2, 4, 8, 16, 32, 64 spermatogonia and, finally, 128 primary spermatocytes. The cells are all clustered in only one group and they evolve gradually and synchronically, remaining connected by cytoplasmic bridges to a central cytoplasmic mass called cytophore. A last division, which is meiotic, gives rise to an isogenic group with 512 spermatids: the cellular body of each one is reduced to 1,3 μm in diameter.

By means of a complex spermiogenic process of cellular differentiation, each body becomes a filiform and helicoid spermatozoon measuring 48 μm in length and 0.3 μm in diameter. The morphogenetic and chronologic sequence of the various regions is the following: flagellum elongation; nucleus spiralization and elongation; mitochondrial region elongation; and finally, spiralization and elongation, first of the posterior acrosome and after of the anterior acrosome.

In the spermatozoon:

1) The posterior acrosome (7 μm) is a right-handed double helix integrated by two fibers: a narrow one and a wide one; each describes seven complete turns.

2) The anterior acrosome (6.5 μm) is a longitudinal axis which has a ribbonshaped expansion describing seven right-handed complete turns around the same axis.

3) The nuclear region (5.5 μm) is also a right-handed double helix integrated by two fibers. On their base they show the same trickness, but this is alternatively altered along it from the base to the other side.

4) The mitochondrial region (8.5 μm) and the flagellar one (20 μm) are externally smooth.

INTRODUCTION

Dina lineata, a very common leech in the rivers and streamlets of Spain, is hermaphrodite and proterandrous. Its male reproductive system shows three very defined regions (fig. 1): testicular sacs, epidydimis and atrium. Spermatogonia are free within the coelomic testicular cavities and they change into spermatozoa following the usual process of spermatogenesis and spermiogenesis.

The study of spermatozoa and spermatogenesis in Hirudinea has interested various authors. The complex mechanism of reproduction (hermaphroditism with traumatic fertilization or with copulation) suggests a high degree of specialization in the gamete, and so,.a complex differentiation process. The works carried out by WISSOCQ & MALECHA (1974, 1975) and by MALECHA (1975) on *Piscicola geometra* (Rhyncobdellae: Piscicolidae); by DAMAS (1968, 1974) on *Glossiphonia complanata* (Rhyncobdellae: Glossiphonidae); and by PASTISSON (1975) on *Hirudo medicinalis* (Gnatobdellae) allow FERRAGUTTI (1983) to propose a morphological model for the spermatozoon of Hirudinea, which is classified within the group of modified spermatozoa. This spermatozoon is characterized by the presence of a long acrosome with two regions; a thin, long and helicoid nucleus, a midpiece with only one mitochondrion, and a long, flexible flagellum. In this model, all studied spermatozoa show features proper to each species. The one we study shows hypodermic traumatic fertilization with presence of spermatophores (NAGAO, 1957), which is a peculiar feature of the reproduction system of leeches classified in the orders Pharyngobdellae and Rhyncobdellae.

