The morphology of the female reproductive structures of *Penonyx excavatus* (Oligochaeta)

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Perionyx excavatus (Oligochaeta) is a vermicomposting earthworm of which little is known regarding its reproductive strategies. This is a second paper by the authors on the morphology of the reproductive structures in an attempt to reconcile the scanty and confusing literature on this topic and to investigate the possibility of self-fertilization. The investigation was done by examining the gross anatomy and histology, using scanning and transmission electron microscopy. The female reproductive system consists of three pairs of spermathecae in segments seven, eight and nine. Each spermatheca consists of one ampulla and four sessile diverticula. The spermathecae open separately but midventrally in the intersegmental groove anterior to the segments in which they occur. One pair of ovaries is found in segment 13. The ova are released into the body cavity and are collected by the two ciliated ovum funnels, also in segment 13. The two short oviducts leading from the ovaries, join into a common oviduct. The common oviduct opens into the female opening, which is situated midventrally, anterior to the ring of body chaetae on segment 14.

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Perionyx excavatus, an earthworm belonging to the family Megascolecidae, is utilised in vermicomposting (Neuhauser, Loehr & Malecki 1988) since it is a very prolific epigeic species. It was initially described in 1872 by Perrier and is endemic to the southern regions of Asia (Gates 1972). Until recently not much was known about the biology of *P. excavatus*. Reinecke & Hallatt (1989) published information on growth and cocoon production and the life cycle was described by Hallatt, Reinecke & Viljoen (1990). Publications on the moisture requirements (Hallatt, Viljoen & Reinecke 1992) and the influence of worm density on cocoon production (Reinecke & Reinecke 1994) also appeared.

Studies on the biology of this species have shown that both paired worms and single specimens, hatched and maintained in isolation, produced viable cocoons. The question arose as to the strategy used by the isolated worms to enable them to produce offspring. One possibility, that of self-fertilization as postulated by Hallatt *et al.* (1990), was examined by studying the morphology of the reproductive organs. The aim was to determine whether any physical connections or other mechanisms might exist enabling sperm to reach the ova of the same worm internally, resulting in self-fertilization.

No comprehensive information regarding the female reproductive structures has been published and the descriptions presented by Beddard (1886, 1892), Edwards & Lofty (1972) and Hanumante (1975) are in some instances contradictory. Ovaries, ovum funnels and oviducts were not mentioned by Beddard (1886, 1892), Edwards & Lofty (1972) or Gates (1972) but were referred to by Hanumante (1975). There are also contradictions concerning the relative position of these organs in the earthworm body.

The morphology of the male reproductive structures has been described (Reinecke & Pieters 1997) and the present paper is concerned with the morphology of the female reproductive structures.

Material and methods

The specimens of *P. excavatus* used during the present study were from a breeding stock kept in the laboratory of the Department of Zoology at Potchefstroom University. This stock originated from cocoons provided by Prof. O. Graff of Braunschweig (Germany). The breeding stock was maintained in an environmental control chamber at 25°C and 80% r.h. in a cattle manure substrate.

To obtain adult worms, cocoons were collected from the breeding containers and incubated in distilled water at 25°C. One-day-old hatchlings were placed into 95 cm^3 glass flasks in groups of three to eight individuals per flask containing a cattle manure substrate. This substrate was prepared by grinding and sieving sun-dried, urine-free cattle droppings to obtain a particle size of 500 µm. This particle size was found to be favourable for the growth of *P. excavatus* (Reinecke & Hallatt 1989; Hallatt *et al.* 1990). Distilled water was added to obtain a moisture content of 80% because this is the moisture content at which these worms grow and reproduce best (Hallatt *et al.* 1990).

As soon as clitellate worms could be obtained, from the fourteenth day after hatching (Hallatt *et al.* 1990), 25 specimens were prepared for dissection to determine the position and number of reproductive organs and pores. Each worm was rinsed in distilled water, killed and fixed in 70% ethanol. The dissections were carried out dorsally using tungsten needles and studied under a Wild Heerbrugg M5A dissecting microscope.

