The morphology of the male reproductive structures of *Perionyx excavatus* (Oligochaeta)

Sophié A. Reinecke
Department of Zoology, Private Box X1, Matieland, 7602 South Africa e-mail: sar@maties.sun.ac.za

*Rialet Pieters
Department of Zoology, Potchefstroom University for CHE, Potchefstroom, 2520 South Africa e-mail: drkrp@puknet.puk.ac.za

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Perionyx excavatus (Oligochaeta) is a vermicomposting earthworm of which the reproductive strategies are little known. In order to obtain more information on this, its reproductive systems were studied. Existing literature on the topic is either contradictory or scant. The investigation was done by examining the gross anatomy and histology, using scanning and transmission electron microscopy. The male reproductive system consists of two pairs of testes in segments 10 and 11 and two pairs of seminal vesicles in segments 11 and 12. In mature worms the developing spermatozoa are released by the testis into the body cavities of segments 10 and 11. The seminal vesicles in a particular segment receive developing spermatozoa from the lumen of the preceding segment. Two pairs of ciliated sperm funnels also occur in segments 10 and 11 and become the ciliated vasa deferentia carrying the sperm. The vasa deferentia on each side fuse to form only one vas deferens. These vasa deferentia reach the pair of prostatic glands in segment 18, each joining the prostatic duct and opening via the ventral male openings adjacent to each other on segment 18. Associated with the male openings are retractable penial chaetae. The cellular composition and organelle distribution of some organs and structures are also described. The positions of the female reproductive structures are illustrated diagrammatically in this paper.

* To whom correspondence should be addressed

Perionyx excavatus is a very prolific epigeic earthworm species, utilized successfully in vermicomposting (Neuhauser, Loehr & Malecki 1988). It was described by Perrier in 1872 and is endemic to the southern regions of Asia and Philippines. Until recently not much was known about the biology of this species. In 1990 the life cycle was described by Hallatt, Reinecke & Viljoen and information on growth and cocoon production (Reinecke & Hallatt 1989), moisture requirements (Hallatt, Viljoen & Reinecke 1992) and the influence of worm density on growth and cocoon production (Reinecke & Reinecke 1994) have also been published. A question concerning the reproductive strategies of *P. excavatus* originated during some of these studies (Hallatt *et al.* 1990), since both paired and worms kept singly had produced viable cocoons. It was not clear whether the viable cocoons produced by single worms were the result of parthenogenesis or of self-fertilisation. No comprehensive information regarding the reproductive structures of this worm has been published and the descriptions presented by Beddard (1886, 1892), Edwards & Lofty (1972), Gates (1972) and Hanumante (1975) are in some instances contradictory. Both Beddard (1892) and Hanumante (1975) described the presence of testis sacs while Gates (1972) did not refer to such structures. Neither Beddard (1886, 1892) nor Edwards & Lofty (1972) nor Gates (1972) referred to ovaria, ovum funnels or oviducts. Hanumante (1975), however, found such structures to be present. There are also contradictions concerning the distribution of these organs in the earthworm body. According to Beddard (1886) the spermathecae are found in segments 8 to 10 while Hanumante (1975) determined them to be in segments six, seven and eight. Beddard (1886) also described one spermatheca per segment while Hanumante (1975) referred to them as paired organs.

During the present study we attempted to elucidate the morphology and position of the reproductive organs and to determine whether the possibility of self-fertilisation exists as a reproductive strategy in the life-cycle of this earthworm species. In this paper the morphology of the male reproductive system will be discussed.

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 the 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% (dry weight basis) because this is the moisture at which these worms grow and reproduce best (Hallatt *et al.* 1990).