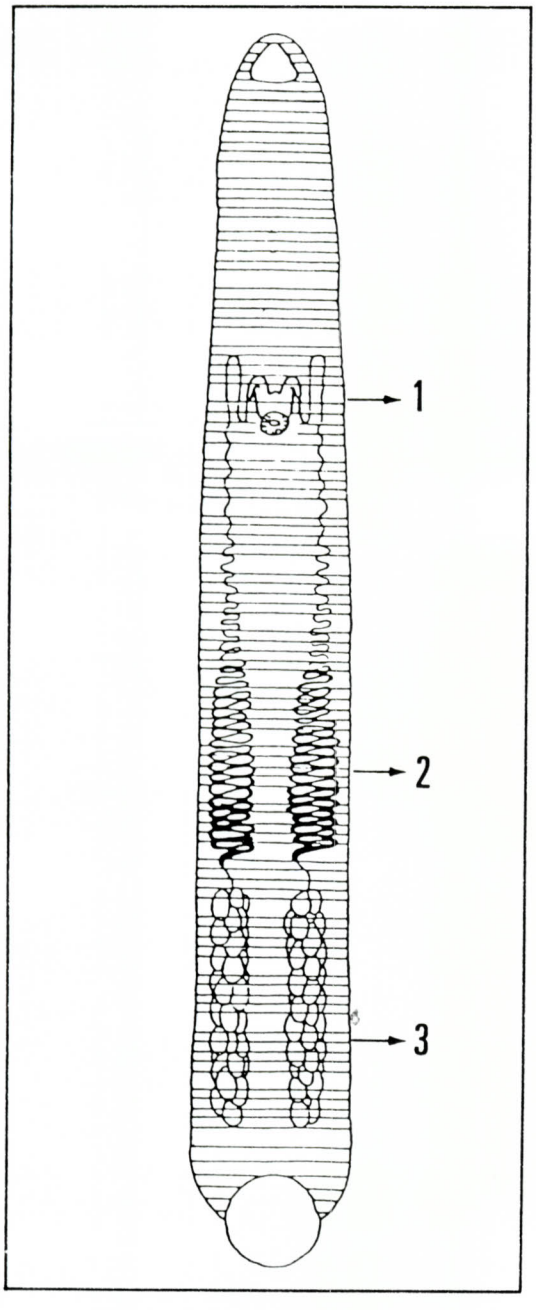


FIG. 1. Diagram of the male genital system of *Dina lineata*. 1) Atrium; 2) Epidydimis; 3) Testicular sacs.
Esquema de l'aparell genital masculí de *Dina lineata*. 1) Regió atrial; 2) Epididim; 3) Sacs testiculars.

MATERIALS AND METHODS

A specimen, measuring about 4 cm in length and with the male reproductive system visible from outside, was anaesthetized within an air-proof recipient containing 25 cc Seltzerwater and 20 drops of etilic alcohol (70°).

A ventral dissection was carried out until the male genitals were exposed. The testicles, placed in the caudal region on both sides of the nervous chain, were put on an excavated microscope-slide containing Sorensen phosphate buffer (0.16 M, pH 7.9). After tearing its delicate cover, we obtained a suspension of germ cells which was taken into a centrifuge tube and was subjected to the following process:

— Fixation in 2.5 % buffered glutaraldehyde and postfixation in 1 % osmium tetroxide; this allows us to visualize better the material and reduces centrifugation time.
— Dehydration by an ascendent gradation of alcohols, and treatment with amyl acetate.
— The suspension is deposited on a Nuclepore filter with a pore diameter of 0.2 μm. After it was subjected to critical point and metallized by the sputtering method during 4 minutes.

Observations have been carried out with a Super III-A ISI in the Electron Microscopy Service of the Authonomons University of Barcelona and with a Cambridge Steroscan S-4 in the Electron Microscopy Service of the University of Barcelona.

We gratefully acknowledge both centers for their help and particularly Profs. J. Egozcue, M. Ponsà, R. Bargalló and J. López, since their teachings have made this work possible. We must also thank Prof. M. Durfort for her help and encouragement.

The testicular spermatozoon (fig. 2)

The spermatozoon of *Dina lineata* (figure 4) is a filiform helicoid cell measuring about 48 μm in length and 0.3 μm in diameter.

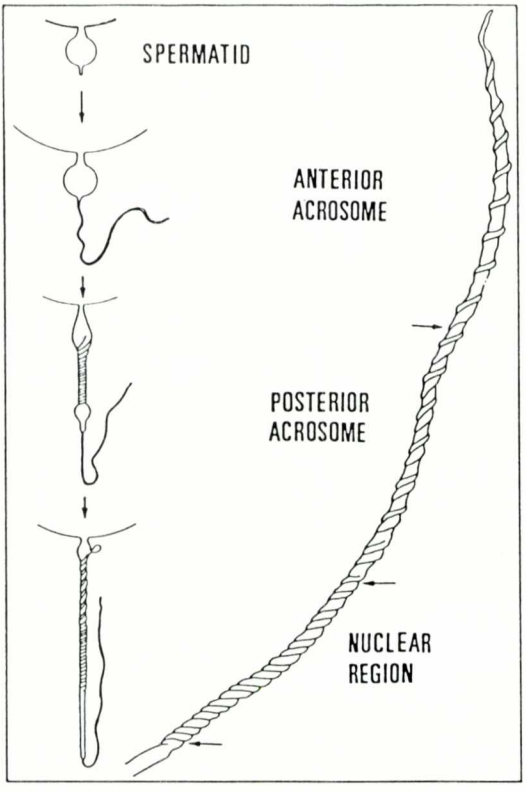


FIG. 2. Diagram showing the process of spermiogenesis (left) and the various helicoid regions in the spermatozoon (right).
Esquema que mostra, a l'esquerra, el procés d'espermiogènesi i, a la dreta, les diverses regions helicoïdals de l'espermatozoide.