That part of the body containing the reproductive organs was used for sectioning for light and electron microscopy. For

light microscopy the worms were rinsed in distilled water and the anterior third of the body, containing the reproductive organs, was fixed in aqueous Bouin's for 3 h (Humason 1979). For embedding in paraffin wax, samples were dehydrated in a graded ethanol series (50%; 70%; 90%; 100%) and placed into a 1:1 ethanol:chloroform mixture before transferring to 100% chloroform. Tissues were exposed to three consecutive paraffin wax baths of which the last one was exposed to a vacuum of 150 cm Hg, before embedding in Histosec. Longitudinal and transverse sections of 5 µm thickness were cut on a '820' Spencer microtome and floated onto slides pre-treated with poly-L-lysine (Huang, Gibson, Facer, Gu & Polak 1983).

Sections were stained with Mallory's trichrome stain (Humason 1979). The prepared slides were studied under a Leitz LS Laborlux S high magnification microscope and photomicrographs were taken by a Wild photographic system using FP4 film.

Material for SEM and TEM was fixed in Todd's fixative for 2 h (Todd 1986) and washed in cacodylate buffer (0.05M; pH = 7.4) twice for 10 min each, postfixed in 1% (w/v) aqueous osmium tetroxide for 1 h (Bullock 1984). The samples were washed in distilled water after which they were stained with 2% uranyl acetate (Tiedt, Jooste & Hamilton-Attwell 1987). The material was washed in distilled water and dehydrated in an acetone series (50%; 75%; 95%; 100%; 100%) for 20 min each.

For TEM studies the samples were placed in a 1:1 mixture of acetone and Spurr resin after dehydration (Spurr 1969), transferred to 100% resin and embedded in fresh Spurr's resin. A Reichert Ultracut E ultra microtome was used to cut sections of 100–130 nm thickness which were stained with 5% uranyl acetate and lead citrate (Reynolds 1963) and studied in a Philips CM 10 transmission electron microscope at 100 kV.

Material was also prepared for SEM to observe the external reproductive organs by critical point drying in liquid carbon dioxide, and after dehydration, mounting on stubs and coating with carbon. These samples were studied under a Cambridge Stereoscan 250 SEM.

Results and discussion

Number and position of female reproductive structures

Reinecke & Pieters (1997) diagrammatically represented the positions of all the reproductive structures (Refer: Figure 1A in Reinecke & Pieters 1997). One pair of ovaries and one pair of ovum funnels connected to one pair of short oviducts are found in segment 13. This confirms the findings of Hanumante (1975). In segment 14 the oviducts join to form a short common oviduct, ending in the single female reproductive opening. The latter is situated in the centre of the segment, ventral and directly anterior to the ring of body chaetae (Figure 1A). The position of the female aperture observed in this study supports the findings of Beddard (1886), and there is thus agreement that the female aperture of *P. excavatus* is on segment 14. All the worms investigated in the present study had a single, central female aperture, however Beddard (1886) reported the female aperture in different segments, and claimed that there were more than one. Each of segments 7, 8 and 9 contain one pair of spermathecae and one pair of spermathecal pores.

Morphology of the female reproductive system

For the sake of simplicity, the female organs and structures are described from anterior to posterior.

Ovaries

The ovaries of *P. excavatus* occur in segment 13, which is one segment removed from the last set of testes (in segment 11). This is the situation for earthworm families in general (Jamieson 1977; Stephenson 1930). The ovaries of *P. excavatus* are attached ventrally to both sides of the alimentary canal, and their narrower ends are directed towards the anterior septum of segment 13. Each ovary branches into numerous stalks with mature oocytes. This is in line with the findings of Hanumante (1975) and Stephenson (1930).

According to Hesse, as quoted by Stephenson (1930), the following three regions can be distinguished in earthworm ovaries, *viz.* (1) the primordial cells are limited to the area of the septum attachment, (2) posterior to the latter is a cell division region, and (3) a region which contains mature oocytes that lies posteriorly to the former one. These regions were also observed for *Fredericia, Lumbricillus, Tubifex* and *Limnodrilus* (Stephenson 1930). Hanumante (1975) described the free ends of the ovaries as lobes containing oocytes of different developing stages in a linear order. During the present histological studies the linear rows could not be distinguished. It was, however, possible to recognise that the oogonial cells occur nearest to the attachment and that the more mature stages occur further away.

