Chapter – VII

Impact of the secretory material of caecilian (Amphibia Gymnophiona) male Mullerian gland on motility of sperm: a study in *Uraeotyphlus narayani*
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**INTRODUCTION**

Caecilians constitute a unique group of subterranean amphibians, with a variety of reproductive modes. The classical biphasic amphibian life cycle of oviparity with an aquatic larval stage is practiced, at one extreme. Viviparity, almost totally skipping an aquatic phase in the life cycle, lies at the other (Wake 1977). Internal fertilization, making use of the eversible phalloseum as the phallus, is unique to the caecilians and is meant for direct sperm transfer into the reproductive tract of the female. The acquisition of the attenuate habitus has been accompanied by changes of morphological features including those of the reproductive system (Wake, 1977). A striking difference in caecilian spermatogenesis from that of the two other amphibian orders is presence of a number of extended testis lobes, varying between species, to produce a large number of spermatozoa (Wake, 1968). Further, the caecilians are known for retention of Mullerian ducts in the adult males as a pair of functional glands. The significance of this retention is to be sought in the elaboration of a secretory material which is believed to play a role as vehicle for transport of sperm and, perhaps, provide nutritional support to the sperm, and that this structure evolved with the acquisition of capacity for internal fertilization (Wake, 1977). In *Uraeotyphlus narayani* we have found that the urinogenital duct and the male Mullerian duct join into a common duct before opening into the cloaca (Chapter III), confirming that the secretion of the Mullerian gland and the sperm mix before ejaculation. Yet, a role for the secretory material of the Mullerian gland (despite elucidation of its formation and composition; see chapters 3 to 6).
Until recently, light microscopic observations of caecilian spermatozoa have been reported for just five of the taxa, *Ichthyophis glutinosis*, *Uraeotyphlus narayani*, *Siphionops annulatus*, *Gegeneophis carnosus* (Seshachar, 1939, 1940, 1943, 1945), and *Chthonerpton indistinctum* (de Sa and Berois, 1986). More recently, Wake (1994) has greatly augmented the number of caecilians (22 genera, 29 species, representing all families) of which spermatozoa have been examined by light microscopy. van der Horst and van der Merve (1991) and van der Horst et al. (1991) made electron microscopic observation of the sperm of *Typhlonectes natans*. Very recently, Sheltinga et al. (2003) made ultrastructural analysis of the sperm of this and three other species of caecilians, viz., *Ichthyophis glutinosis*, *Ichthyophis beddomei* and *Gegeneophis ramaswamii*. According to these various studies the caecilian spermatozoon is filiform and, as seen with light microscope, formed of a head, midpiece and tail. The head is formed of an acrosome and the nucleus. Ultrastructural studies have shown that the acrosome is in the form of a complex composed of an acrosome vesicle surrounding the dense acrosome rod, also known as perforatorium. The nucleus has a cone-shaped depression, the endonuclear canal, into which the perforatorium of the acrosome extends for most of the former’s length. The midpiece consists of centrioles and the anterior part of the tail. The two centrioles are close together, closer to the nucleus, and the distal centriole forms the basal body of the axoneme. An axial fibre also arises from this region. The centrioles, axial fibre and the anterior part of the axoneme, all in the midpiece, are surrounded by mitochondria. The tail consists of the axoneme and axial fibre both enclosed by plasma membrane. More anteriorly, the two are held close together. Beyond the distal part of the midpiece, the two are separated by an undulating membrane (van der Horst and van der Merwe, 1991; van der Horst et al., 1991; Jamieson 1999, Scheltinga et al., 2003).

All these studies made use of testis lobes fixed in various fixatives. To the best of our knowledge there is no study ever observing the live sperm of any caecilian. Studies using live sperm of caecilians will be highly relevant in the context of the concept that the unique Mullerian gland is the source of a substance which forms the vehicle of transport for the sperm during ejaculation, and also provides nutritional support thereafter. Therefore, the present study in *Uraeotyphlus narayani* was set forth to test the following hypotheses: i) like in the other vertebrate groups practicing
internal fertilization, in the caecilians also the spermatozoa require to be initiated into
motility subsequent to ejaculation into the female tract; ii) the caecilian spermatozoa
require nutritional support in the form of energy substrates and/or constituents that
would contribute to an optimum osmolarity for sperm motility; and iii) the secretory
material of Mullerian gland would fulfill both these roles. In other words, it was
hypothesized that the Mullerian gland is a provision for internal fertilization in the
context of attempt towards terrestrialization in the amphibian stage of evolution itself
and it is the physiological forerunner of the epididymis and the male accessory
reproductive glands of the amniotic vertebrates. The study shows that the sperm
released directly from the testis are motile and, therefore, the secretory material of
Mullerian gland does not play any role in the initiation of sperm motility proving the
hypothesis 1 in the negative. The study also shows that the secretory material of
Mullerian gland contributes to sustenance of speed as well as duration of motility of
the spermatozoa, proving hypothesis 2 in full and hypothesis 3 in part.

