Contribution of the Secretory Material of Caecilian (Amphibia: Gymnophiona) Male Mullerian Gland to Motility of Sperm: A Study in *Uraeotyphlus narayani*

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ABSTRACT Caecilians are a unique group of limbless burrowing amphibians with discontinuous distribution. Several caecilian species are viviparous, and all practice internal fertilization. In amniotic vertebrates the sperm undergo post-testicular physiological maturation when they are initiated into motility under the influence of an epididymal secretion. Further, during ejaculation mammalian sperm are suspended in a fluid secreted by the male accessory sex glands, viz., prostate gland and seminal vesicles. Caecilians lack comparable glands, but still practice internal fertilization. Uniquely, male caecilians retain the Mullerian ducts in the adults as a pair of functional glands. It has long been hypothesized, based on indirect evidence, that the Mullerian gland would be a male accessory sex gland, secreting a fluid in which sperm are suspended during ejaculation and which would also provide nutritional support to the ejaculated sperm. In the present study, the secretory material of the Mullerian gland of *Uraeotyphlus narayani* was mixed with sperm obtained from the testis, and the changes in motility were recorded. *Uraeotyphlus narayani* sperm possess a perforatorium of the acrosome proceeding deep into the endonuclear canal of the nucleus. The midpiece is characterized by closely applied centrioles, the anterior ends of the axoneme and axial fiber, and a mitochondrial sheath. The long tail has an undulating membrane on one side, supported by the axoneme and an axial fiber. The live sperm possess a mitochondrial vesicle, also known as the cytoplasmic droplet, anywhere along the head and the midpiece, as in anuran sperm, which is shed from sperm that have ceased motility. *Uraeotyphlus narayani* sperm are motile the moment they are released directly from the testis, indicating that the sperm do not require post-testicular physiological maturation. On being mixed with the secretory material of the Mullerian gland, the spermatozoa are enhanced in speed as well as duration of motility. Therefore, the caecilian male Mullerian gland is considered to be the male accessory sex gland. J. Morphol. 263:227–237, 2005. © 2004 Wiley-Liss, Inc.

KEY WORDS: caecilian; sperm; Mullerian gland; male accessory sex gland

Caecilians are a unique group of subterranean or semi- to aquatic limbless amphibians with a variety of reproductive modes. At one extreme is the classical biphasic amphibian life cycle of oviparity with an aquatic larval stage, and at the other extreme is viviparity in which an aquatic phase in the life cycle is absent (Wake, 1977). All caecilians practice internal fertilization, making use of the eversible phallodeum as the phallus, wherein the spermatozoa are transferred directly into the reproductive tract of the female. Although many urodeles also practice internal fertilization, sperm packed as spermatophores are presented to the female and quiescently stored in spermatheca for a long duration and directly inseminated onto the egg at fertilization (Wake and Dickie, 1998; Onitake et al., 2000; Itoh et al., 2002; Watanabe et al., 2003). The sperm are motile within the spermatophore but there is little or no motility in spermatheca or vas deferens (Hardy and Dent, 1986). In the newt *Cynops pyrrhogaster*, the egg-jelly contains sperm motility-inducing substance (Ukita et al., 1999) and the sperm are initiated within 3 min (Ukita et al., 1999; Watanabe et al., 2003). Internal fertilization has also been recorded in a few anuran species (Sever et al., 2002), but little is known about the initiation of sperm motility in these species.

Caecilians, in which fertilization is internal, lack vas deferens and spermatheca, and the sperm are not delivered to the female packaged in spermatophores (Wake, 1977, 1981). Thus, the manner in which the sperm are transferred to the females in the caecilians is not known. Also, nothing is known about motility of sperm in caecilians and the factors that contribute to it. Uniquely, caecilians are known

Contract grant sponsor: University Grants Commission, Govt. of India; Contract grant number: F3-209/2001(SR-II) (to M.A.A.); Department of Science and Technology, Govt. of India, under the FIST Scheme (to M.A.A., O.V.O.).

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Published online 16 December 2004 in
Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/jmor.10300

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for retention of the Mullerian duct, the progenitor of the female duct, in adult males as a pair of functional glands (Tonutti, 1931; Wake, 1970, 1977, 1981; Exbrayat, 1985, 1986, 1992). The significance of this retention is to be sought in the elaboration of a secretory material, which is believed to play a role as a vehicle for transport of sperm and, perhaps, in providing nutritional support to the sperm in the context of internal fertilization (Wake, 1977, 1981). In *Uraeotyphlus narayani* we found that the urinogenital duct and the male Mullerian duct join to form a common duct before opening into the cloaca (George et al., 2004), strengthening the view that Mullerian gland secretions and sperm mix before ejaculation. Yet a role for the secretory material of the caecilian male Mullerian gland has remained only speculative.