Stephenson (1930) reported ovary sacs for some of the perichaete Megascolecidae, but no ovary sacs have been observed in subsequent studies on *P. excavatus* (Gates 1972; Hanumante 1975).

The ovum funnels, oviducts and female apertures

The ovum funnels of *P. excavatus* are in the same segment as the ovaries and this corresponds with the general position for ovum funnels of earthworms (Stephenson 1930). They are attached ventrally to the posterior septum of segment 13. Hanumante (1975) described them as saucer-shaped with ciliated edges. The ovum funnels are revealed by histological studies only (Figure 1B).

TEM micrographs showed that the ovum funnels of *P. excavatus* consist of a single layer of ciliated columnar epithelium. These cells are arranged with their ciliated ends pointing to the opening of the funnel and their nuclei are basally situated (Figure 1C). The cytoplasm of these cells clearly reveals endoplasmic reticula and mitochondria. A large number of Golgi complexes are present, as well as secretory vesicles (Figure 1D). An amorphous substance is also observed between the cilia.

The oviducts, originating in the funnels, pass through the posterior septum of segment 13 and unite as a common oviduct that opens midventrally on segment 14. The position of the opening coincides with what Stephenson (1930) described. Although the ovaries are posterior to the testes, the female opening is anterior to the male ones.

The oviducts of *P. excavatus* consist of a single layer of

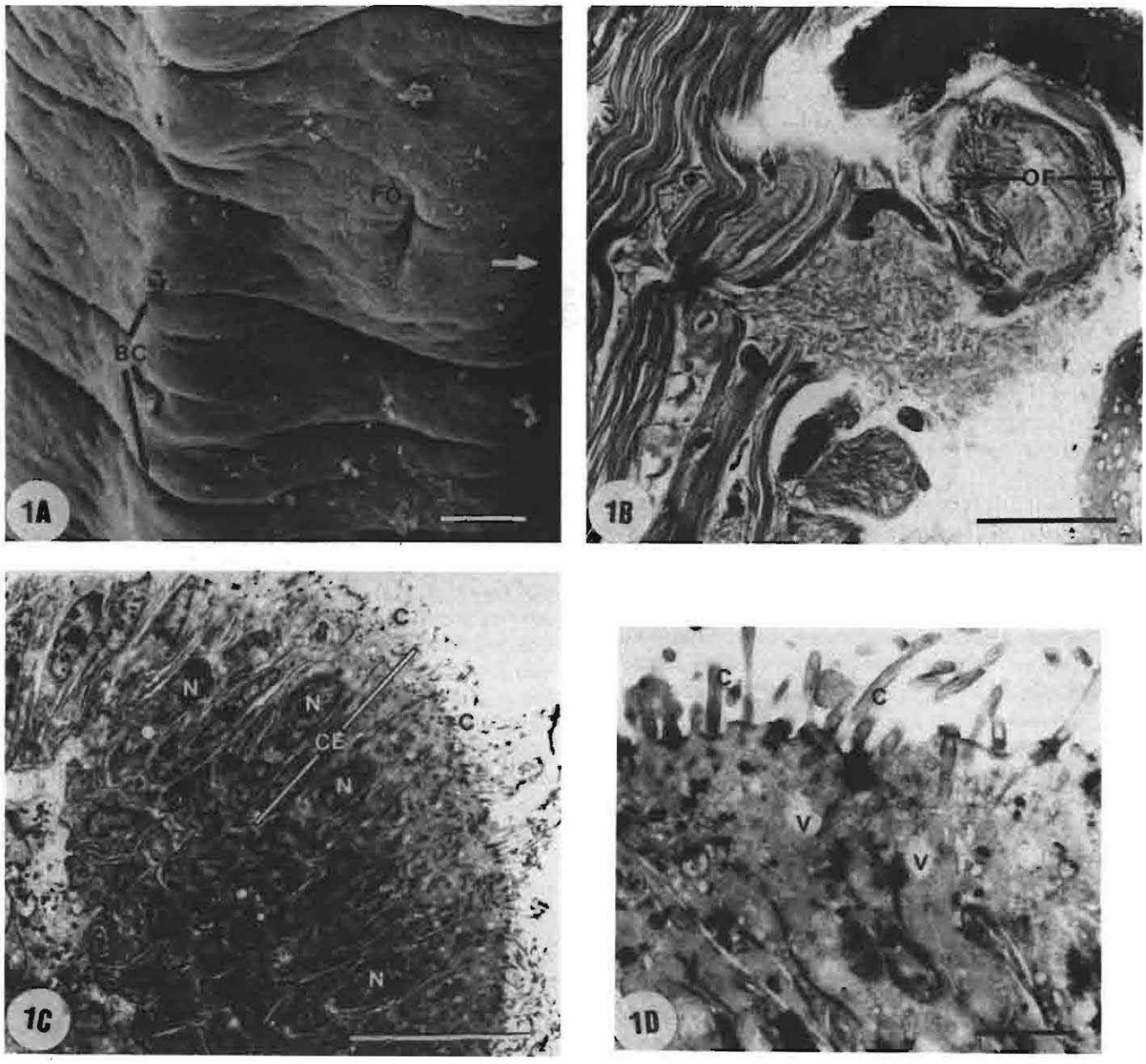

