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Reproductive system and spermatozoa of *Paraturbanella teissieri* (Gastrotricha, Macrodasyida): implications for sperm transfer modality in Turbanellidae

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Abstract The morphology of the reproductive apparatus of several species of Turbanellidae, which are sequential hermaphrodites, has been studied for a comparison with that of other Gastrotricha Macrodasyida, which are simultaneous hermaphrodites. The common structural plan of the genital system of Turbanellidae includes two testes which extend into two sperm ducts turning anteriorly and fusing in a midventral pore, two ovaries with oocytes maturing in a cephalic direction and only one accessory organ, a seminal receptacle, provided with an external pore. A possible sperm transfer modality alternative to that described in the literature is advanced. Spermatological characters of *Paraturbanella teissieri* have been compared with those of the two *Turbanella* species studied up to date. Turbanellidae share with other Macrodasyida the general model of spermatozoon, but are the only representatives of this taxon known so far which have sperm devoid of the striated cylinder around the axoneme. Both the structure of the reproductive apparatus and the fine morphology of the spermatozoa of Turbanellidae species agree with the evolutionary view, recently supported by morphological and molecular data, which puts this taxon on a separate clade, early divergent from the stem lineage of Macrodasyida.

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Introduction

Within Gastrotricha, Macrodasyida and Chaetonotida strongly differ in morphology, reproductive biology and ecology (Balsamo 1992). Macrodasyida, almost exclusively marine, have a complex sexual apparatus of various morphology, likely related to their hermaphroditism and internal fertilisation, whereas Chaetonotida, mostly freshwater dwelling, are mainly parthenogenic with a very simple genital system (but see Weiss 2001). Macrodasyida are basically protandric hermaphrodite, their testes maturing before the ovaries, but then they become simultaneous hermaphrodites with spermatozoa and oocytes maturing at the same time. Two accessory sexual structures, called the frontal and the caudal organ, are generally present with the function of seminal receptacle and copulatory organ, respectively; they are supposed to be part of the primitive plan of the genital system of Macrodasyida (Ruppert and Shaw 1977).

Turbanellidae and Dactylopodolidae are an exception among Macrodasyida, since at least *Paraturbanella* and *Turbanella* species in the first taxon and *Dactylopodola* species in the second one alternate male and female phases (Teuchert 1968). This sexual condition is probably shared with the species of the other four genera of Turbanellidae, for which, however, data are insufficient or even absent. Current knowledge about the genital system and the reproductive biology of Turbanellidae mainly consists of the detailed ultrastructural studies by Teuchert (1968, 1975, 1976, 1977) on *Turbanella cornuta* Remane, 1924. This species has two simple, elongated testes, lateral to the anterior intestine and peculiar for turning anteriorly and fusing in a single midventral pore, and two ovaries lying at the sides of the caudal gut oocytes maturing in a cephalic direction. Teuchert (1977) described also two accessory structures, the so-called ‘gland organ of the intestinal region’, adjacent to the fully grown egg and having a possible secretory function, and an oblong, dorsal organ next to the posterior end. The latter was interpreted by Teuchert as a seminal receptacle, from which spermatozoa should migrate through the so-called "oviduct" (actually a tissue strand) towards the mature oocyte. However, Ruppert (1991) reinterpreted the anatomy of *T. cornuta* suggesting that the anterior organ actually functions as a seminal receptacle whereas the caudal structure is a copulatory organ. Thus he hypothesised a sperm transfer modality similar to that known for *Macrodasys* sp., which fills its caudal organ with spermatozoa collected from the male pore by means of sharp, ventral flexions of the body (Ruppert 1978). Maguire (1976), reporting the sporadic presence of putative 'spermatophores' in mature individuals of *Turbanella varians* Maguire, 1976, suggested that these spermatophores transfer sperm from the testes of the male individual to the sac-like organ interpreted as a 'bursa' of the female individual. The few, scattered observations available on some other species of Turbanellidae are in line with the structural plan of *T. cornuta* (see Remane 1927; Clausen 1965, 1996, 2000; Maguire 1976; Kisielewski 1987; Evans and Hummon 1991; Ruppert 1991; Evans 1992). Nothing is known about the genital system of *Pseudoturbanella stylifera* d'Hondt, 1968.