Light microscopic observations of caecilian spermatozoa have been reported for several taxa (Seshachar, 1939, 1940, 1943, 1945; de Sa and Berois, 1986; Wake, 1994). Van der Horst and Van der Merwe (1991) and van der Horst et al. (1991) made electron microscopic observations of the sperm of *Typhlonectes natans.* Recently, Sheltinga et al. (2003) made ultrastructural analysis of the sperm of that and three other species of caecilians, viz., *Ichthyophis glutinosus, Ichthyophis beddomei,* and *Gegeneophis ramaswamii*. Smita et al. (2004a, b) described spermatogenesis, spermiogenesis, and ultrastructure of sperm of *Ichthyophis tricolor* and *Uraeotyphlus narayani*. According to these studies the caecilian sperm is filiform and, as seen with the light microscope, formed of a head, midpiece, and tail. The head consists of an acrosome and nucleus. Ultrastructural studies have shown that the acrosome is a complex composed of an acrosomal vesicle surrounding the dense acrosome rod, also known as the perforatorium. The nucleus has an endonuclear canal into which the perforatorium of the acrosome extends for most of its length. The midpiece consists of centrioles, axoneme, axial fiber, and a sheath of mitochondria. The tail consists of axoneme and axial fiber. While in the midpiece the axoneme and axial fiber are held closely together, beyond the midpiece the two are separated by an undulating membrane on one side (Van der Horst and Van der Merwe, 1991; Van der Horst et al., 1991; Jamieson, 1999; Scheltinga et al., 2003; Smita et al., 2004b).

All these studies on the morphology of caecilian sperm made use of testis lobes fixed in various fixatives. To the best of our knowledge, there has been no study observing live sperm of a 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 sperm during ejaculation, and also provides nutritional support thereafter. Therefore, the present study in *Uraeotyphlus narayani* was designed to test the following hypotheses: 1) in caecilians, as in the other vertebrate groups that practice internal fertilization, spermatozoa require initiation into motility subsequent to ejaculation into the female tract; 2) 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 3) the secretory material of the Mullerian gland would fulfill both these roles. In other words, we hypothesize that the Mullerian gland is a provision for internal fertilization in the context of a primitive form of terrestrialization, and it is the physiological forerunner of the epididymis and the male accessory reproductive glands of amniotic vertebrates. This study shows that sperm released in an amphibian physiological saline solution directly from the testis are motile and, therefore, the secretory material of the Mullerian gland does not play any role in the initiation of sperm motility, proving Hypothesis 1 in the negative. The study also shows that the secretory material of the Mullerian gland contributes to enhancing the speed as well as duration of motility of the spermatozoa, confirming Hypothesis 2 in full and Hypothesis 3 in part.

MATERIALS AND METHODS

Animal Collection and Maintenance

Male *Uraeotyphlus narayani,* weighing ~22–26 g, and measuring 20–27 cm, were chosen for the study. Animals were collected from the coconut groves and rubber plantations in and 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, Smita et al., 2003). In the laboratory, the animals were kept alive in moist soil and fed live earthworms. Five male animals were used in the investigation.

Light Microscopic and Experimental Studies With Sperm

The animals were sacrificed using MS222 and dissected immediately. The testis lobes and Mullerian glands were removed and washed 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 of 50 μl of the sample were made for observation in brightfield, darkfield, and phase-contrast illuminations in a Leica Axioscop 2 Plus research microscope (Leica, Germany). Pattern, speed, and duration of motility of the spermatozoa were observed and recorded by two independent observers.

The Mullerian gland on one side was washed thoroughly in the amphibian physiological saline solution and cut transversely into three or four pieces. The contents of the gland were released by running a needle over the tissue, applying mild pressure, into 1 ml of the saline solution, and thoroughly mixed. Ten μl volume of this preparation was added onto the hanging drop preparation of the sperm. An equal volume of the saline solution alone was added to the control. The differences in the pattern, speed, and duration of motility of spermatozoa were observed and recorded. For obtaining visual evidence for motility of the spermatozoa, keeping a nonmotile germ cell as the positional indicator, the same spermatozoa were photographed at 3–30-sec intervals.