MATERIALS AND METHODS

Animal collection and maintenance

Male Uraeotyphlus narayani, weighing approximately 22-26g, and measuring
a length of 20-27cm, were chosen for the study. Animals were collected from the
coconut groves and rubber plantations in an around Thodupuzha in Idukki District,
Kerala, India (Lat 09° 53' 52" N Long 76° 42' 29" E), during October-November
(period of peak active spermatogenesis), 2003, among which three were males. In the
laboratory, the animals were kept alive in moist soil and fed on live earthworm.

Light microscopic and experimental studies with the sperm

The animals were dissected under mild MS222 anesthesia. Testis lobes and
Mullerian glands were dissected and transferred to petri dishes. After washing
thoroughly in Amphibian physiological saline solution (pH 7.4) (Dubin and Dionne,
1994), one or two testis lobes were macerated in the same solution taken in an embryo
cup. After thorough dispersion of the cells using a blowpipe, hanging drop preparations were made for observation under bright field, dark field and phase-contrast illuminations in a Leitz Diaplan research microscope (Leica, Germany). Patterns, speed and duration of motility were observed and recorded by two independent observers.

The Mullerian glands were washed thoroughly in the Amphibian physiological saline solution and cut transversely into three or four pieces. The content of the gland was released, by running a needle over the tissue applying mild pressure, into the saline solution and thoroughly mixed. Drops of this preparation were added on to the hanging drop preparation of the sperm from the testis. The difference in the pattern, speed and duration of motility of spermatozoa were observed and recorded.

The sperm preparations before and after mixing with the material from the Mullerian gland were also prepared into thin smears, fixed in methanol and stained in 1% eosin, 1% eosin and 10% nigrosine or Giemsa's. The images of the sperm and the granules present in the material of the Mullerian gland, from a Carl Zeiss Axioscope 2 research microscope (Carl Zeiss, Germany), were captured in a computer through a CCD camera (Sony, Japan). Measurements were made using Carl Zeiss Axiovision Image Analysis software (Carl Zeiss, Germany). The data were used to calculate the respective means and the standard deviations (M ± SD).

RESULTS

Morphology of the sperm

The pyriform spermatozoa of *Uraeotyphlus narayani* measure a length of 104.54 ± 2.87 μm from the tip of the acrosome to the tip of the tail. The acrosome vesicle ahead of the nucleus measures a length of 4.13 ± 0.35 μm. The nucleus is 18.44 ± 0.37 μm long and 1.21 ± 0.08 μm dia. The midpiece is 7.63 ± 1.15 μm long and 1.86 ± 0.39 μm dia. The tail is 74.31 ± 2.75 μm long and the undulating membrane is 1.26 ± 0.39 μm wide (Fig. 1). A lobe of granular cytoplasm occurring anywhere along the
length of the sperm from the anterior end of the nucleus to the posterior end of the midpiece was noticed (4.93 ± 0.64μm dia; 5.81 ± 0.86μm long), more frequently around the midpiece (Figs. 1, 2). The spermatozoa which were engaged in prolonged motility and those which were ceased in motility lacked this structure (Fig. 2).

Sperm motility

Since the time they were released in amphibian saline solution, the spermatozoa of *U. narayani* were motile. Three kinds of motility were noticed: i) slow to rapid forward progression (Fig. 3); ii) sidewise lashing of the tail (Fig. 4); and iii) very rapid cork screw-like movement of the flagellum, beginning from the midpiece and ending at the tip of the tail

Interestingly, spermatozoa from cysts which were not spermiated also had their flagella in lashing movement (Fig. 5). Since in the study only the testis lobes were macerated, in some cases spermatozoa were found held together in two or more numbers, and in such sperm also violent flagellar beat was noticed (Fig. 6).