As soon as clitellate worms could be obtained, after about the fourteenth day after hatcing (Hallatt *et al.* 1990), some 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 tung-

sten 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, fixed in aqueous Bouin's for 3 h (Humason 1979). For embedding in paraffin, 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. They were exposed to three consecutive paraffin baths of which the last one was exposed to a vacuum of 150 cmHg. The paraffin Histosec (Merck) was used at a temperature of 56–58°C. Paraffin blocks, containing three samples each, were cast in paper moulds. Longitudinal and transverse sections of 5 µm thickness were cut on an '820' Spencer microtome and floated on slides pre-treated with poly-L-lysine (Huang, Gibson, Facer, Gu & Polak 1983).

The wax was dissolved by two submersions in xylene and the sections hydrated in a graded ethanol series (100%; 90%; 70%) before staining with Mallory's trichrome stain (Humason 1979). Differentiation of colours took place in 90% ethanol, dehydration in an ethanol graded series and elucidating in xylene. Cover slips were mounted with Entellan. 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, post-fixed in 4% 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 drying critically in liquid carbon dioxide, 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 all reproductive structures

The distribution and number of the reproductive structures are schematically presented in Figure 1A. The position of the reproductive structures are reported as follows:

segment 7–9: one pair of spermathecae and one pair of spermatheca apertures

segment 10: one pair of testes and one pair of sperm funnels

segment 11: one pair of testes, one pair of seminal vesicles and one pair of sperm funnels

segment 12: one pair of seminal vesicles

segment 13: one pair of ovaries, one pair of ovum funnels connected to one pair of short oviducts

segment 14: one common oviduct and one female reproductive opening

segment 18: one pair of prostate glands, one pair of male reproductive openings and associated penial chaetae

segment 10–18: one or two pair(s) vasa deferentia running through

During the present study the testes could not be detected by dissecting stereoscopically but evidence thereof was found during the histological studies. This study therefore showed that *P. excavatus* is holandric, which coincides with the statements of Edwards & Lofty (1972) that most species of the families Lumbricidae, Megascolecidae and Glossoscolecidae are holandric. Hanumante (1975) also found testes in segments 10 and 11.

Both Beddard (1892) and Hanumante (1975) referred to the presence of testis sacs, but no evidence of this could be found during the present study. Gates (1972) also did not mention testis sacs.

We found that *P. excavatus* has two pairs of seminal vesicles, one pair per segment. Both Hanumante (1975) and Gates (1972) also reported two pairs of seminal vesicles. Hanumante (1975) reported them to be in segments 10 and 11 and Gates (1972) in segments 11 and 12 with a protrusion of the pair in segment 12 into segment 13.

Hanumante (1975) referred to sperm funnels in *P. excavatus* and found them in the same segments as we did during the present study. He also reported only two pairs, one pair per segment. Each of these sperm funnels proceeds into a vas deferens which later joins to form only one vas deferens on each side.

The position of the single pair of prostatic glands as described by Gates (1972) is confirmed by the results of our study. Hanumante (1975), in contrast found the prostatic glands occurring from segment 16/17 up to segment 20/21.

Morphology of the male reproductive system

The male reproductive organs and structures are described as they are situated from anterior to posterior.

Testes

The testes were found ventral and directly posterior to the anterior septums of the different segments. The pair in segment 11 were therefore ventral to the seminal vesicles also included in that segment. No connection between the testis and seminal vesicle in the following segment was observed. The region that is described in this study as the testis, consists of homogenous cells of the same shape and size as compared to the heterogeneous cells in the seminal vesicle. The homogenous cells are presumed to be spermatogonia. According to