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

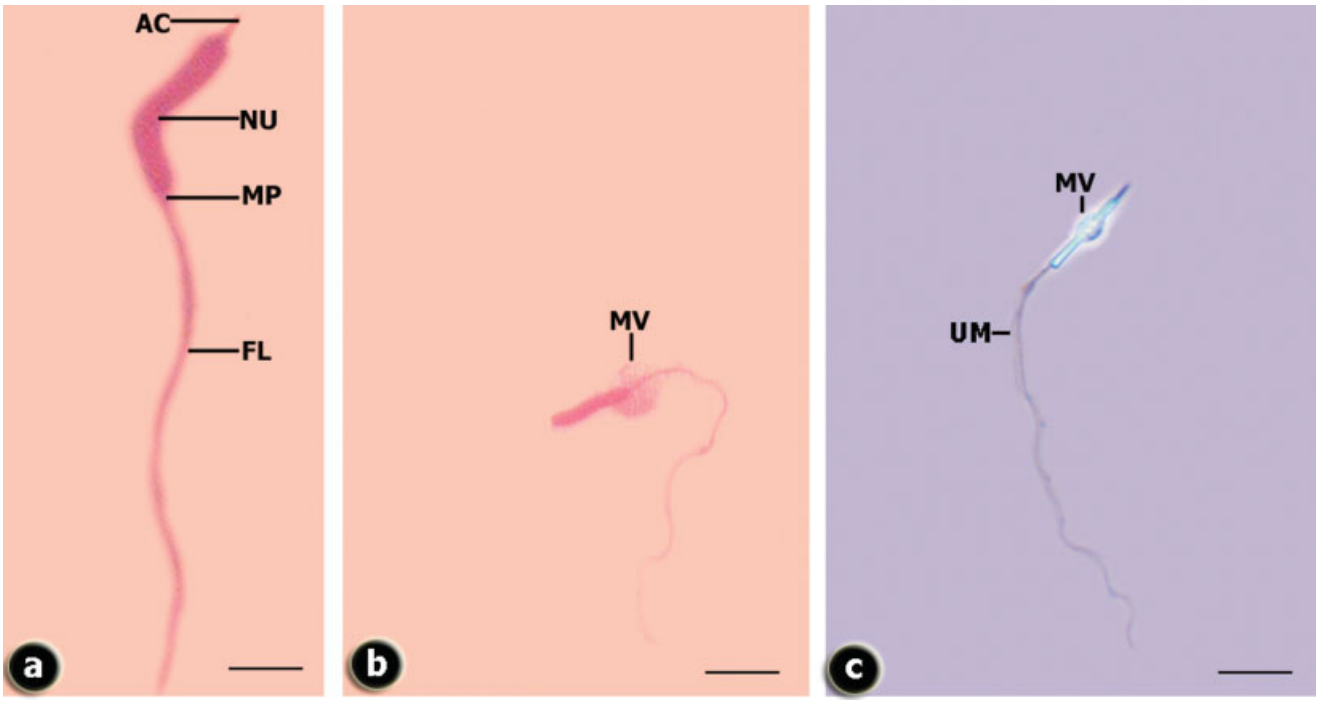

Fig. 1. **a:** Morphology of the sperm (nonmotile) of *Uraeotyphlus narayani* as seen in an eosin-nigrosine-stained preparation. **b:** Eosin-stained sperm of *U. narayani* showing a prominent mitochondrial vesicle. **c:** Unstained sperm of *U. narayani* in phase-contrast illumination, showing the prominent acrosome and mitochondrial vesicle. AC, acrosome; FL, flagellum; MP, midpiece; NU, nucleus; UM, undulating membrane. Scale bar in **a** = 9 μm; in **b**, 20 μm; in **c**, 15 μm.

granules present in the material of the Mullerian gland were captured using a Carl Zeiss Axioscop 2 Plus research microscope (Carl Zeiss, Germany) in a computer through a CCD camera (Sony, Japan). Measurements were made using Carl Zeiss Axiovision Image Analysis software. The data were used to calculate the means and the standard deviations (M ± SD) of length of the sperm, length of the acrosome anterior to the nucleus, length and diameter of the nucleus, length and diameter of the midpiece, length of the tail, width of the undulating membrane, and length and diameter of the mitochondrial vesicle (cytoplasmic droplet). Data on duration and rate of sperm motility were also treated thus, and a paired sample *t*-test was conducted between the respective control and experimental values.

RESULTS

Morphology of Sperm

The pyriform spermatozoa of *Uraeotyphlus narayani* are 104.54 ± 2.87 μm long from the tip of the acrosome to the tip of the tail. The acrosome vesicle anterior to 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 in diameter. The midpiece is 7.63 ± 1.15 μm long and 1.86 ± 0.39 μm in diameter. 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 observed (4.93 ± 0.64 μm dia; 5.81 ± 0.86 μm long), more frequently around the midpiece (Figs. 1, 2). The spermatozoa that were engaged in prolonged motility or ceased motility lacked this structure (Fig. 2).

Sperm Motility

From the moment they were released in amphibian physiological saline solution, the spermatozoa of *Uraeotyphlus narayani* were motile. Three kinds of motility were noticed: i) slow to rapid forward progression (Fig. 3); ii) side-wise lashing of the tail (Fig. 4); and iii) very rapid corkscrew-like movement of the flagellum, beginning from the midpiece and ending at the tip of the tail

Spermatozoa from cysts that were not spermiated also had flagella that exhibited a lashing movement (Fig. 5). Because in the study only the testis lobes were macerated to obtain sperm, in some cases sperm were found held together in pairs or larger aggregations, and in such sperm a violent flagellar beat was also 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 the preparation of the Mullerian gland substance are given in Table 1. The data reveal that with mixing of the Mullerian gland substance, the sperm are enhanced in speed as well as