Studying its external morphology, we can distinguish four regions: acrosomal, nuclear, mitochondrial and flagellar. Observations by means of P.A.S., Feulgen and Papanicolau techniques have allowed us to identify every one of these zones.

ACROSOMAL REGION

The acrosome measures 13.5 μm in length and shows two zones in it: anterior acrosome and posterior acrosome. The anterior acrosome is viewed as a rectilinear conical axis which emits a ribbon-shaped expansion twisted around it in a right-handed helicoid path. This ribbon completes seven turns with a stretch of 0.7 μm and it ends the anterior acrosome, which gradually grows thin and pointed. This zone has a total length of 6.5 μm and an approximate diameter of 0.24 μm.

The posterior acrosome is a right-handed double helix made up by two elements of different thickness: a thick fiber measuring 0.45 μm and a thin fiber of 0.25 μm. Both have a stretch of 0.83 μm and describe an angle of 135° with the longitudinal axis. They complete seven turns around this axis. At the same time that this double helix moves away from the nucleus, the thick fiber base, in contact with the thin fiber, enlarges and becomes an helicoid ribbon measuring 0.08 μm in diameter; it will continue along the anterior acrosome. The posterior acrosome as a whole has an approximate diameter of 0.33 μm and a total length of 7 μm.

NUCLEAR REGION

The nuclear region measures 5.5 μm in length and 0.35 μm in diameter. Its structure is also helicoid and it is made up by two strands with an helicoid path. On its base, near to the mitochondrial region, the double helix is very compressed. The stretch and thickness of each fiber are the same as for the other region: 0.46 and 0.17 μm respectively.

At the same time that we get nearer to the anterior acrosome, the external nuclear morphology is slightly modified. Both fibers alter their thickness in alternate turns, so that in the very end of this region we observe a false figure consisting of a braid made up by three fibers: one thick fiber and two thin ones. The thick fiber ends the nuclear region and is also the beginning of the posterior acrosome.

The mitochondrial region measures circa 0.5 μm in length and 0.3 μm in diameter. It roughly ends the nuclear double helix. On its external surface, a simple helix with a stretch of 0.65 μm is insinuated. As we move away from the nuclear region this helix attenuates and finally disappears.

The flagellar region is 20 μm long and when viewed by scanning electron microscopy it appears as a long filament with a smooth surface. Its considerable flexibility contrasts with the greater rigidity we find in the other regions.