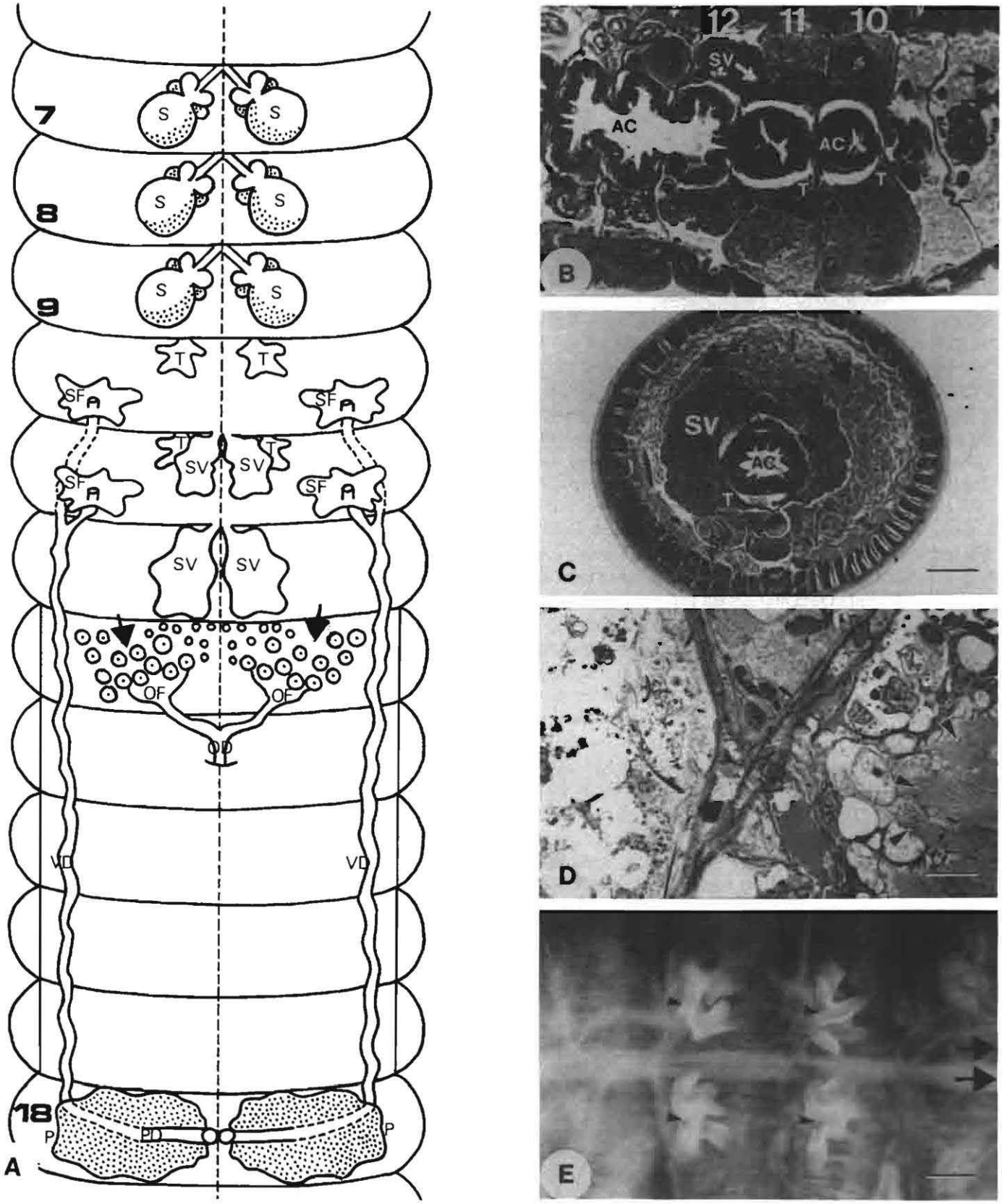

Figure 1 **(A)** A diagrammatic representation, not according to scale, of all the reproductive structures of *Perionyx excavatus*, no. 7–18 = segment numbers, arrows = ovaries. (B–E) TEM and light micrographs of *P. excavatus*. **(B)** A longitudinal section through the segments 10, 11 and 12 that contain the testes and seminal vesicles (arrow); bar = 70 μm. **(C)** A cross section through the centre of segment 11 containing the seminal vesicles; sperm funnels and developing sperm, arrow = developing sperm; bar = 70 μm. **(D)** A section through the wall of a seminal vesicle, arrow = muscle fibres; bar = 6.2 μm. **(E)** A section through segments 10 and 11 showing the sperm funnels, arrow = sperm funnels; double arrows = anterior of worm; bar = 8.6 μm. AC = alimentary canal, NC = nerve chord, OD = oviduct, OF = ovum funnel, P = prostatic gland, PD = prostatic duct, S = spermatheca, SF = sperm funnel, SV = seminal vesicle, T = testis, VD = vas deferens.