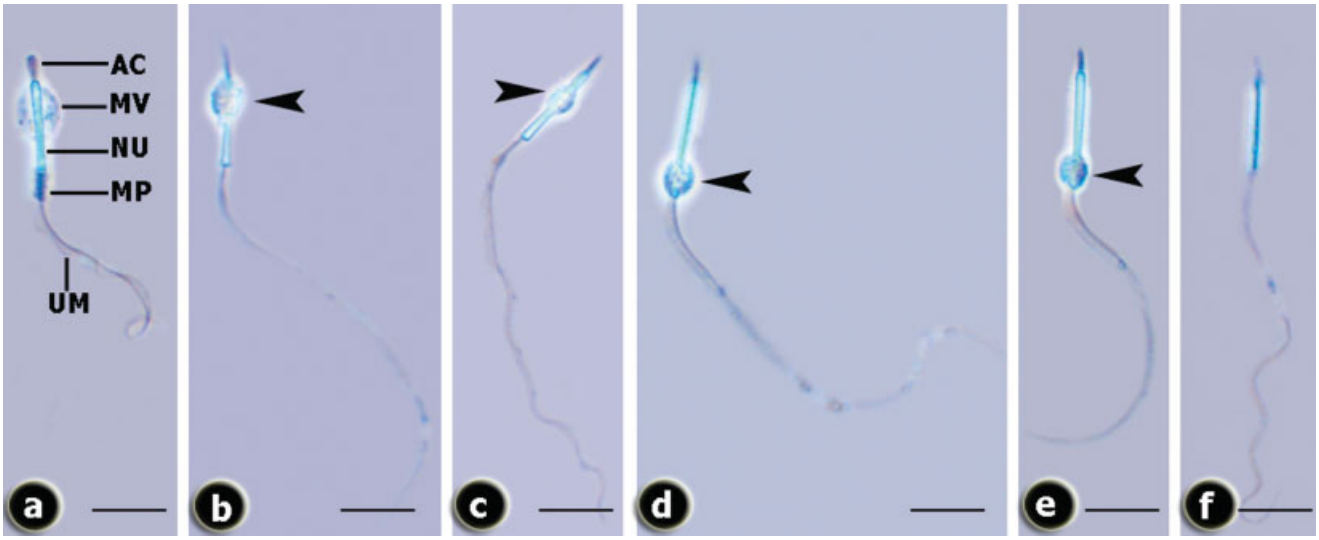

Fig. 2. **a–f:** Unstained sperm of *Uraeotyphlus narayani* in phase-contrast illumination, showing the mitochondrial vesicle localized at different levels. In **f**, the sperm has lost the mitochondrial vesicle. AC, acrosome; MP, midpiece; MV, arrowhead, mitochondrial vesicle; NU, nucleus; UM, undulating membrane. Scale bar = 15 μm.

duration of motility. In fact, in the sperm preparation to which the Mullerian gland substance was added, the spermatozoa remained motile more than 10 h (the period of close observation).

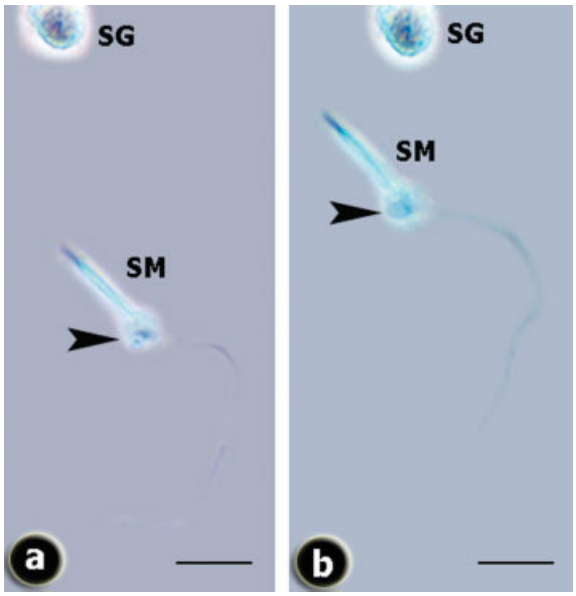

Fig. 3. Unstained sperm of *Uraeotyphlus narayani* in phase-contrast illumination, showing slow, forward progression. The photographs of the same sperm and the field were obtained at a time interval of 30 sec. The loose spermatogenic cell (SG) is static and the sperm (SM) has made forward progression. Note the intact prominent mitochondrial vesicle (arrowhead). Scale bar = 15 μm.

Nature of Secretory Material of Mullerian Gland

The moment the preparation of the Mullerian gland substance was added to the sperm suspension, the medium became turbid. Microscopic observation revealed the presence of spherical granules remaining suspended in the medium (Fig. 7). The granules increased in size, proportional 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 Among Spermatozoa

Because in the present study the spermatozoa were obtained by macerating lobes of the testis, mature spermatozoa that exhibited motility were found among immature germ cells (Fig. 9a,b). The latter were easily distinguished from cells that were unique in being extremely large, possessing blunt processes, containing a very large nucleus, and possessing cytoplasm containing minute granules (Fig. 9c). These are not germ cells. The blunt processes identify them as ameboid cells.

DISCUSSION

Morphology of Sperm

All descriptions so far of the structure of caecilian sperm are based on light or electron microscopic

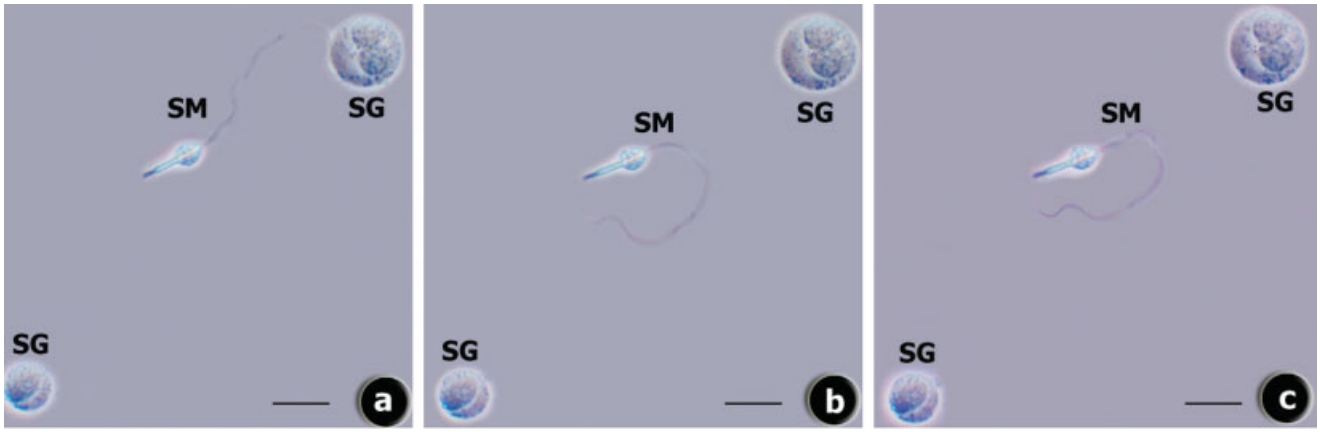

Fig. 4. Unstained sperm of *Uraeotyphlus narayani* in phase-contrast illumination. Photographs are of the same sperm and field, each at a time interval of 3 sec. The spermatogenic cells (SG) are static whereas the sperm (SM) has lashed the tail laterally. Scale bar = 20 μm.