Figure 1 (A) TEM micrograph of ventral view of segment 14, showing the position of the female reproductive opening (FO), (BC = body chaetae); bar = 1.0 μm. **(B)** Light micrograph of longitudinal section through segment 14 showing an ovum funnel (OF); bar = 10.0 μm. **(C)** TEM micrograph of an ovum funnel displaying the ciliated columnar epithelial cells (CE) with nuclei (N), (C = cilia); bar = 10.0 μm. **(D)** TEM micrograph depicting vesicles (V) and cilia (C) in the ovum funnel; bar = 1.0 μm.

ciliated epithelium and this observation confirms that of Stephenson (1930). The epithelial layer is surrounded by connective tissue. Muscle fibres are present in close proximity to the connective tissue (Figure 2A). These muscle fibres may aid the cilia in transporting the ova.

Although the female opening is single (Figure 1A), sections showed that it is formed by the two separate oviducts which are joined together (Figure 2B).

Spermathecae and spermathecal pores

A pair of spermathecae are situated ventrally and on both sides of the digestive tract (Figure 2C), in each of three segments. Although Beddard (1886) described only two pairs of spermathecae Hanumante (1975) mentioned three pairs. The latter finding is supported by the present study.

Our findings as to the relative position of the spermathecal pores agree with those of Beddard (1886) and Gates (1972) who also found them to be situated midventrally. We observed the pores in the intersegmental fold (Figure 2D), anterior to the segment in which the spermathecae occur. Hanumante (1975), however, described the openings as ventrolateral. The spermathecae open close together but clearly separate from each other (Figure 2D). The spermathecal pores seen during this investigation have no cilia, chaetae or surface ornamentation.

Each opening gives rise to a small canal, the spermathecal duct. This duct widens into a large ampulla, as described by Stephenson (1930). At the base of the ampulla there are four