Contribution of secretory material of Mullerian gland to motility of spermatozoa

Data on motility of the spermatozoa without and with mixing of preparation of the Mullerian gland substance are in the table 1. The data clearly reveal that with the mixing of the Mullerian gland substance, the sperm are enhanced in speed as well as duration of motility. In fact, in the sperm preparation to which Mullerian gland substance was added the spermatozoa remained motile much beyond 5hr (the period of observation).

Nature of secretory material of the Mullerian gland

The moment preparation of Mullerian gland substance was added to the sperm suspension, the medium became turbid. Microscopic observation revealed presence of spherical granules remaining suspended in the saline medium (Fig. 7). The granules increased in size, proportionate to the period of standing. This prompted us to prepare
methanol-fixed, eosin-stained smears of the preparation of the Mullerian gland substance in the amphibian physiological saline solution, and it was found that comparable granules, but very large in size, were present (Fig. 8).

**Occurrence of Large Irregularly-Shaped Cells Amongst Spermatozoa**

Since in the present study the spermatozoa were obtained by macerating lobes of the testis, understandably, mature spermatozoa that exhibited motility were found amongst immature germ cells (Figs. 9a, b). The latter could be easily distinguished from cells that were unique in being extremely large, possessed blunt processes, contained a very large nucleus and possessed cytoplasm containing minute granules (Fig. 9c). These are not germ cells. The blunt processes characterize them to be ameboid cells.

**DISCUSSION**

**Morphology of Sperm**

All descriptions, so far, of the structure of caecilian sperm are based on light or electron microscopic observations of sperm obtained from the testis fixed in some fluid (Seshachar, 1939, 1940, 1943, 1945; de Sa and Berois, 1986; van der Horst et al., 1991; van der Horst and van der Merwe 1991; Wake 1994; Jamieson, 1999; Scheltinga et al., 2003; Smita et al., 2004). Thus, the present report is the first ever description of caecilian sperm based on observation of live sperm. The morphological characteristics of the spermatozoa of *Uraeotyphlus narayani*, with spatulate acrosome, cylindrical nucleus, slightly expanded mid-piece and long flagellum provided with an undulating membrane on one side of it, conform by and large to the descriptions referred *vide supra*. There is already one report on the dimensions of the sperm of *Uraeotyphlus narayani* (Scheltinga et al., 2003), according to which the total length is 120 μm, head length 16.6 μm, acrosome vesicle length 5.5 μm, nucleus length 11.1 μm, nucleus width 4.4 μm, mid-piece length 5.0 μm and tail length 90.4 μm. The measurements in the present report,
using an image analysis software, differ a little in respect of each, i.e., 1.4 μm, 22.57μm, 4.13μm, 18.44μm, 1.2μm, 7.6 μm and 74.31μm, respectively (all, mean values). The two results reveal that the measurements may differ between individuals of the same species.

One major discovery with regard to the structure of sperm in the present study is the occurrence of a vesicular structure, with a lightly staining content, present anywhere along the length of the head or the mid-piece. Interestingly, this structure is absent in the spermatozoa that were ceased in motility. Scheltinga and Jamieson (2002a), in their review on the spermatozoa of urodeles, describe a cytoplasmic droplet on the spermatozoon of most salamanders observed at various locations anywhere from the head to the posterior end of the mid-piece. Lee and Jamieson (1992) reported the occurrence of such a droplet in the sperm of the frog *Myxophyes*, and perhaps *Rheobatrachus*. As mentioned in the review of Scheltinga and Jamieson (2002b), in the testicular sperm of the leptodactylid anurans *Caudiverbera caudiverbera* and *Pleuroderma thaul* also the mitochondria occur in a cytoplasmic droplet. The droplet is lost during the final stages of maturation of the spermatozoa. The most notable feature of the droplet is the presence of mitochondria and a characteristic structure composed of several concentric membranes, together with vesicles containing a granular material, cisternal structures and osmiophilic bodies. In internally fertilizing urodeles the droplet becomes detached in most of the spermatozoa found in spermathecae. It has been suggested that the droplet is functional and could play a role in the maturation of spermatozoa (see, Scheltinga and Jamieson, 2002a,b).