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., 2004b). Thus, the present report is the first description of caecilian sperm based on observations of live sperm. The morphological characteristics of the spermatozoa of *Uraeotyphlus narayani,* with a spatulate acrosome, cylindrical nucleus, slightly ex-

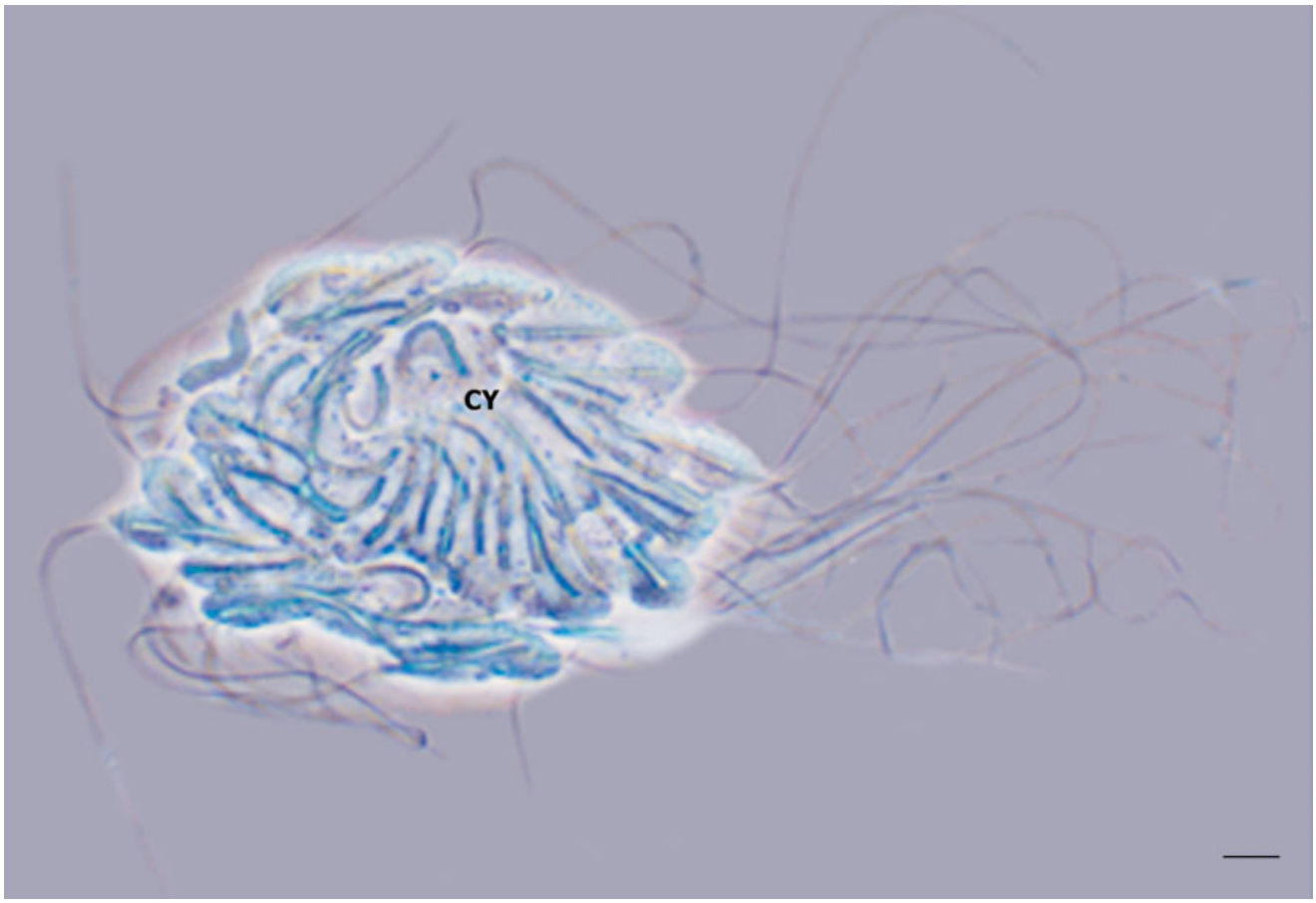

Fig. 5. An unstained mature sperm cyst (CY) of *Uraeotyphlus narayani* in phase-contrast illumination showing lashing of the tail of the sperm (SM). Scale bar = 12 μm.