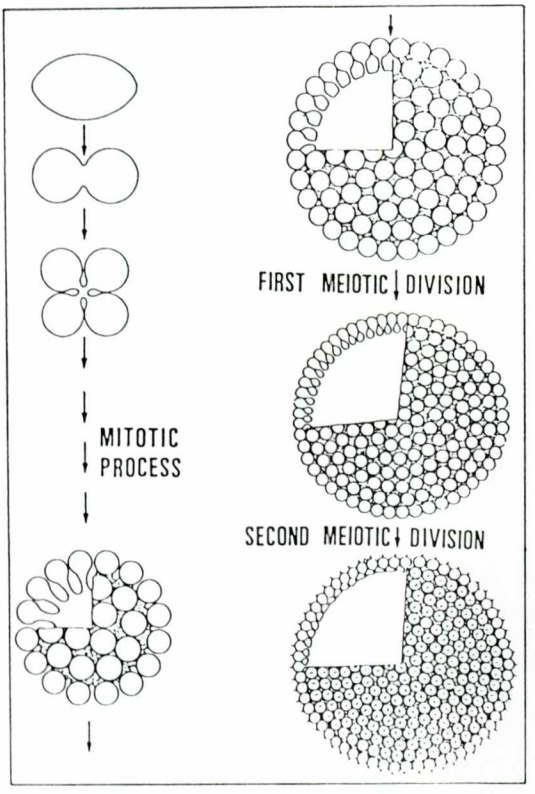


FIG. 3. Schematic drawing of spermatogenesis. Each mature spermatogonium gives rise, after seven mitotic divisions, to a group of 128 primary spermatocytes. They produce 512 spermatids. The cluster remains joined by cytoplasmic bridges to a common central mass, the cytophore.
Dibuix esquemàtic de l'espermatogènesi. Cada espermatogònia madura origina, mitjançant set divisions mitòtiques, un conjunt de 128 espermatòcits primaris. Aquests produeixen 512 espermàtides. El grup roman unit per ponts citoplasmàtics a una massa central comuna, el citòfor.

Spermatogenesis (fig. 3)

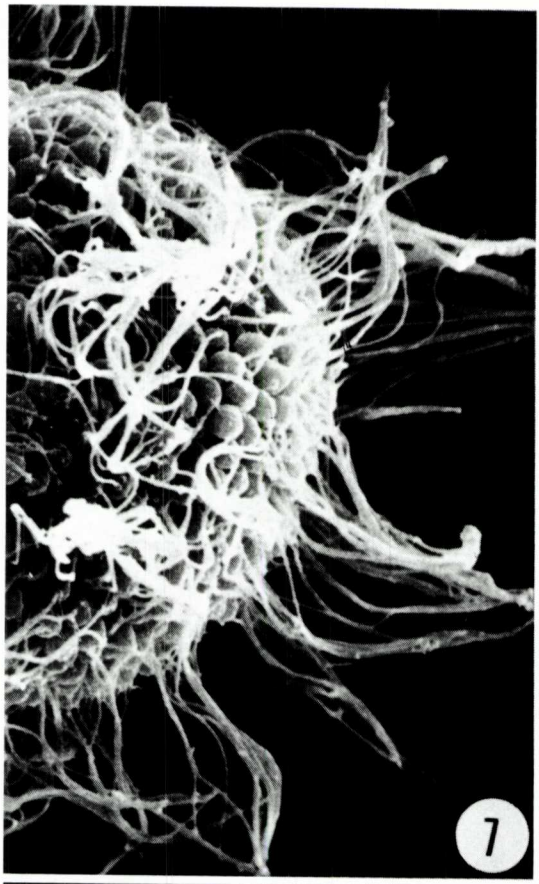
A mature spermatogonia is an oval cell which measures 10.6 and 6 μm in major and minor diameter respectively.

This cell begins a mitotic process which does not lead to a complete cytoplasmic division, but to the formation of two daughter cells which will remain joined by a common cytoplasmic bridge. A new anticlinal division will give rise to a group of four spermatogonia. We call it an isogenic group of four cells, because all of them arise from a primitive cell and they will follow a process synchronically, connected by a cytoplasmic bridge.

One division after another will lead to isogenic groups with 8, 16, 32 and 64 spermatogonia (fig. 5). The diameter of the clusted will progressively grow until it reaches 15 μm. The cellular body will reduce to 2.3 μm; within it, we practically only find the nucleus and the Golgi complex, which is the precursor of the acrosomic vesicle. Cytoplasmic bridges will grow longer and thinner in the first stages and then will go radially towards the geometrical center of the cluster. These bridges, however, get in contact long before reaching the geometrical center —the longer before, the more the cellular population of the cluster grows— so that we find a common central cytoplasmic mass, the cytophore. This new structure allows the bridges to reduce in length, while, theoretically, they should continue enlarging. Consequently, the architectural stability of the cluster increases and intercellular communication is quicker. Moreover, since the cytophore contains a great number of cytoplasmic organelles, the cellular bodies can reduce and they only contain the nucleus and the Golgi complex. The bridges shorten and only contain mitochondria and the basal body. Micrographs obtained by PASTISSON (1975) from *Hirudo medicinalis* and by MALECHA (1975) from *Piscicola geometra*, confirm all this. These micrographs show the presence of a great number of mitochondria, endoplasmic reticulum and glycogen granules in the cytophore; these organelles and inclusions are related with regard to nutritive and energetic processes.