The morphology and the ultrastructure of the filiform, flagellate spermatozoa of *T. cornuta* (see Teuchert 1975, 1976) and of *T. ambronensis* Remane, 1943 (see Ferraguti and Balsamo 1995) fit well the model of Macrodasyida sperm (a long, narrow, and helicoidal acrosome composed of two recognisable regions, a coiled nucleus wound around a mitochondrial axis and a flagellum with a conventional 9+2 axoneme enclosed by a striated cylinder), except for the absence of the striated cylinder around the axoneme (Ferraguti and Balsamo 1994). No ultrastructural data are available for spermatozoa of any other species of Turbanellidae.

In this paper we describe in detail the genital system of *Paraturbanella teissieri* Swedmark, 1954, and give new information about other three *Paraturbanella* species as well as of an additional *Turbanella* species. We discuss the basic plan of the genital apparatus of Turbanellidae in the light of these new data and propose an alternative, possible, sperm transfer modality in the family. The ultrastructure of the spermatozoon of *P. teissieri* is described and compared with that of the sperm of *Turbanella* species.

Materials and methods

Sexually mature specimens of three species of *Paraturbanella* (*P. dohrni* Remane, 1927, *P. pallida* Luporini, Magagnini and Tongiorgi, 1973, and *P. teissieri*) and two of *Turbanella* (*T. cornuta* and *T. ambronensis*) were extracted by decantation and narcotisation with $MgCl_2$ 7% aqueous solution from sandy sediments collected during 2000 and 2001 along the Tyrrhenian and the Adriatic coasts (Italy), while specimens of a fourth species, *P. aggregotubulata* Evans, 1992, were obtained from sand samples taken at Central Padre Island (Texas, USA).The analysis of specimens was performed in vivo by means of a Leitz Dialux 20 microscope equipped with Nomarski interference contrast. Locations of the principal morphological characters along the body length are reported in percentage units (U), measured from the anterior to the posterior end. Specimens were prepared for TEM study by fixation in a 0.1 M phosphate-buffered (pH 7.2) solution of paraformaldehyde, glutaraldehyde and picric acid, with added sucrose (SPAFG, Ermak and Eakin 1976), postfixation in 2% aqueous osmium tetroxide followed by washing in 0.1 M phosphate buffer, dehydration in a graded acetone series, prestaining in uranyl acetate in acetone 70% and embedding in Araldite. Sections were cut with an LKB Ultratome Nova, double stained with alcoholic uranyl acetate and lead citrate, and observed with a Philips 300 TEM. Several 1 µm sections were stained with toluidine blue and observed under an Olympus Vanox optical microscope. For SEM analysis specimens were fixed in 10% borax-buffered formalin, dehydrated in a graded ethanol series and critically point dried with CO_2. Stubs were coated with gold-palladium and observed with a Philips XL 40 SEM.