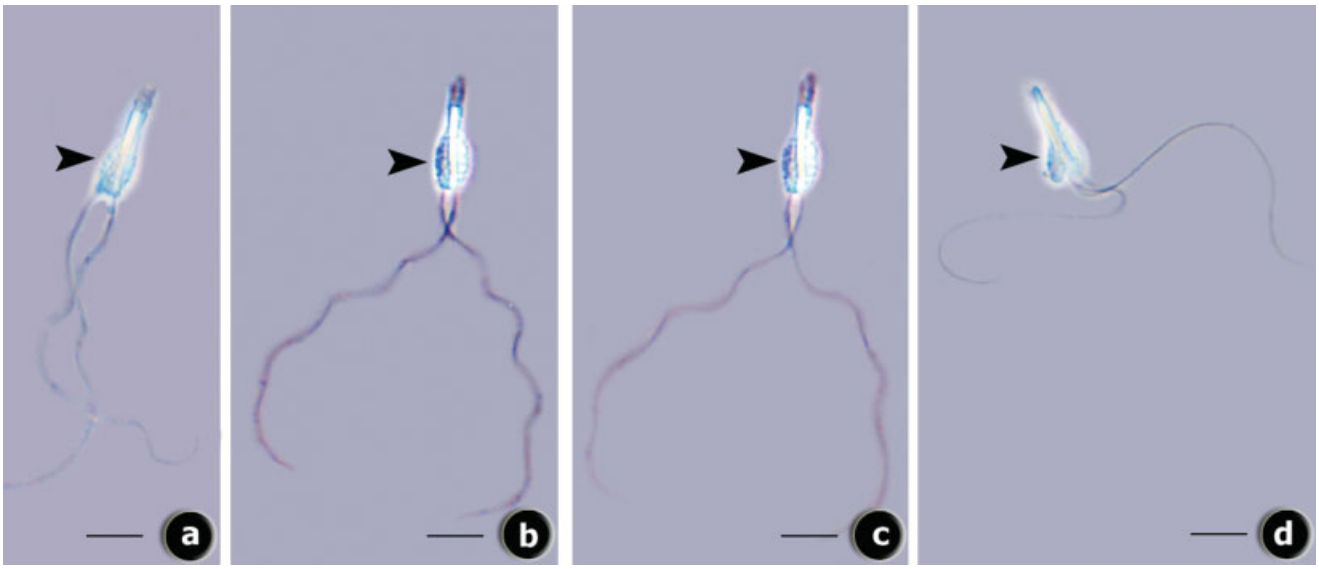

Fig. 6. Unstained sperm of *Uraeotyphlus narayani* in phase-contrast illumination. Maceration resulted in dissociation of sperm held together in pairs or larger aggregations. Photographs are of a duplex sperm exposed at 3-sec intervals, each demonstrating lashing of the flagellum. Note the duplex sperm retaining the mitochondrial vesicle (arrowhead). Scale bar = 12 μm.

panded midpiece, and long flagellum provided with an undulating membrane on one side of it conform by and large to the descriptions referred to above.

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, that occurs anywhere along the length of the head or the midpiece, depending on the spermatozoon examined. This structure is absent in the spermatozoa that had ceased motility. Scheltinga and Jamieson (2002a), in their review on the spermatozoon 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 midpiece. Lee and Jamieson (1992) reported the occurrence of such a droplet in the sperm of the frog *Myxophyes,* and, perhaps, *Rheobatrachus* also. As mentioned in the review of Scheltinga and Jamieson (2002b), in the testicular sperm of the leptodactylid anurans *Caudiverbera caudiverbera* and *Pleuroderma thaul* the mitochondria also 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 the internally fertilizing urodeles the droplet becomes detached in most of the spermatozoa lodged in the spermathecae. It has been suggested that the droplet is functional and could play a role in the maturation of spermatozoa (Scheltinga and Jamieson, 2002a, b). According to Kouba et al. (2003), the sperm of the toad *Bufo americanus* possesses a mitochondrial vesicle which in electron microscopy and fluorescent staining was confirmed to contain a large number of active mitochondria with very few other cytoplasmic structures. Nearly all spermatozoa exhibiting motility had an intact mitochondrial vesicle, and dissociation of this structure was related to motility loss. The sperm of this toad were inactivated when put in simplified amphibian Ringer's solution and this resulted in a reduced number of sperm with intact mitochondrial vesicles with the passage of time. The cytoplasmic droplet described in the review of Scheltinga and Jamieson (2002a, b), the mitochondrial vesicle reported by Kouba et al. (2003), and the vesicular structure reported here appear to be the same. According to Scheltinga and Jamieson (2002a,b) the cytoplasmic droplet is observed in the spermatids and often in the mature sperm of caecilians. However, to the best of our

TABLE 1. Motility of spermatozoa of Uraeotyphlus narayani *with and without the male Mullerian gland substance*

	Sperm motility	
Experimental condition	Duration (min)	Rate (μm/sec)
Amphibian physiological saline alone	45.28 ± 3.81	1.82 ± 0.21
Amphibian physiological saline + Mullerian gland secretory material	600 (and beyond)*	7.52 ± 0.47*

Values are M ± SD of three observations, each in duplicate.
*$P < 0.001$.