Hanumante (1975) the testes are ventral from the alimentary canal, on both sides of the nerve chord. The testes are attached posteriorly to the anterior septum of its segment. This concurs with the present study (Figure 1B & 1C). Hanumante (1975) described the testes as white, lobed organs with a compact base carrying four to eight arms of spermatogonial cells.

Both Beddard (1892) and Hanumante (1975) mentioned testis sacs for *P. excavatus*, but no testis sacs were present in the worms examined during the present study. Developing sperm released by the testes fill the entire body cavities of both segments 10 and 11 (Figure 1B & 1C). This is common for earthworms lacking testis sacs (Stephenson 1930).

Seminal vesicles

Edwards & Lofty (1972) described seminal vesicles as the largest and most conspicuous structures of the reproductive system of the Oligochaeta. According to these authors seminal vesicles do not occur in the same segment as the testes or testis sacs, but in the present study it was found that these structures do occur in the same segment. Compare segment 11 that contains one pair of testes as well as one pair of seminal vesicles.

The seminal vesicles of *P. excavatus* seem to be connective tissue proliferations of the anterior septa of the appropriate segments. Since these proliferations have a small opening to the preceding segments, they also contain developing sperm. This corresponds with statements by Stephenson (1930). These connections explain the presence of developing male reproductive cells in the second pair of seminal vesicles although that segment does not contain testes. The existence of comparting connective tissue in earthworm seminal vesicles that was mentioned by Edwards & Lofty (1972) was confirmed by this study. The individual compartments are connected to each other.

Stephenson (1930) advanced theories trying to explain how the spermatogonial cells from the body cavities reach the seminal vesicles. According to one theory the activity of the elastic muscle fibres in the wall of the seminal vesicle causes the spermatogonia to be sucked into the seminal vesicle. During the present study TEM work revealed that the muscle fibres form one of the three layers found in the walls (Figure 1D). On contraction these fibres could create pressure differences, sucking the spermatogonia from the testis into the seminal vesicles.

Sperm funnels

The sperm funnels of most earthworms are orientated in such a way that their narrow bases are attached to the posterior septum of the segment in which they occur, opposite the anteriorly situated testes (Stephenson 1930). This was also found to be the case for *P. excavatus* (Figure 1E).

The sperm funnels appear as white, iridescent rosettes with multiple folds surrounding a central opening leading to the vas deferens. The iridescence is caused by the presence of sperm, lying with their heads between the cilia of the funnel epithelium, arranged parallel to each other (Figure 2A).

The ciliary end of the cells in the funnel wall is richly supplied with mitochondria (Figure 2A). The opposite end contains many secretory granules. The secretions from these cells are collected in vesicles which open on the surface of the funnels (Figure 2B), between the cilia. According to Stephenson (1930) sperm funnels do not have a secretory function but in *P. excavatus* the ciliated epithelium was found to secrete.

Vasa deferentia

The male ducts or vasa deferentia are connected to the sperm funnels and run posteriorly. The vasa deferentia first proceed ventrally from the funnels until they reach the longitudinal muscle layer. From there they continue parallel to the ventral nerve chord, through the consecutive segments until they reach the prostate glands. The vasa deferentia of *P. excavatus* are convoluted but not as variously coiled as those of some of the Tubificidae and Enchytraeidae (Figure 2C) (Stephenson 1930).

The two male ducts of each side run in close proximity to each other, the one above the other, and then unite to form a single duct. According to Stephenson (1930) the fusion most commonly takes place in the twelfth segment of holandric species. Hanumante (1975) claimed that the vasa deferentia of *P. excavatus* on one side remain separate and open separately to the outside. We found that a single duct enters the prostate gland on each side.