Finally, another mitotic division leads to the formation of an isogenic group of 128 primary spermatocytes. The diameter of the cluster can attain 20 μm.

The first reductional meiotic division gives rise to a group of 256 secondary spermatocytes. The second division is equatorial and it ends the increasing geometrical progression (ratio 2), giving rise to an isogenic group of 512 spermatids. The diameter of the cluster will not increase any more, but its density will do, because the cellular bodies reduce (1.3 μm in diameter).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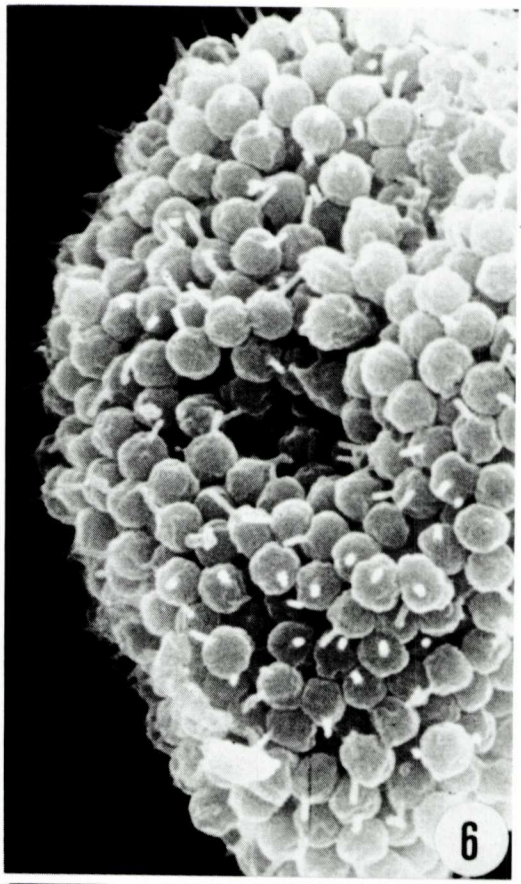
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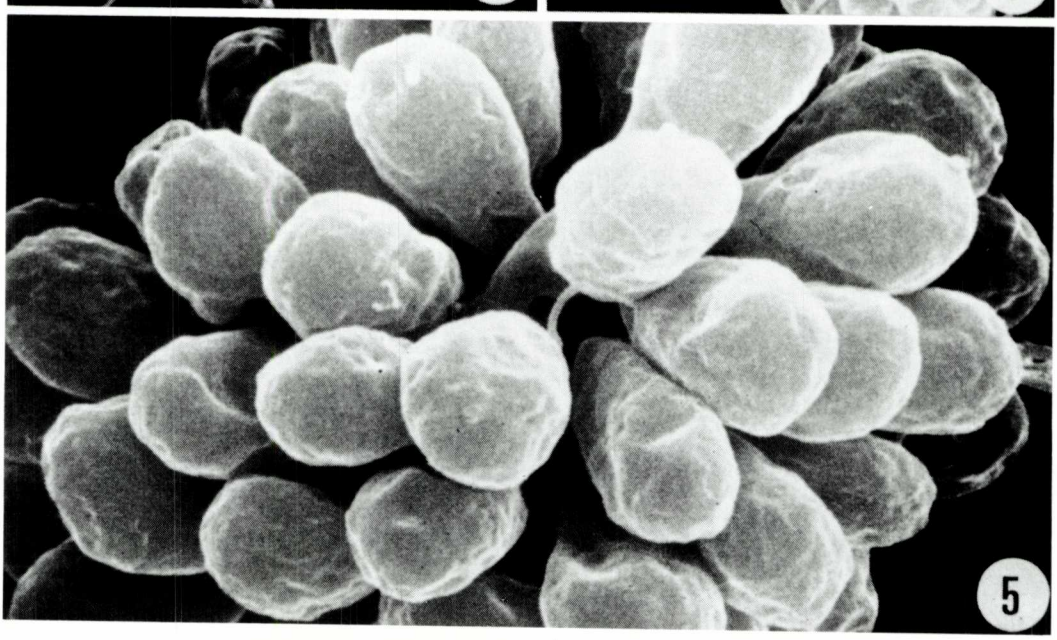
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Spermiogenesis (fig. 2)

Immediately, a cellular differentiation process begins. A nearly spherical spermatid, which measures 1:3 μm in diameter, will become an helicoid filiform spermatozoon measuring 47.5 μm in length and 0.3 μm in diameter.