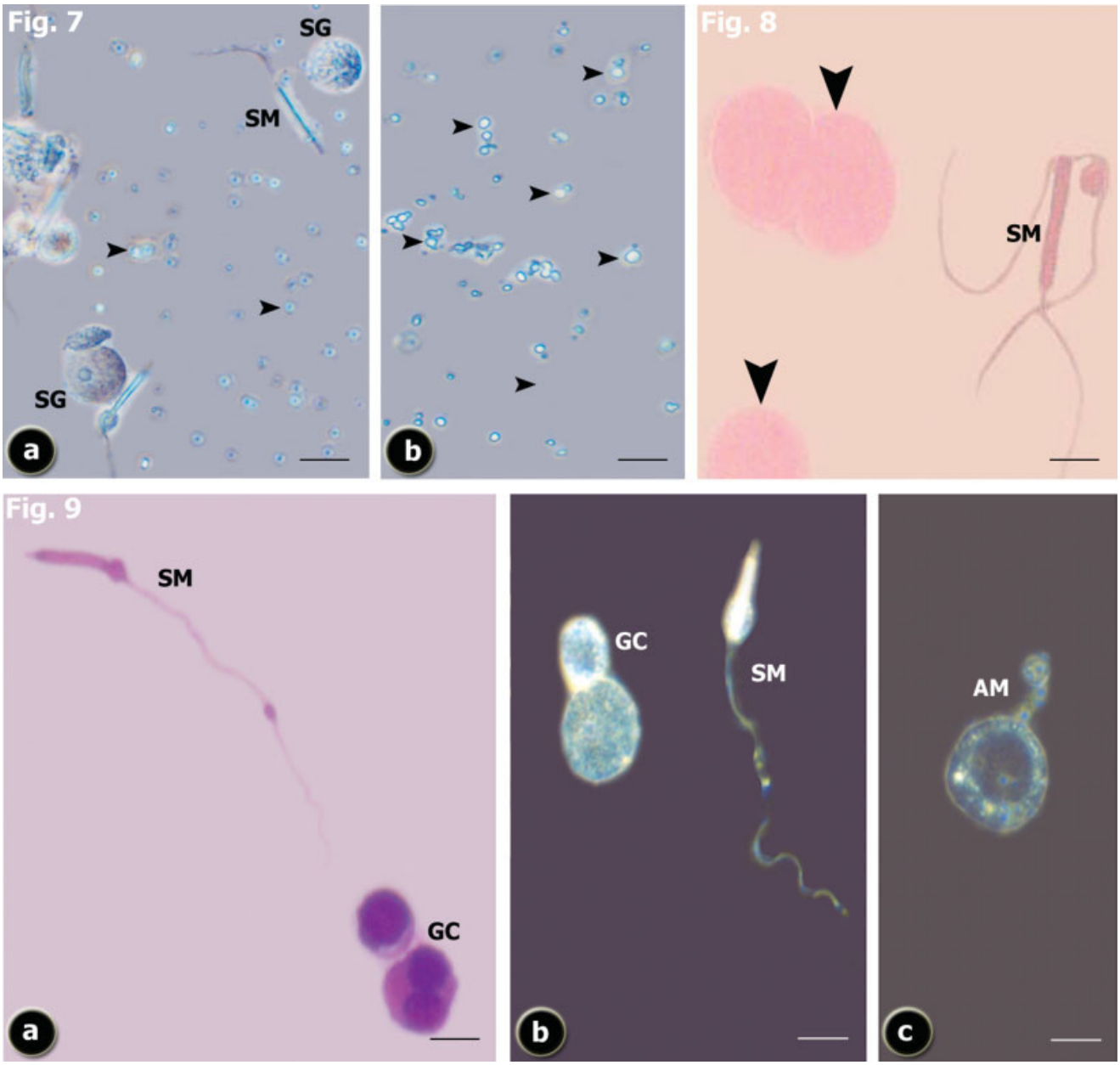

Fig. 7. **a:** Unstained preparation of sperm of *Uraeotyphlus narayani* mixed with the preparation of Mullerian gland secretory material which includes secretory granules. SG, spermatogenic cell; SM, sperm; arrowheads point to secretory granules of the Mullerian gland. **b:** Unstained preparation of secretory material of the Mullerian gland alone of *U. narayani* showing the granular nature. Scale bar = 20 μm.

Fig. 8. Eosin-stained smear of sperm (SM) suspension of *Uraeotyphlus narayani*, mixed with the secretory material of the Mullerian gland (arrowheads). Having been prepared long after extraction standing, the granules of the Mullerian gland secretory material have considerably increased in size. Scale bar = 10 μm.

Fig. 9. Photomicrographs to illustrate the difference between immature germ cells (GC) and ameboid cell (AM) present amidst *Uraeotyphlus narayani* sperm (SM) suspension. **a:** Eosin-nigrosine-stained, brightfield illumination. **b,c:** Unstained preparation, darkfield illumination. Scale bar = 20 μm.

knowledge there is no original report on the occurrence of the cytoplasmic droplet or mitochondrial vesicle in any caecilian species. Perhaps this structure in caecilian sperm does not show up in TEM-fixed specimens (B.G.M. Jamieson, pers. commun.). Thus, pending further study, it is proposed that *Uraeotyphlus narayani* sperm also possess a cytoplasmic droplet or mitochondrial vesicle, and that the structure is dissociated when the spermatozoa cease motility.

In 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 behind the head of the spermatozoa. Subsequently, it moves posteriorly along the midpiece until it reaches the point of annulus and then is shed when the spermatozoa leave the corpus epididymidis (Hermo et al., 1988). The cytoplasm of this droplet contains membrane-bound vesicles and lamellae, the origin of which is not yet definitely known, although it has been speculated that they are remnants of the Golgi apparatus (Oko et al., 1993). The occurrence of a cytoplasmic droplet in the sperm has also been reported in the painted turtle, *Chrysemys picta*. In turtles, the cytoplasmic 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 anurans and caecilians (Amphibia), it essentially consists of functional mitochondria (Scheltinga and Jamieson, 2002a,b; Kouba et al., 2003). In turtles (Reptilia) it contains mitochondrial fragments (Gist et al., 1992). In mammals, although the cytoplasmic droplet is still retained, there is no indication of derivation of any of its parts from mitochondria (Oko et al., 1993; Hermo et al., 1988).