According to Kouba et al. (2003), the sperm of the toad *Bufo americanus* possesses a mitochondria vesicle, which in electron microscopy and fluorescent staining confirmed to contain a large number of active mitochondria with very few other cytoplasmic structures. Nearly all spermatozoa exhibiting motility had an intact...
mitochondria vesicle, and dissociation of this structure was related to motility loss. The sperm of this toad were inactivated when put in simplified amphibian Ringer solution and this resulted in lesser number of sperm with intact mitochondria vesicle with passage of time. The cytoplasmic droplet described in the review of Jamieson and Scheltinga (2002a,b), the mitochondria vesicle reported by Kouba et al. (2003) and the vesicular structure reported by us in this paper appear to be the same. Scheltinga and Jamieson (2002b) ate that the cytoplasmic droplet is observed in spermatids and often in the mature sperm of caecilians. However, to the best of our knowledge there is no original report on the occurrence of cytoplasmic droplet or mitochondria vesicle in any of the caecilian species. Perhaps, this structure in the caecilian sperm does not show up in TEM-fixed specimens (B.G.M. Jamieson, personal communication).

In the mammals, the spermatozoa carry a small droplet of cytoplasm, the cytoplasmic droplet, while leaving the testis (Oko et al., 1993; Hermo et al., 1988; Akbarsha et al., 2000). When in the testis, the cytoplasmic droplet is located rear to the head of the spermatozoa. Subsequently, it moves posteriorly along with the mid-piece until it reaches the point of annulus and then is shed when the spermatozoa leave the corpus epididymidis (Hermo et al., 1988). The cytoplasm at this droplet contains membrane-bound vesicles and lamelle, the origin of which is not yet definitely known, though it has been speculated that they are remnants of Golgi apparatus (Oko et al., 1993). Occurrence of cytoplasmic droplet on the sperm has been reported in painted turtle, Chrysemys picta, also. In the turtle, the droplet contains a large quantity of lipid droplets in addition to hollow vesicles and degenerate mitochondrial fragments (Gist et al., 1992). Thus, it appears that there is an evolutionary trend in the organization and derivation of the constituent structures of the cytoplasmic droplet. In the Amphibia, it essentially consists of functional mitochondria (Scheltinga and Jamieson, 2006b; Kuba
et al., 2003). In the turtle (Reptilia) it contains mitochondrial fragments (Gist et al., 1992). In the mammals, though the cytoplasmic droplet is still retained, there is no indication of derivation of any of its parts from the mitochondria (Oko et al., 1993; Hermo et al., 1988).

**Motility of Sperm Obtained from Testis**

The observations in the present study unambiguously show that spermatozoa of *Uraeotyphlus narayani*, released in an appropriate medium directly from the testis, exhibit motility including rapid, forward progression, a situation comparable to that in the anurans which practice external fertilization (Katagiri, 1987; Wake and Dickie, 1998). On the other hand, it is known that the newts generally practice internal fertilization through the spermatophores and the sperm are released towards the surface of the egg-jelly. In *Cynops pyrrhogaster*, the spermatozoa are released on the egg-jelly, when sperm motility is induced on its surface (Watanabe et al., 2003). Thus, in the case of the newt the spermatozoa require to be initiated into motility, and the factor that contributes to the initiation lies in the osmolarity of the environment of the sperm, which in turn is determined by the egg-jelly. A few anuran species belonging to the genera *Ascaphus* and *Leiopelma* also practice internal fertilization. Sperm taken from the oviduct of *Ascaphus* were highly motile when placed in saline (Sever et al., 2002). Thus, the spermatozoa of *U. narayani* appear to differ in that the testicular spermatozoa are initiated into motility by being prepared in an amphibian physiological saline solution with osmolarity 281 mOsm at pH 7.4. The interesting observation of two or more spermatozoa held together exhibiting motility indicates that the sperm acquire the capability to motility much before spermiation. In this regard, the spermatozoa of caecilians differ from those of the amniotes because in the latter the sperm collected from the testis are not motile (Cooper, 1995), and those collected from the point of ejaculation are motile when diluted in an
appropriate medium (Harrison et al., 1978; Shahul Hamid and Akbaarsha, 1990). Thus, in
the caecilian, which lacks a separate duct system to transport the sperm, the latter do not
appear to require post-testicular maturation process unlike in the amniotes which possess
a separate male reproductive tract, and the epididymis secretes a material that plays one
or more roles in initiating sperm motility. This is known for all the three classes of
amniotes, the Reptilia (Depeiges and Dufaure, 1977, 1983; Depeiges and Dacheux, 1985;
Shahul Hamid and Akbaarsha, 1989; Manimekalai and Akbaarsha, 1992), Aves (Birkhead,