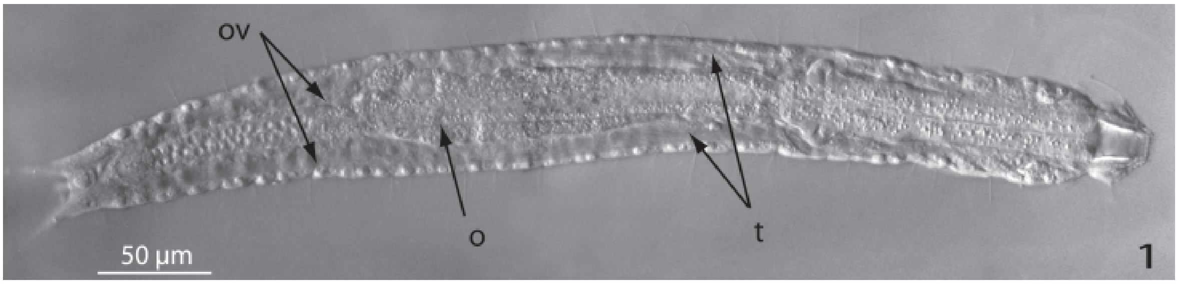
Results

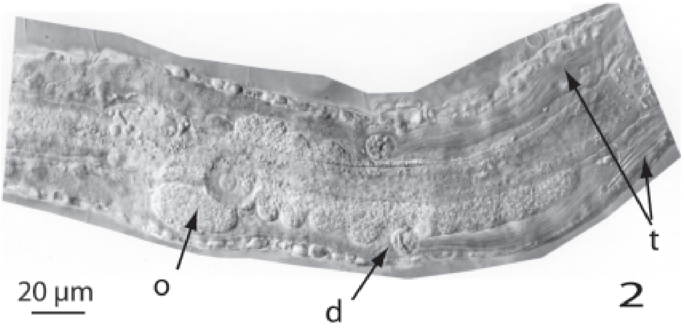
Reproductive system

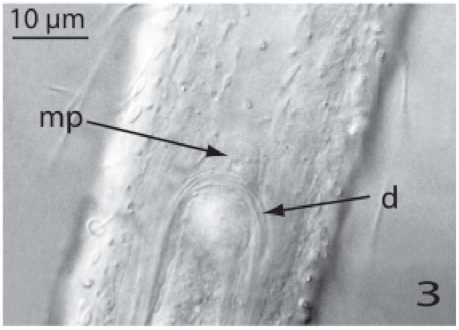
The following description refers to *P. teissieri*, unless otherwise specified. Two club-shaped elongate testes lie laterally to the anterior intestine, beginning just below the pharyngointestinal junction (U38) and extending up to U46, where they gradually narrow into two sperm ducts. A small, refringent, rounded structure ('cap-cell' *sensu* Teuchert 1968) is visible under the optical microscope in the foremost part of each testis (Figs. 1, 2). A cellular sheath surrounds each male gonad extending also to the proximal part of the sperm duct. The germinal epithelium is a narrow cellular band which lies dorsolaterally along the whole length of the testis lumen, and gradually becomes thinner and lateral. Successive stages of spermatogenesis follow each other from the caudal portion of each testis towards its anterior end where spermatozoa complete their maturation in a centripetal direction, gather in the testis lumen and gradually shift into the sperm duct, all with the head oriented towards the posterior body end. During the male phase bundles of motionless spermatozoa, parallel to each other, are clearly visible within the sperm ducts; these abruptly bend anteriorly (U52) and join together into a common midventral pore (U40), which is often hardly visible even under Nomarski optics and is surrounded by a glandular muff (Fig. 3). The SEM analysis of sexually mature specimens of *T. cornuta* has shown that the male pore looks quite different in morphology during each sexual phase; it is evident and surrounded by a protruding cuticular ring in the male phase, whereas it is a barely visible, transversal slit in the female phase (Fig. 4a, b).

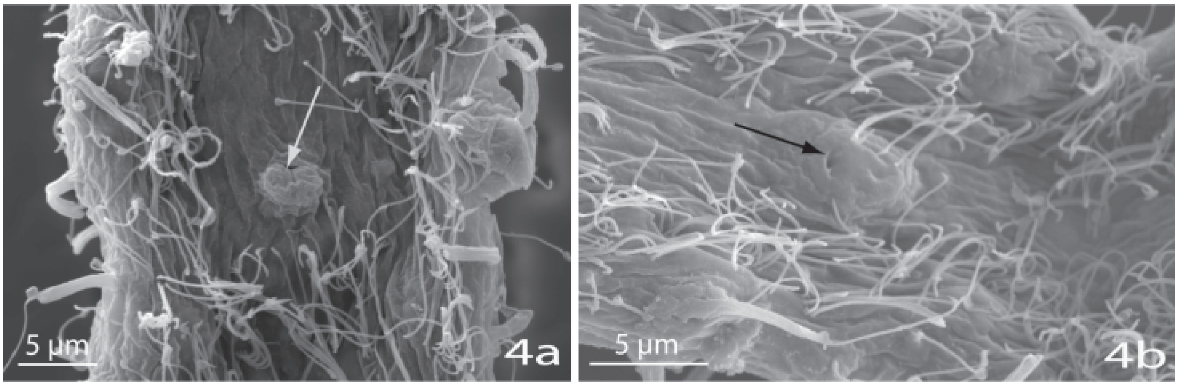
In mature female specimens of *P. teissieri* two ovaries lie along the sides of the caudal intestine, extending anteriorly from U84 to U64. Since oocytes mature one at a time in a cephalic direction from each ovary alternately, the anteriormost, full-grown oocyte is usually visible during the female phase, gradually shifting dorsally and extending from U68 up to U56 (Figs. 1, 2). No distinct cellular sheath covering the ovaries, nor an oviduct and a female genital pore are present.