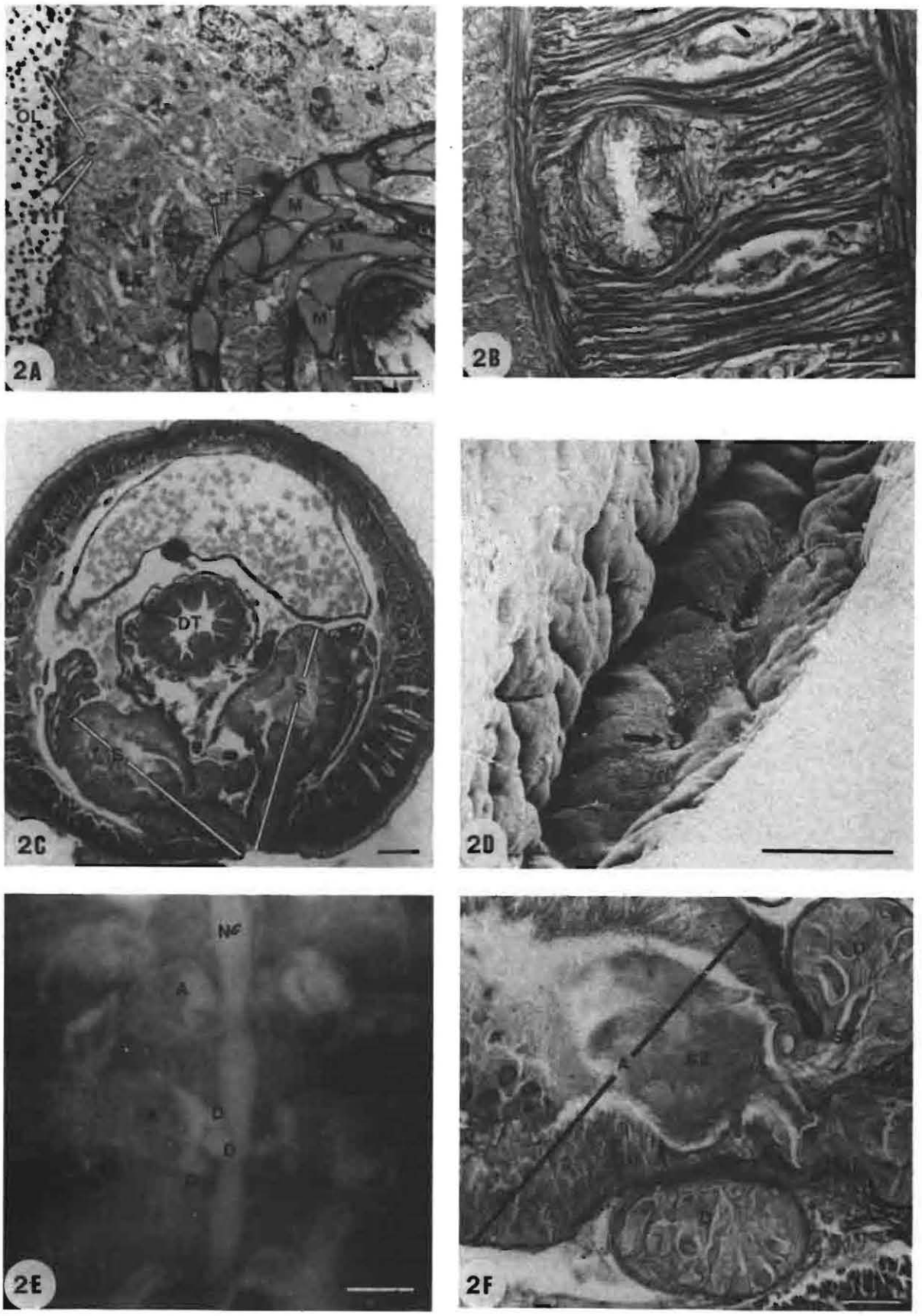

Figure 2 (A) TEM micrograph of oviduct wall of *P. excavatus* with lumen (OL) on the left, (CT = connective tissue; M= muscle fibres); bar = 2.0 μm. **(B)** Light micrograph of longitudinal section through female opening showing its double nature (↑); bar = 10.0 μm. **(C)** Light micrograph of cross section through a segment containing one pair of spermathecae (S), (DT = digestive tract); bar = 50.0 μm. **(D)** SEM micrograph of ventral side of an intersegmental groove showing the position of the spermathecal pores (↑); bar = 10.0 μm. **(E)** Light micrograph of dissected *P excavatus* specimen, showing the relative positions of the spermathecal diverticula (D) and spermathecal ampullae (A), (NC = ventral nerve cord). (The ampullae have been emptied to make the underlying diverticula more visible); bar = 10.0 μm. **(F)** Light micrograph of ampulla (A) and connected diverticula (D) of a spermatheca, (SZ = spermatozoa); bar = 2.0 μm.

diverticula (Figure 2E). According to Stephenson (1930) the presence of these diverticula is common for most members of the Megascolecidae, as well as many Enchytraeidae and a few Lumbricidae. Some diverticula may be simple sac-like evaginations attached directly to the base of the ampulla or they may be connected by a slender stalk. The diverticula of *P. excavatus* are without stalks (Figure 2F). Dissections of mated specimens of *P. excavatus* reveal these diverticula as white, glossy structures, clearly distinguishable from the dull surface of the ampullae.