Role of Mullerian Gland Secretion in Motility of Spermatozoa

The data generated in the present study clearly substantiate a role for the secretory
material of the Mullerian gland of *Uraeotyphlus narayani* (and perhaps, all caecilians) in
sustenance of motility of spermatozoa. It was Tonutti (1931) who, for the first time,
suggested that caecilian male Mullerian gland secretion would form a medium in which
the sperm would be suspended during ejaculation. It was further suggested that the
secretory material, on being added on to the sperm, would provide nutritional support for
the latter for their motility (Wake, 1981). Our earlier observation in *Uraeotyphlus
narayani* that the terminal portions of the male Mullerian duct and the male urinogenital
duct join to form a common duct before opening into the cloaca (George et al., 2004) has
strengthened the concept that the secretion of the male Mullerian gland would be added
on to the spermatozoa much before they arrive at the cloaca.

The enhanced duration as well as speed of motility of *Uraeotyphlus narayani*
sperm mixed in the medium with the secretory material of the male Mullerian gland
supports the hypothesis of Wake (1981) atleast to the extent of the secretory material
contributing to sustenance of sperm motility. Thus, we have found a definite role to the
secretory material of the male Mullerian gland of a caecilian in sperm motility. The
contribution of the male Mullerian gland secretion to motility of sperm could be one or even more. Earlier studies have shown that the male Mullerian gland secretion is rich in proteins, glycoproteins, acid phosphatase and a monosaccharide (Wake, 1981). It is an established fact that each one of these seminal constituents contributes in one way or the other to sustenance of motility of spermatozoa in the amniotes (Luke and Coffey, 1994). In the mammals the prostate gland and seminal vesicles secrete these various substances, and are added on to the sperm only at the time of ejaculation. Therefore, the hypothesis of Wake (1981) that the male Mullerian gland of caecilians is a stage in evolution towards secretion of a seminal plasma, a requirement for internal fertilization in the context of a primitive form of terrestrialization, which in the mammals is taken care of by the prostate gland and seminal vesicles, is supported by the findings of the present paper. Tonutti (1931) for the first time suggested that the Mullerian gland might be a functional analog of mammalian prostate gland. Wake (1981) attempted to trace the evolution of a part of the mammalian prostate gland from the Mullerian duct. Studies on the protein profile of the secretion of Mullerian gland and the antigenic homogeneity of some of these proteins with secretory proteins of male accessory glands of amniotes, currently in progress, will throw light on evolutionary relationship between caecilian male Mullerian gland and the amniotic male accessory sex glands.

Physical Nature of the Secretory Material

An earlier paper (George et al., 2004) has shown that the microvillated secretory cells of the tubular glands of *Uraeotyphlus narayani* Mullerian gland secrete a material, which is released in the form of structured granules. Histological and ultrastructural evidences were also produced to show their dissolution or flocculation in the central duct of the gland. Curiously enough, granules that are not flocculated are always present amidst the flocculated material in the lumen of the duct. It led to a speculation that the
secretory material could still remain as granules at the time of ejaculation. The observations in the present study substantiate this hypothesis. Clear evidence of a portion of the secretory material maintaining its identity as structured granules even after mixing with the sperm has been obtained. There is a comparable situation prevailing in the lizards, snakes and turtles among the reptiles. It is known that in these reptiles the epithelium of epididymis secretes material in the form of structured granules, which mix with the sperm (Depeiges and Dufaure, 1977, 1981; 1983; Dufaure and Saint-Girons, 1984; Manimekalai and Akbarsha, 1992; Akbarsha and Manimekalai, 1999).

Interestingly, these reptiles lack prostate gland and seminal vesicles, which evolved for the first time only in the mammals, but possess an ampulla ductus deferentis (Akbarsha and Meeran, 1995; Daisy et al., 2000) and a renal sex segment (Bishop, 1959; Sarkar and Shivanandappa, 1989). Thus, internal fertilization being a requirement for terrestrialization, there have been different stages in evolution to provide a medium for sperm transport and nutritional support to the sperm for motility, which may be sequenced as follows:

1. The Mullerian gland secreting the substance in the form of discrete granules, which partly flocculate after discharge into the lumen, and partly remain granular, for suspending and supporting the sperm in the caecilians.

2. The ductus epididymidis secreting proteins/glycoproteins, partly solublized and partly as discrete granules, and the male accessory glands viz., the ampulla ductus deferentis and renal sex segment also secreting large amounts of mucoid substances and enzymes, all of which play vital roles in the initiation and sustenance of sperm motility in several reptiles.