The morphogenesis of the various structures follows this chronological order:

1) Flagellum elongation.
2) Nucleus elongation and spiralization.
3) Mitochondrial region elongation.
4) Posterior acrosome elongation and spiralization.
5) Anterior acrosome elongation and spiralization.

An isogenic group with 512 spermatids (fig. 6) measures about 20 μm in diameter. From each cellular body comes out a microtubular structure covered by a membrane. When the flagellum appears, it is the very beginning of a complex morphogenetic process:

1) The rudimentary flagellum measures 0.19 μm in diameter and 0.85 μm in length. The elongation takes place quickly (fig. 8) and, at the end of it, the isogenic group seems a hairy morula (fig. 7).
2) The nucleus enlarges progressively. Its spiralization begins just at one end of the cellular body and it becomes progressively narrower along it (fig. 9).
3) The mitochondrion placed between the nucleus and the flagellum enlarges progressively without spiralizing. The diameter of the globular chondriome measures about 0.6 μm. When elongation finishes, it reduces to 0.22 μm.
4) The last stage is acrosomic region differentiation. First, the posterior acrosome differentiates closing more and more its helix the nearer it is to the nucleus. Later, a cytoplasmic expansion (acrosomic cap), which contains the acrosomic substance, will establish the limit between the posterior and the anterior acrosome. After its contents have been emptied into the posterior acrosome (MALECHA, 1975), the anterior will go on with its elongation until it reaches about 6.5 μm. Finally, the cytophore degenerates and spermatozoa, still arranged in group, pass into the wide epidydimis.

DISCUSSION

In fact, it does not exist any work carried out by SEM about spermatogenesis, spermiogenesis and morphology of spermatozoa in leeches. All works have been carried out from sections observed by TEM. We can mention the ones ated in the Introduction.

In outline, spermatozoa from all studied leeches have a common origin, morphogenesis and basic structure. However, some morphological differences exist between spermatozoa from leeches classified in different orders or families: nummer of fibers in the nuclear helix (from 1 to 3), an equal or different thickness in the helically twisted acrosomic fibers, the stretch between the elements of this helix, relative length of the regions, etc. The model of modified spermatozoon proper to Hirudinea has in *Dina lineata* some specific features. These morphological details are only little variations in the same structural plan.

In Hirudinea two types of fertilization are known. Although leeches in the order Gnatobdellae show an erectile penis and direct fertilization by copulation, the ones in the orders Rhyncobdellae and Pharyn-

FIG. 4. Testicular spermatozoon. 7000 x.
Espermatozoide testicular. 7000 x.

FIG. 5. Isogenic group of 64 spermatogonia. 5500 x.
Grup isogènic de 64 espermatogònies. 5500 x.

FIG. 6. Isogenic group of 512 spermatids before taking place flagellum elongation. 5500 x.
Grup isogènic de 512 espermàtides abans de produir-se l'elongació del flagel. 5500 x.