The iridescent character is attributed to the presence of sperm exchanged by mating, and was only present in worms that were cultured in batches. It was never observed in worms confined singly. In histological sections sperm were also observed in the bases of the ampullae of mated worms (Figure 2F).

The walls of the spermathecal ducts consist of a stratified epithelium surrounded by connective tissue which is in agreement with Jamieson's (1981) findings. The cell layer bordering on the lumen has many microvilli (Figure 3A). A layer of amorphous substance is seen to occur between the microvilli. There are no secretory granules visible in the cells comprising the duct wall, but many vesicles are seen, and the membranes of some of them fuse with the apical cell membrane (Figure 3B). In contrast, epithelial cells of the spermathecal duct of *Tubifex tubifex* have distinct secretory granules (Jamieson

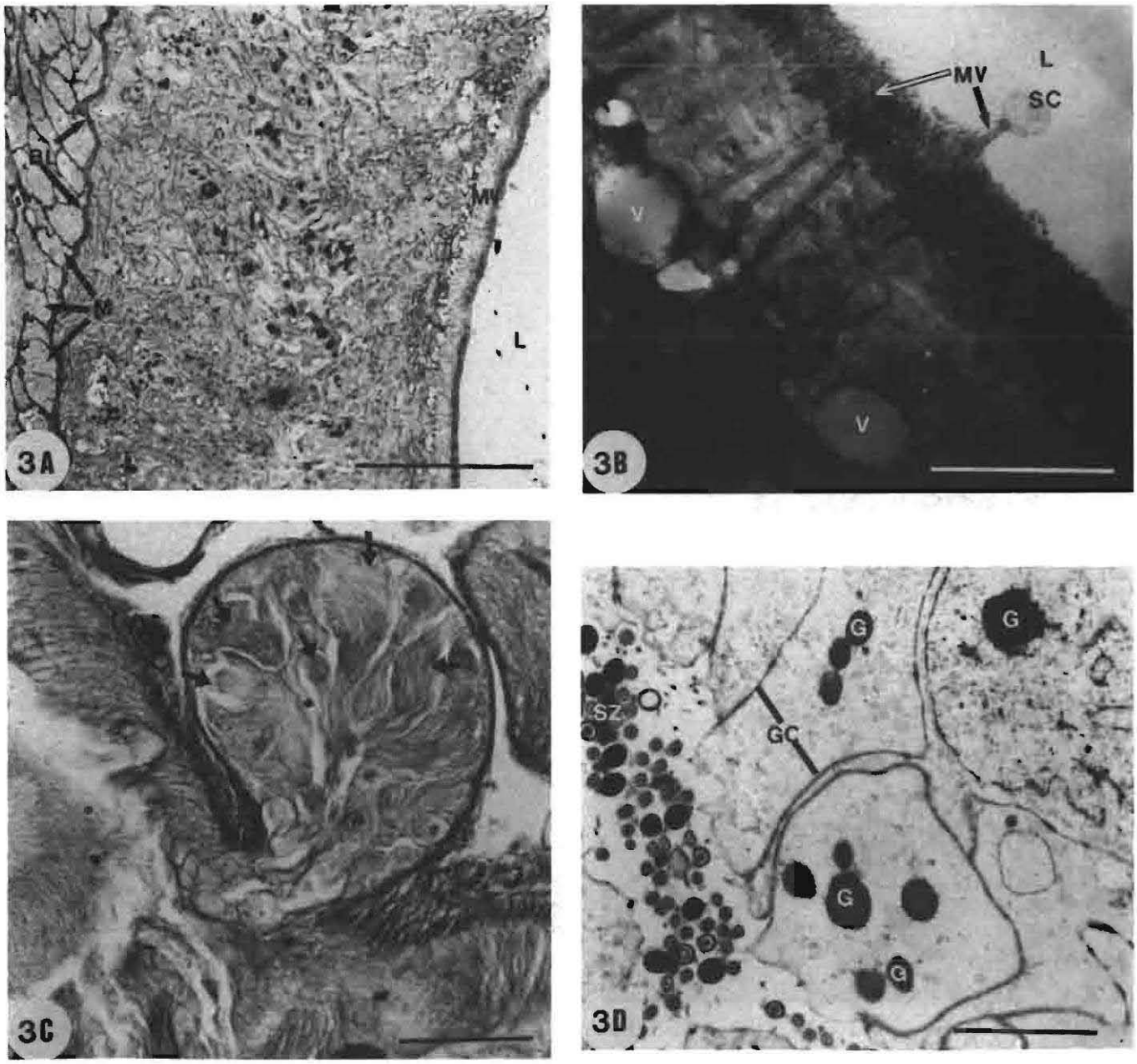

Figure 3 (A) TEM micrograph of longitudinal section through a spermathecal duct; bar = 10.0 μm. **(B)** TEM micrograph of the spermathecal duct, shown in (A) at higher magnification, showing villi and secretion in the lumen; bar = 1.0 μm. **(C)** Light micrograph of spermathecal diverticulum showing the slit-like spaces (↑) filled with spermatozoa; bar = 10.0 μm. **(D)** TEM micrograph of the contents of a spermathecal diverticulum; bar = 2.0 μm. (BL = basal lamina, G = granules, GC = granular cell, L = spermathecal duct lumen, M = muscle tissue, MV = microvilli, SC = secretion, SZ = spermatozoa, V = vescicles.)

1981). Surrounding the basal lamina of the spermathecal duct is a circular muscle layer probably responsible for the entrance and exit of the mating partner's sperm into and out of the spermatheca (Figure 3A). This muscle layer stretches from the spermathecal pore up to the ventral base of the ampulla.

Each diverticulum is spherically shaped and consists mainly of secretory cells. It has a fine canal system interspaced with atria (Figure 3C). Each atrium is filled with an amorphous substance of unknown origin, which may be a secretory product of the cells (Figure 3D). According to Stephenson (1930) the secretion has a nutritious