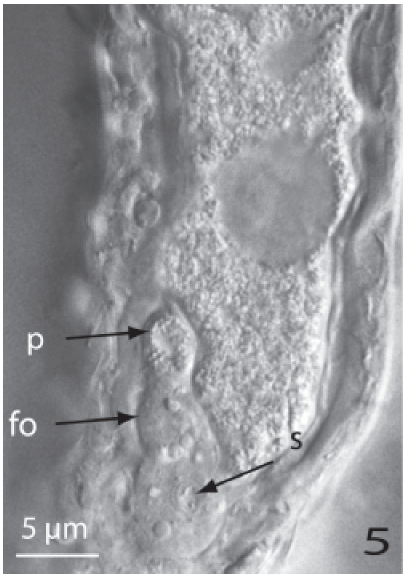
An elongate, pear-shaped organ ('frontal organ'), 43–56 µm long, lies dorsally to the intestine from about U56 to U64 (Fig. 5) and appears to be formed just at the

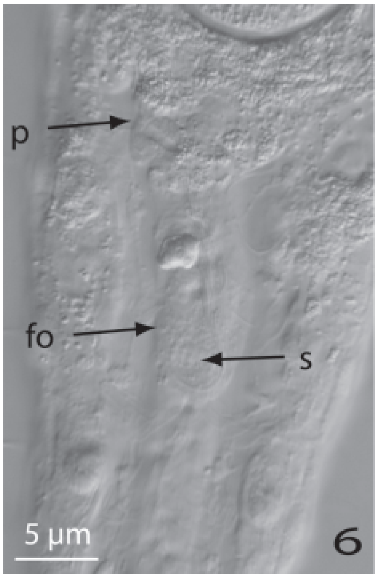


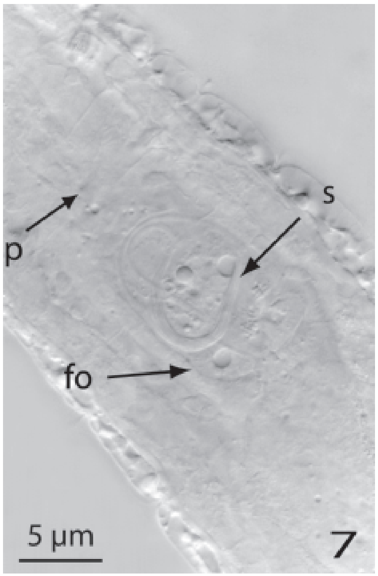












Figs. 1–3 *Paraturbanella teissieri* (Nomarski interference contrast)

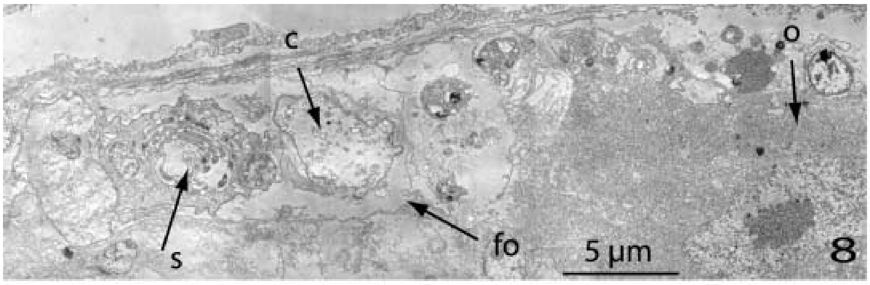
Fig. 1 Habitus of a mature adult in the female phase in vivo, showing the ovaries (*ov*), a vitellogenic oocyte (*o*) and the inactive testes (*t*)

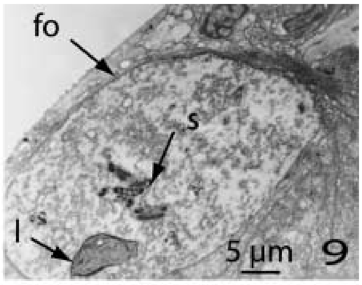
Fig. 2 Detail of the genital system of a mature adult in the female phase in vivo, showing the testes (*t*), the sperm ducts (*d*) and a mature oocyte (*o*)