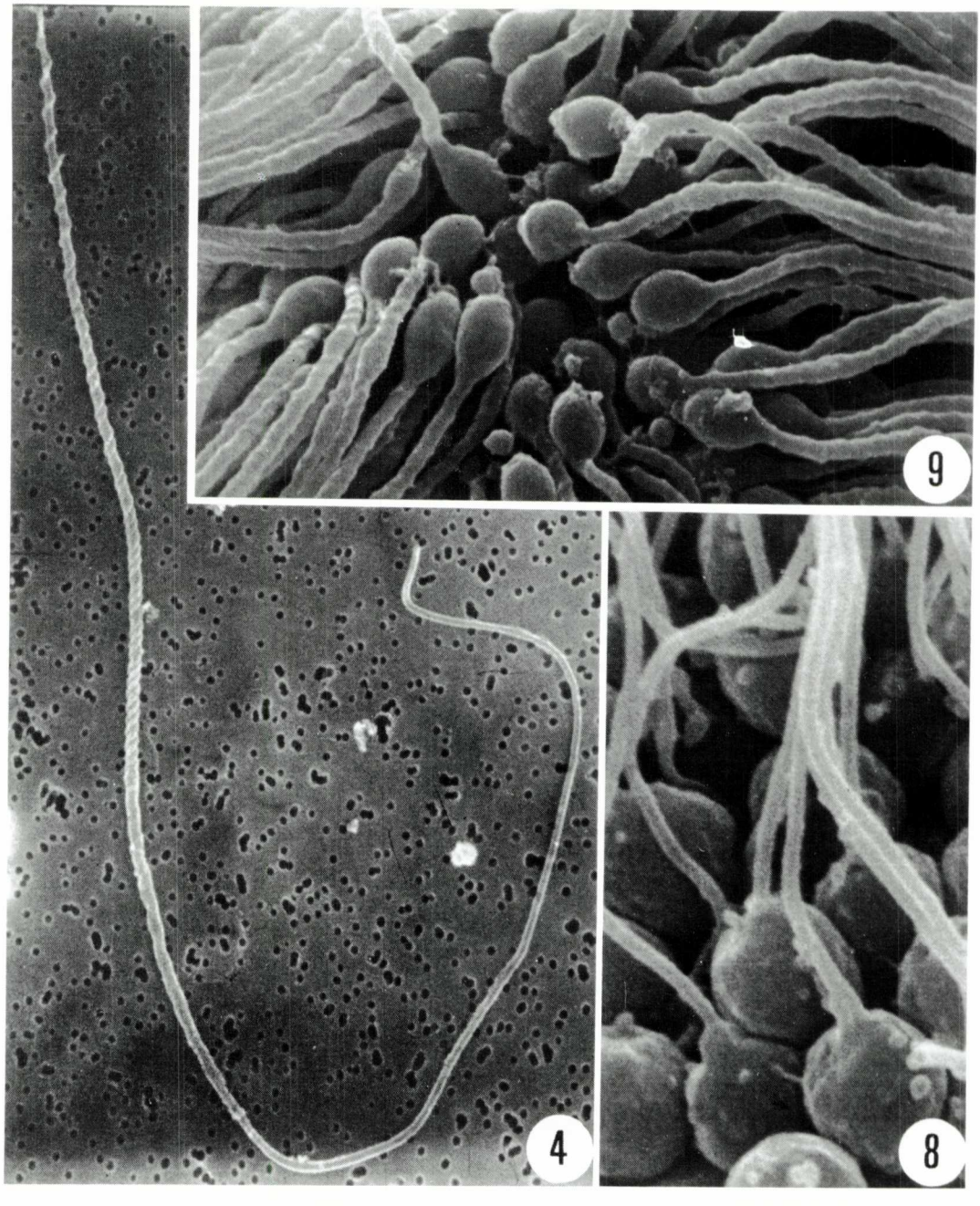

FIG. 7. Isogenic group of 512 spermatids after has taken place flagellum elongation. 3600 x.
Grup isogènic de 512 espermàtides després de l'elongació del flagel. 3600 x.

FIG. 8. Quick elongation of the flagellum from each cellular body. 18000 x.
Elongació ràpida del flagel a partir de cada cos cel·lular. 18000 x.

FIG. 9. Nuclear morphogenesis. 9000 x.
Morfogènesi nuclear. 9000 x.

gobdellae show spermatophores instead of penis and they mate in many cases with hypodermic traumatic fertilization. External morphology in both groups does not suggest the existence of any mechanical properties which were sufficiently different as to change spermatozoa behaviour when the medium changes: from the dense conjuntive stroma (present in traumatic fertilization) to a more fluid medium, the female receptaculum (present in copulation). The study of morphogenesis, which are the changes produced, and which are the involved organs, is part of the way in order to know the final biological meaning of these changes.

From a morphological point of view, we find differences in size, arrangement and composition of the elements, among spermatozoa of the various studied species. It seems to be that these morphological differences are the only ones we can emphasize.

With no doubt, specific adaptation to each fertilization system will render, if not into important changes in their structural model, then into quantitative and qualitative changes concerning acrosomic contents and maduration process.

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