function, since sperm are stored there until cocoon formation commences. Varute & More (1971) found that the mucous substance secreted by the glandular mucosa of the spermathecae of *Pheretima elongata* and *Haplochaetella powelli* contain glycogen and carboxyl-containing mucopolysaccharides, the former probably functioning as energy reserve for stored spermatozoa and the latter in preparing the necessary chemical environment for sperm storage.

The wall of the ampulla is lined with a stratified epithelium and consists of granular cells, which according to Stephenson (1930) have a secreting function. It seems that the mucus containing cells are of the apocrine secretory type. The ampulla is surrounded by connective tissue. The function of the ampulla, other than for additional storage of sperm, is unknown in *P. excavatus*. According to Richards & Fleming (1982) the epithelium of spermathecae of *Dendrobaena subrubicunda*, three *Allolobophora* species and *Lumbricus rubellus* phagocytoses the sperm, but no evidence of this could be found in *P. excavatus*.

Conclusion

During this study, several aspects concerning the female reproductive system of *P. excavatus* were elucidated. However, after investigating the female structures in this study, as well as the male organs (Reinecke & Pieters 1997), no morphological grounds could be found for self-fertilization.

The possibility that sperm cells could migrate through the septa into adjacent ova-containing segments and fertilise these ova before being deposited into cocoons cannot be ruled out. According to Stephenson (1930), the migration of sperm through the septa occurs in many members of the Eudrilidae. During the present study, however, no sperm cells were found in the diverticula of worms raised singly. The only other possible explanation for the fact that single individuals of this species form viable cocoons without having any contact with other individuals during their lifetime, could be parthenogenesis. This has been found for *Pheretima*-species, some Tubificidae, Enchytraeidae and Lumbricidae (Christensen 1980; Reynolds 1974) and needs to be studied further in *P. excavatus*.

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