The lumen of each duct is lined with ciliated epithelium. The cilia are long and directed posteriorly. The distal ends of the epithelial cells are similar to the cells of the sperm funnels, richly supplied with mitochondria (Figure 2D). This probably indicates active involvement of the cilia in the transportation of sperm. These cells do not contain any secretory granules and any secretions that may be present originate from the sperm funnels.

The vas deferens of each side enters the wall of the prostate gland where the prostatic duct leaves the gland (ventrally), runs close to the prostatic duct, and opens into the lumen. Throughout its passage in the wall of the prostate gland, the vas deferens seems to be completely isolated from the tissue of the prostate glands.

Prostatic glands

The two prostatic glands in segment 18 are situated laterally to the alimentary canal, and are attached to the ventral side via the prostatic ducts (Figure 2E). The fact that the glands are confined to one segment contradicts Hanumante's (1975) findings of glands stretching from segment 16/17 to segment 20/21. According to Hanumante (1975) they consist of a large glandular region and a smaller non-glandular region but during our study we found that they only consist of glandular tissue.

According to Stephenson (1930) representatives of the Megascolecidae have one of two types of prostatic glands: tubular or lobular. The tubular gland looks like a duct, repeatedly folded, and the whole structure has a central lumen. The lobular gland consists of lobules, each with its own small duct. The classification of the prostatic glands of *P. excavatus* as lobular or tubular seems to depend on the magnification of the microscope. Under a light microscope the glands appear rich in canals with many compartments. TEM studies revealed that the compartments are filled with glandular cells. Different glandular cells are alternatively arranged around a central opening (Figure 3A) making these glands probably