3. The ductus epididymidis secreting proteins and glycoproteins in solublized form, thereby playing a critical role in the post-testicular physiological
maturation of sperm, contributing to initiation of sperm motility and acquisition of fertilizing ability, and the accessory glands such as prostate gland and seminal vesicles each secreting a fluidic substance with a variety of constituents for forming the medium of transport and for sustenance of sperm motility in the female tract in the case of mammals.

The packaging of the secretory material into granules in the caecilian is, perhaps, an adaptation for seasonal reproduction (Smita et al., 2003; George et al., 2004), and has a specific purpose of releasing their content in a phased manner commensurating with the requirement of proteins, sugar and enzymes during the storage and/or motility of sperm in the female reproductive tract. We have observed the presence of ameboid cells amongst the caecilian sperm earlier in tissue-fixed preparations (Smita et al., 2002, 2003). Their significance and origin will be discussed elsewhere.

Thus, the paper reports for the first time occurrence of a mitochondria vesicle/cytoplasmic droplet in the live sperm, collected from the testis, of a caecilian and qualifies the caecilian male Mullerian gland unambiguously to be a male accessory sex gland.

REFERENCES


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Table 1. Data on motility of spermatozoa of *U. narayani* with and without the male Mullerian gland substance. Values are M ± SD of three observations each.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Sperm motility</th>
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<tr>
<td></td>
<td>Duration (min)</td>
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<tr>
<td>1. Amphibian physiological saline alone.</td>
<td>45.28±3.81</td>
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<tr>
<td>2. Amphibian physiological saline + Mullerian gland extract.</td>
<td>600 (and beyond) * (in all 3x2 observations)</td>
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* *p*<0.001
FIGURE LEGENDS

Fig. 1a. Morphology of the sperm (ceased in motility) of U. narayani. As seen in eosin-nigrosine-stained preparation. AC, acrosome; NU, Nucleus; MP, Midpiece; FL, flagellum; UM, undulating membrane (x1000).

Fig. 1b. Eosin-stained sperm showing a prominent mitochondria vesicle (x 400).

Fig. 1c. Unstained sperm under phase-contrast illumination, showing the prominent acrosome and mitochondria vesicle. AC, acrosome; NU, Nucleus; MP, Midpiece (x 400).

Fig. 2 a-f. Unstained sperm of U. narayani under phase-contrast illumination, showing the mitochondria vesicle localized at different levels. In figure f the sperm had lost the mitochondria vesicle. Abbreviations, same as in figure 1a, except that the mitochondria vesicle is indicated by arrowhead (x 400).

Fig. 3. Unstained sperm under phase-contrast illumination, showing slow, forward progression. The photographs of the same sperm and the field were obtained at a gap of 15 sec. The loose spermatogenic cell (SG) is static and the sperm (SM) has made forward progression. Note the intact prominent mitochondria vesicle (arrowhead) (x 400).

Fig. 4 Unstained sperm under phase contrast illumination. Photographs are of the same sperm and field, each at a gap of 3 sec. The spermatogenic cells (SG) are static whereas the sperm (SM) has lashed the tail side-wise (x 400).

Fig. 5 An unstained mature sperm cyst (CY) under phase-contrast illumination showing lashing of the tail of the sperm (SM) (x 400).

Fig. 6 Unstained sperm (SM) under phase-contrast illumination. Maceration resulted in dissociation of sperm held together in two or more numbers. These are the photographs of a same duplex sperm exposed at a gap of 3 sec each demonstrating lashing of the flagellum. Note the duplex sperm retaining the mitochondria vesicle (arrowhead) (x 400).

Fig. 7a Unstained preparation of sperm mixed with the preparation of the Mullerian gland secretory material which are secretory granules. SG, spermatogenic cell; SM, sperm; arrowheads point to secretory granules of the Mullerian gland.
Fig. 7b Unstained preparation of secretory material of Mullerian gland alone showing the structured granular nature (x 400).

Fig. 8 Eosin-stained smear of sperm (SM) suspension mixed with the secretory material of the Mullerian gland. This having been prepared after a long standing, the structures of granules of Mullerian gland secretory material have considerably increased in size.

Figs. 9 a-c. Photomicrographs to illustrate the difference between immature germ cells (GC) and amoeboid cell (AM) a. eosin-nigrosine stained bright field illumination; b, c, unstained preparation under dark field illumination (x 400).