Motility of Sperm Obtained From Testis

Our observations 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 anurans that practice external fertilization (Katagiri, 1987; Wake and Dickie, 1998). On the other hand, it is known that newts generally practice internal fertilization through spermatophores and the spermatozoa are released towards the surface of 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). In *Cynops pyrrhogaster,* sperm motility is induced by a factor in the egg-jelly that alters the osmolarity of the sperm (Watanabe et al., 2003). Thus, spermatozoa of *U. narayani* appear to differ in that they are initiated into motility by being prepared in an amphibian physiological saline solution with osmolarity 281 mOsm at pH 7.4. The observation of two or more spermatozoa held together and exhibiting motility indicates that the sperm acquire the capability to move even before spermiation. In this regard, spermatozoa of caecilians differ from those of amniotes because in the latter 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 Akbarsha, 1990). Thus, in caecilians, which lack a separate duct system to transport sperm, the latter do not appear to require a post-testicular maturation process, unlike in 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 three classes of amniotes, Reptilia (Depeiges and Dufaure, 1977, 1983; Depeiges and Dacheux, 1985; Shahul Hamid and Akbarsha, 1989; Manimekalai and Akbarsha, 1992), Aves (Birkhead, 1998), and Mammalia (Cooper, 1995, 1998).

Role of Mullerian Gland Secretion in Motility of Spermatozoa

Our data substantiate a role for the secretory material of the Mullerian gland of *Uraeotyphlus narayani* (and, perhaps, all caecilians) in sustenance of motility of spermatozoa. Tonutti (1931), for the first time, suggested that caecilian male Mullerian gland secretion formed a medium in which sperm would be suspended during ejaculation. It was further suggested that the secretory material, on being added to the sperm, would provide nutritional support for the latter for their motility (Wake, 1981). Our earlier observation in *U. 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 well 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), at least to the extent of the secretory material contributing to sustenance of sperm motility. Thus, we have found a definite role for the secretory material of the caecilian male Mullerian gland in sperm motility. The contribution of the male Mullerian gland secretion to this motility of sperm could involve more than one factor. Earlier studies have shown that the male Mullerian gland secretion is rich in proteins, glycoproteins, acid phosphatase, and a monosaccharide (Wake, 1981). Each of these seminal constituents contributes in one way or the other to sustenance of motility of spermatozoa in amniotes (Luke and Coffey, 1994). In mammals the prostate gland and seminal vesicles secrete these various substances, and are added to the sperm only at the time of ejaculation. Therefore, the hypothesis of Wake (1981) that the caecilian male Mullerian gland is an attempt 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 performed by the prostate gland and seminal vesicles, is supported by our data. Tonutti (1931) initially suggested that the Mullerian gland might be a functional analog of the 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 the 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 the evolutionary relationship between caecilian male Mullerian gland and amniotic male accessory sex glands.

Physical Nature of the Secretory Material

An earlier study (George et al., 2004) showed that the microvillated secretory cells of the tubular glands of the *Uraeotyphlus narayani* Mullerian gland secrete a material that is released in the form of structured granules. Histological and ultrastructural evidence showed their dissolution or flocculation in the central duct of the gland. Granules that are not flocculated are always present amidst the flocculated material in the lumen of the duct. This led to our speculation that the secretory material could still remain granular at ejaculation. The observations in the present study substantiate this hypothesis. We obtained evidence of a portion of the secretory material maintaining its identity as structured granules even after mixing with sperm. There is a comparable situation prevailing in lizards, snakes, and turtles. It is known that in these reptiles the epithelium of the 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). These reptiles lack a prostate gland and seminal vesicles, which evolved in 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, we infer that in the evolution of internal fertilization in vertebrates the medium for sperm transport and nutritional support to the sperm for motility appeared in the following sequence:

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 sperm in caecilians.
2. The ductus epididymidis secreting proteins/glycoproteins, partly solubilized 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 solubilized 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 the prostate gland and seminal vesicles, each secreting a fluid 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 caecilians is, perhaps, an adaptation for seasonal reproduction (Smita et al., 2003; George et al., 2004) and, if so, would have a specific purpose of releasing their content in a phased manner commensurate 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 among caecilian sperm earlier in tissue-fixed preparations (Smita et al., 2002, 2003). Their significance and origin will be discussed elsewhere.

Thus, we report 1) the occurrence of a mitochondrial vesicle or cytoplasmic droplet in the live sperm of a caecilian, and 2) evidence that qualifies the caecilian male Mullerian gland unambiguously to be a male accessory sex gland.

ACKNOWLEDGMENTS

We acknowledge the permission to make use of the ultracut and transmission electron microscope facilities of the Department of Gastro-intestinal Sciences, The Wellcome Trust Research Laboratory, Christian Medical College and Hospital, Vellore, India. We thank Dr. B.G.M. Jamieson, Professor Emeritus, Department of Zoology and Entomology, University of Queensland, Brisbane, Queensland 4072, Australia, for suggestions to improve the manuscript.

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