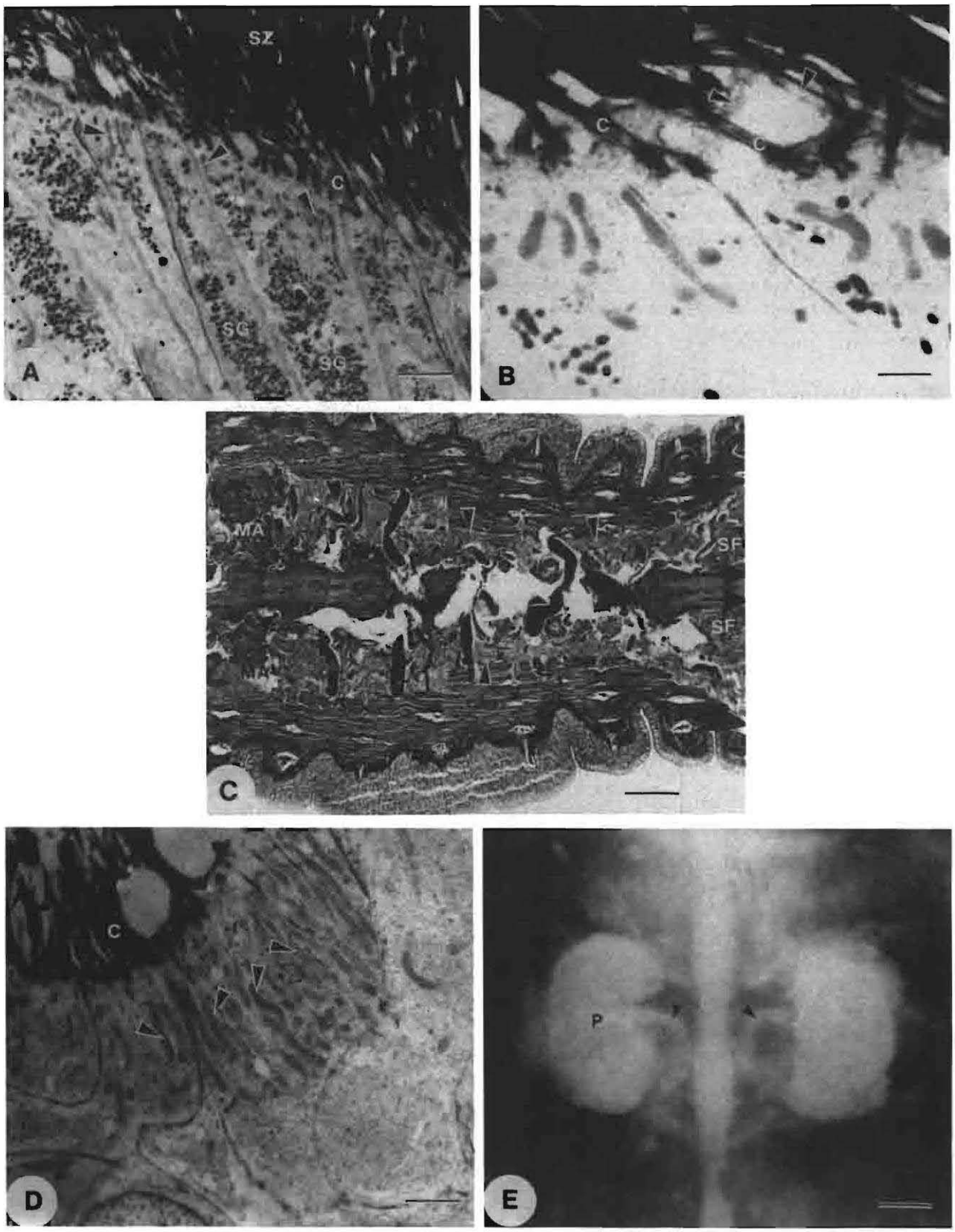

Figure 2 TEM and light micrographs of *P excavatus*. **(A)** The free-end of the sperm funnel wall, arrow = mitochondria; bar = 1.9 µm. **(B)** A higher magnification of the free-end of the sperm funnel, arrow = secretion; bar = 0.5 µm. **(C)** A longitudinal section through the worm to indicate the path of the vasa deferentia (arrow); bar = 70 µm. **(D)** A cross section through a vas deferens showing the tissue surrounding the lumen, arrow = mitochondria; bar = 1.1 µm. **(E)** A section through segment 18 to show the prostatic glands, arrow = prostatic duct; bar = 7.6 µm, C = cilia, MA = male aperture, P = prostatic gland, SF = sperm funnel, SG = secretory granules, SZ = spermatozoa, double arrow = anterior.

lobular. The different cells forming a lobule are not defined by a surrounding membrane nor are the canaliculi lined with epithelium or cilia (Figure 3A).