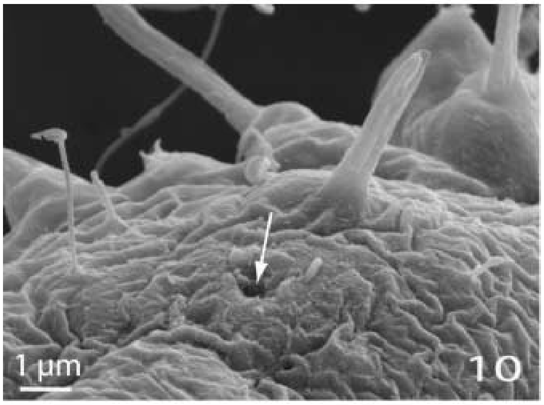
Fig. 3 Detail of the confluence of the sperm ducts (*d*) into a single medioventral male pore (*mp*)

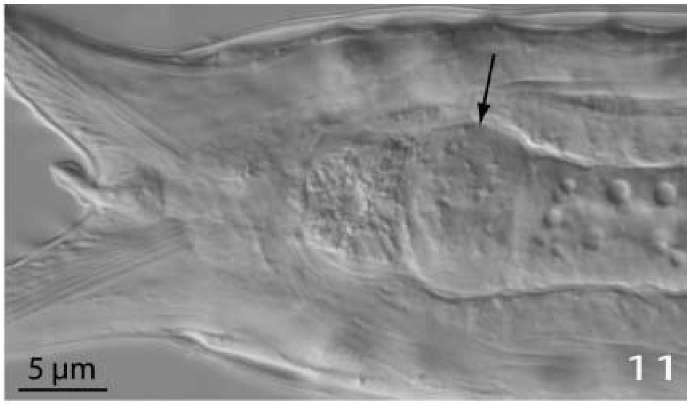
Fig. 4a, b *Turbanella cornuta*. SEM views of the ventral side of the trunk region of mature individuals, showing the single male genital pore protruding during the male phase (**a**) and flat during the female phase (**b**) (*arrow*)

Figs. 5–7 Detail of the frontal organ (*fo*) of mature individuals of *Paraturbanella* species in the female phase, showing the external pore (*p*) and spermatozoa (*s*) in the inner cavity (Nomarski interference contrast) **Fig. 5** *P. teissieri*; **Fig. 6** *Paraturbanella pallida*; **Fig. 7** *Paraturbanella aggregotubulata*