Three different kinds of cells can be distinguished in the

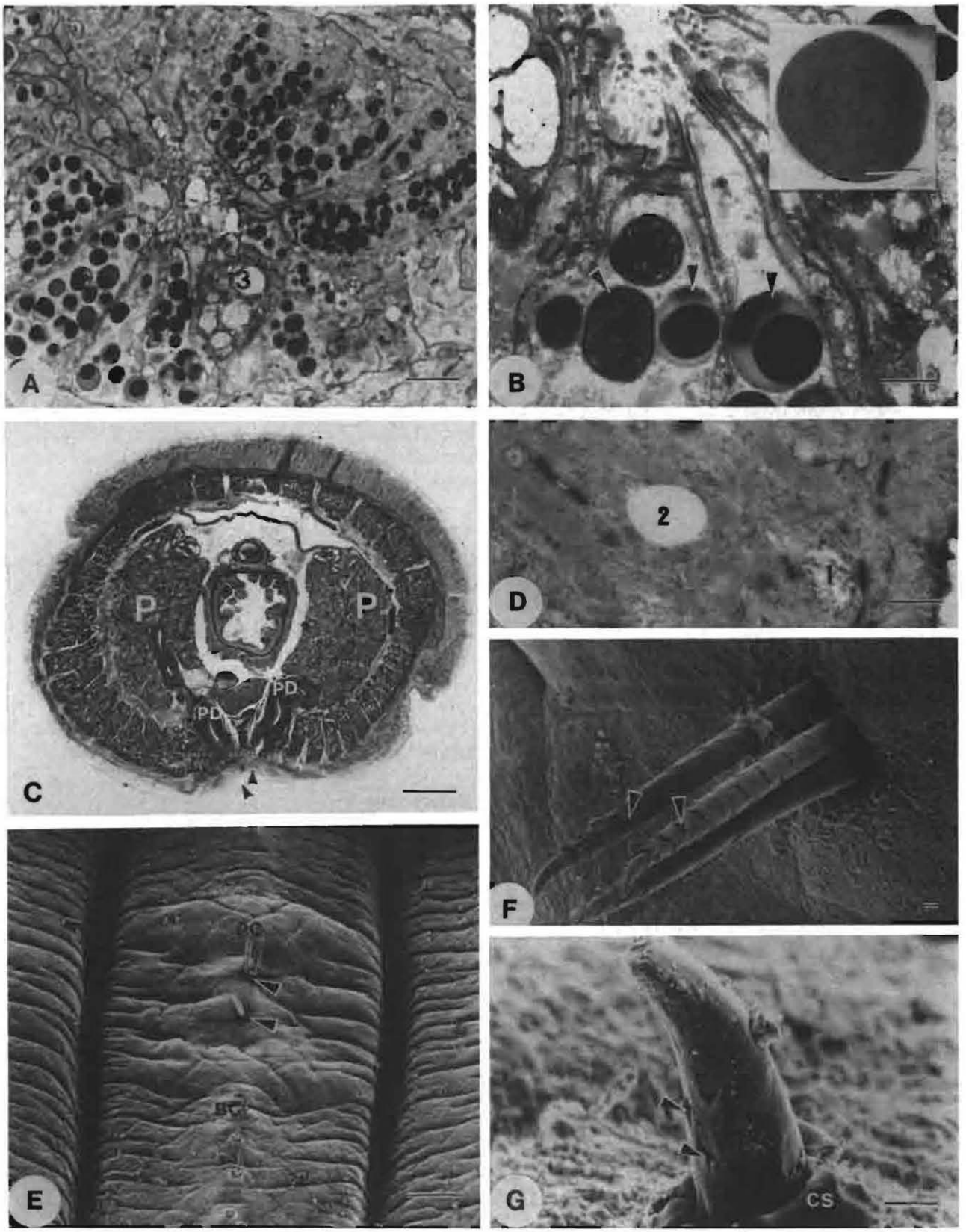

Figure 3 TEM, SEM and light micrographs of *P. excavatus*. **(A)** One of the lobules found in the prostatic glands, 1 = homogeneous glandula cell, 2 = heterogeneous glandular cell, 3 = glandular cells filled with vesicles only; bar = 1.9 µm. **(B)** A higher magnification of the heteroge neous glandular cell of the prostatic glands, arrow = granule; bar = 0.5 µm. The insert shows a close-up of the concentric circles of the gran ules of such a heterogeneous cell; bar = 0.2 µm. **(C)** A cross section through segment 18 to show the two prostatic glands (P), the prostati ducts (PD) as well as the associated male openings (arrow); bar = 70 µm. **(D)** The two types of vesicles, granular (1) and electron-lucid (2) found in the wall of the prostatic ducts; bar = 0.5 µm. **(E)** Segment 18 with external male apertures and associated penial chaetae (PC) on th tumefaction (arrow) and body chaetae (BC). **(F)** A higher magnification of the penial chaetae, arrow = combs; bar = 3.6 µm. **(G)** One of th conical shaped body chaetae displaying its combs (arrow) and collar structure (CS); bar = 0.9 µm.

lobules of the prostatic gland. One of them could be classified as heterogeneous and another as homogeneous in reference to Jamieson's (1981) description of the glandular cells of *T. tubifex* (Figure 3A). The homogeneous glandular cell contains smooth electron-dense granules. In cross sections of the granules of the heterogeneous glandular cells concentric circles can be observed (Figure 3B inset). The pattern depends on the orientation of these cells because sometimes a scribble pattern is observed (Figure 3B). It is possible that these granules are structurally the same as Golgi apparatus and that the scribble pattern is a section through the vesicles and the concentric pattern a section through an enveloping canal system (one tube fitting into another). The gland cells have nuclei. The third kind of cell found in the prostatic gland is less numerous than the other two kinds. These cells seem to be filled with vesicles whilst organelles such as endoplasmic reticulum and nuclei are absent. Muscle fibres occur between the lobules which might explain the manner in which the secretion reaches the canaliculi and flows further.

According to Stephenson (1930) secretions of the prostatic gland probably act as nutrients for the sperm as well as a lubricant for the prostatic duct through which the sperm leaves the body.

The prostate duct originates from the central region of the gland (Figure 3C), travels for a short distance in the direction of the alimentary canal, turns ventrally and opens midventrally on the body wall. The duct wall consists of a layer of cells similar to cylindrical epithelium. Seen in cross section these cells simulate the spokes of a bicycle wheel with a central lumen. The microvilli of the cell membranes line the lumen and the nuclei are peripheral. A large number of vesicles, probably produced by the Golgi apparatus (Jamieson 1981), occur in the duct. Some appear electron-lucid and may contain lipids (Figure 3D).

The male apertures and the associated penial chaetae

The male openings are midventral on segment 18. They are separate but close together on a tumefaction or papillae (Figure 3E). The individuals that were studied under the SEM had lateral and/or transverse grooves clearly defining the tumefaction and not only transverse grooves as mentioned by Gates (1972). Beddard (1886 & 1892) described papillae in an oval-shaped indentation, but none of these specifications could be observed during the present study.

The chaetae associated with the male apertures appear to be longer and thinner than the conical shaped body chaetae (Figures 3E & 3F). Beddard (1892) coined the phrase 'penial chaetae' which differs from 'copulatory chaetae' in that, although the latter are also modified chaetae, they are not associated with the male aperture.

The penial chaetae of *P. excavatus* protrude through the male apertures. The openings are in line with the perichaetal somatic chaetae but the chaetal ring is interrupted on both sides of the tumefaction. No more than three (sometimes fewer) penial chaetae per male aperture were found during our study in comparison to four to nine found by Gates (1972) and five to six found by Beddard (1892). Both male apertures on the same individual do not necessarily have the same number of penial chaetae (Figure 3E).

The length of the penial chaetae varied from 0.4 to 0.8 mm which compares well with lengths reported by Gates (1972). However, the diameter at the base (15–25 μm) differs greatly from the 6.45 μm reported by Gates (1972). The ornamentation on the penial chaetae can be described as comb-like with teeth facing away from the earthworm body. The somatic chaetae also have these little combs (Figure 3F) but they seem to be less sharp. The somatic chaetae protrude through a collar structure bordering the openings while a similar structure is absent from the penial chaetae (Figure 3F & 3G).

The penial chaetae of *P. excavatus* are protrusible which is proven by the different stages of evagination during fixation. It could be possible that all the penial chaetae associated with one male aperture act in unison forming a canal between them through which sperm can be transported. Ismail (1982) reported a single chaeta with three lobes with such a mechanism for *Lampito mauritii*. The comb-like structures on the penial chaetae of *P. excavatus* could facilitate the transport of sperm. Since no mucous bands are formed during copulation and no clasping structures are present in *P. excavatus*, it is possible that the sperm could be transported in these proposed ways.

Conclusion

The aims of this study were to clear up the contradictory information concerning the reproductive structures of *P. excavatus* and whether self-fertilisation is possible for this species. The above information is clear on the morphology and structure of specifically the male reproductive system, but can not give a verdict on the possibility of self-fertilisation. Theoretically self-fertilisation is structurally possible and we could not find any structural barriers preventing self-fertilisation. Further studies are needed in this regard.

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