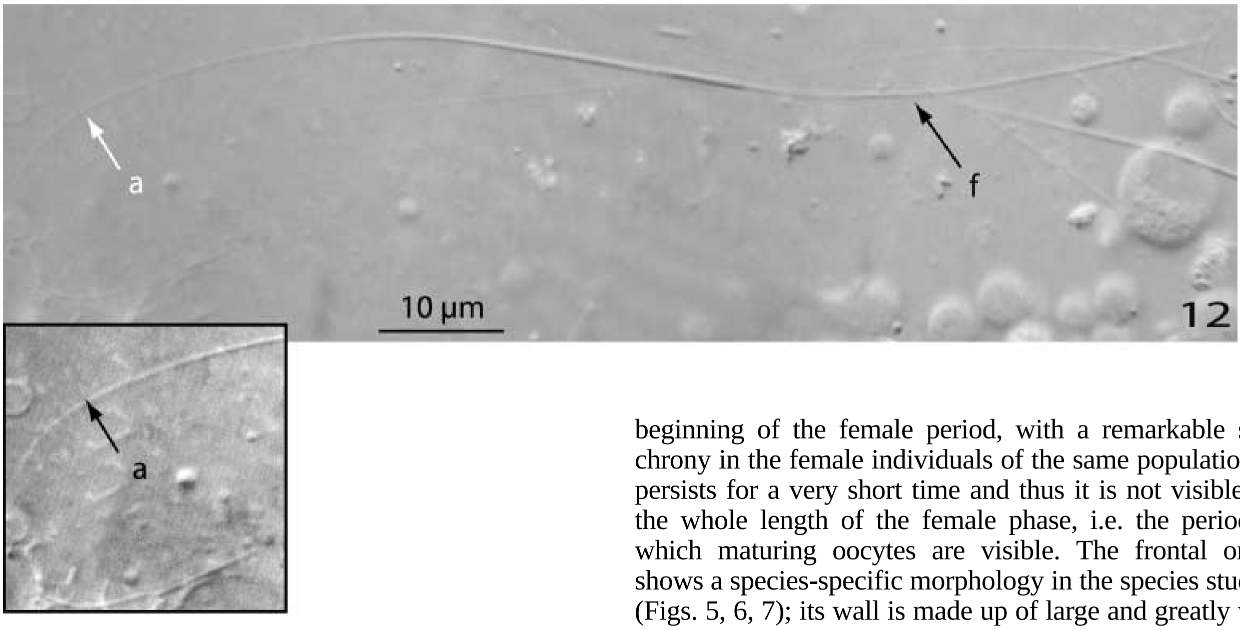


Fig. 8 *Paraturbanella teissieri*. Longitudinal TEM section of a 'frontal organ' of a mature individual in the female phase; the inner cavity (*c*) of the organ containing allosperm (*s*) and the adjacent vitellogenic oocyte (*o*) are shown

Fig. 9 TEM section of a regressing frontal organ (*fo*); degenerating spermatozoa (*s*) and lysosomes (*l*) are visible

Fig. 10 *Turbanella cornuta*. SEM view of the dorsolateral opening of the frontal organ in a mature specimen in female phase (*arrow*)

Fig. 11 *Paraturbanella pallida*. The small, compact organ adhering ventrally to the caudal intestine (Nomarski interference contrast; *arrow*)

Fig. 12 An isolated spermatozoon of *P. pallida* and a detail of the acrosome (Nomarski interference contrast)

beginning of the female period, with a remarkable synchrony in the female individuals of the same population. It persists for a very short time and thus it is not visible for the whole length of the female phase, i.e. the period in which maturing oocytes are visible. The frontal organ shows a species-specific morphology in the species studied (Figs. 5, 6, 7); its wall is made up of large and greatly vacuolated cells containing scattered, rounded and refringent inclusions, while its lumen is mostly filled with granular material and generally shows actively moving spermatozoa, which are initially numerous and then gradually decrease in number (Figs. 5, 6, 7, 8). No distinct internal connection of the frontal organ with the adjacent full-grown egg nor with the previtellogenic oocytes was seen. In a single specimen of *P. teissieri* the frontal organ was apparently reduced to a single large cell containing lysosomes and very few spermatozoa undergoing degeneration (Fig. 9). The frontal organ is connected to the exterior through a poorly differentiated pore, which has been observed in many mature female animals, opening dorsolaterally at U72, where the morphology of the intestine changes from the anterior, large and pigmented zone to the narrower and

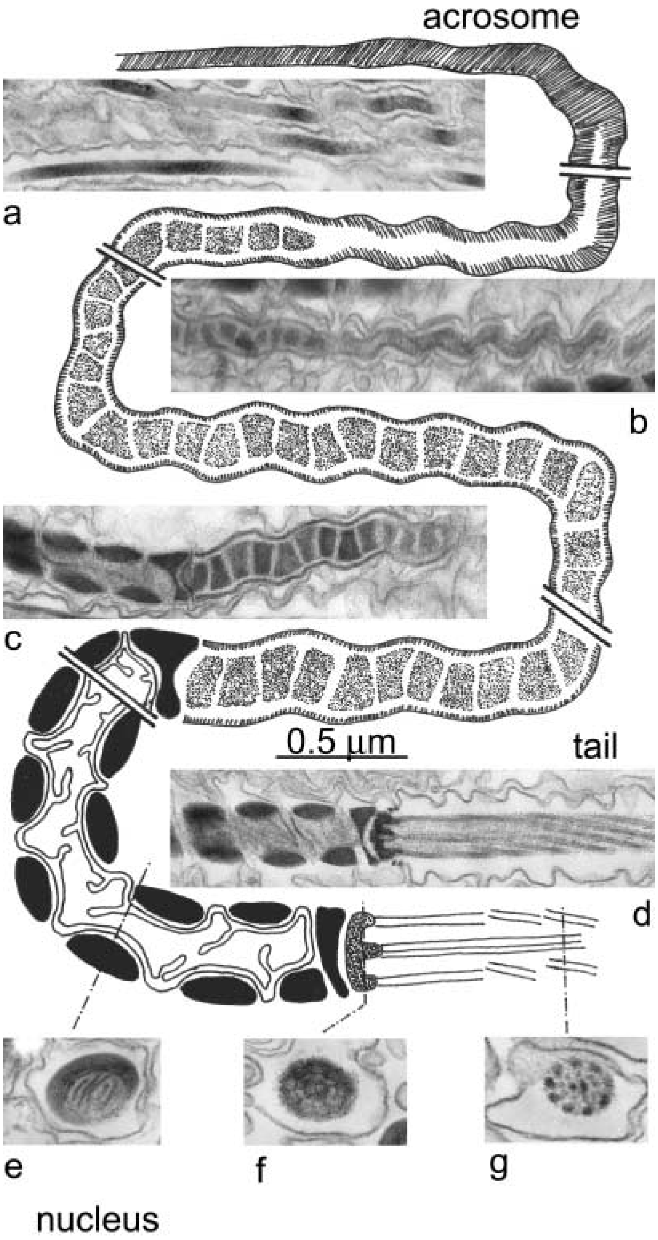


Fig. 13a–g Spermatozoon of *P. teissieri*. In this figure, a schematic drawing of the whole sperm is accompanied by detailed TEM photographic views of some portions. Note that the schematic drawing does not respect the proportions of the details, whereas the micrographs are all at the same magnification. The plasma membrane is omitted. **a** Longitudinal section of the anterior portion of the acrosome showing its paracrystalline structure. **b** The transition area between the anterior (*right*) and the basal (*left*) portion of the acrosome; the paracrystalline portion of the acrosome continues as a sheath involving the organelle. **c** Longitudinal section of the transition area between acrosome (*right*) and nucleus (*left*); the nucleus forms a helix around the mitochondrial axis. **d** Base of the nucleus (*left*) and beginning of the flagellum (*right*). Note the twisted appearance of the axoneme. **e** Cross-sections of the nuclear helix surrounding the mitochondrion. **f** Cross-section of the 'basal body' area. **g** Cross-section of the main portion of the flagellum

vacuolated caudal one (Figs. 5, 6, 7). The edge of the pore appears double under Nomarski optics because it is surrounded by a large glandular ring; in a specimen of *P. pallida* the inner and outer pore diameters measured 4.5 and 8.4 µm, respectively, and a small duct, 1.5 µm long, was seen connecting the pore with the frontal organ (Fig. 6). Similar observations were also carried out on specimens of *T. ambronensis* and *T. cornuta* in the female phase, which contained an evident frontal organ. At SEM observation, several individuals of the latter species showed a dorsolateral, small opening corresponding to that described in *Paraturbanella* species (Fig. 10); the pore often appeared partially occluded by secretory products.

A small, rounded and compact structure adhering ventrally to the terminal intestine was seen in some specimens of *P. teissieri* and *P. dohrni*; it did not show spermatozoa nor a proper external opening in any case (Fig. 11).

Spermatozoa

The spermatozoon of *P. teissieri* is a long, filiform cell, formed by an acrosome, a nucleus surrounding a single mitochondrion and a tail. The total length of the spermatozoon is 120 µm, about 40 µm of which belonging to the flagellum. Both in vivo and under TEM the spermatozoon appears clearly divided into an anterior region, corresponding to the acrosome and the nucleus, and a posterior, rectilinear tail, corresponding to the flagellum (Figs. 12, 13a–g). The acrosome occupies the anteriormost portion of the cell and shows two clearly recognisable regions: the anterior one is thin, 0.1 µm in diameter, rectilinear, and formed by an obliquely striated material, and the posterior one is twisted, with a progressively larger diameter up to 0.2 µm, and is formed by a sort of thick, striated sheath containing a regular series of dense bodies. The two portions of the acrosome are not clear cut, but are connected by a part in which the obliquely striated anterior region becomes progressively more twisted and apparently joins with the posterior, helical one (Fig. 13a–c). The nucleus is spring-shaped and delimits a central area containing a single, long, coiled and column-shaped mitochondrion (Fig. 13c–e). The plasma membrane of the tail is widely spaced from the 9+2 axoneme, which is characterised by doublets extremely twisted around the longitudinal axis. No true basal body is present, and the axoneme base is embedded in a dense material only crossed by the two central tubules which extend to touch the nucleus (Fig. 13f). Testicular spermatozoa are motionless, whereas those contained in the 'frontal organ' move actively.

Discussion

Reproductive system

The structural plan of the reproductive system of *Paraturbanella* species appears basically similar to that of

Turbanella species, also in showing a frontal organ provided with a dorsal pore (Teuchert 1968; Ruppert 1991). The temporary appearance and the cyclic morphological variation of the frontal organ as well as the different morphology of the male pore showed by *T. cornuta* during each sexual phase suggest an alternative hypothesis to that proposed by Ruppert (1991) about the sperm transfer modality in Turbanellidae. We believe that the real copulatory organ of Turbanellidae species is the male pore, which during the male phase protrudes for transferring sperm by direct contact to the dorsal pore of the partner's frontal organ, and during the following female phase becomes flat again. The frontal organ develops at the beginning of the female phase and, once filled with allosperm, starts a gradual but quick regression (1–2 days) during the same sexual phase.

If the sexual condition of Turbanellidae really consists of more than one male and female phase regularly alternating, the brief cycle of the frontal organ (appearance–function–regression) should be repeated during the lifespan of the same individual, however, the somatic euthely of gastrotrichs makes this hypothesis unlikely (Remane 1936). Although some somatic regeneration capacity has been reported in a species of *Turbanella* (Manylov 1995), it appears not to be sufficient in our opinion to form this organ in a short time, and perhaps more than once.

From our observations on a number of specimens of several species belonging to both major genera of Turbanellidae, we rather believe that only two sexual phases follow each other, the male preceding the female one. During the male phase the animal shows bundles of mature filiform spermatozoa filling the testes and the sperm ducts; they are presumably transferred to the frontal organ of an individual in female phase by means of the action of the protruding male pore into the external pore of the partner's frontal organ. Later, at the beginning of the female phase, the frontal organ develops, receives allosperm, and after fertilisation regresses in a short time reabsorbing the unused spermatozoa. The number of spermatozoa contained in the frontal organ appears to be sufficient to fertilise all the maturing oocytes which are visible in the two ovaries; the fertilisation must be a very rapid process, considering the brevity of the period during which the frontal organ is visible. The fertilised oocytes are subsequently laid one at a time by rupture of the body wall.

This hypothesis of fertilisation modality is supported by the observations on *T. varians* by Maguire (1976); however, none of the specimens that we have examined showed structures like the 'spermatophores' described in *T. varians*.

No defined 'caudal organ' was seen in any individual of the species of *Paraturbanella* (*P. teissieri*, *P. dohrni*, *P. pallida*, *P. aggregotubulata*) nor of *Turbanella* (*T. cornuta*, *T. ambronensis*, *T. bocquet*, Kaplan, 1958, *T*. sp.) that we have observed up to date. The posterior structure sporadically observed in some specimens of these species only apparently corresponded in its position to the putative 'caudal organ' reported in the literature (Teuchert 1968; Ruppert 1991; Clausen 1996), but it was ventral, not dorsal, to the intestine. We believe that this structure cannot be homologous to the 'caudal organ' present in most Macrodasyida because it shows a compact, nonmuscular structure, and it never contains spermatozoa; the function of this structure remains unclear, but it does not appear related to the reproductive function at all.

Spermatozoa

The spermatozoa of the *Paraturbanella* species share with the general model of gastrotrich sperm the complex helical structure of the acrosome, the spring-shaped nucleus involving the mitochondria and the absence of a true basal body (Balsamo et al. 1998; Fregni 1998). However, Turbanellidae are the only representatives of Macrodasyida known so far which have spermatozoa devoid of the striated cylinder around the axoneme (Teuchert 1976). Recent phylogenetic analyses suggest that Dactylopodolidae and Turbanellidae are plesiomorphic clades among Macrodasyida (Hochberg and Litvaitis 2000, 2001), and consequently the striated cylinder may not belong to the 'basic plan' of the macrodasyid spermatozoon. Ultrastructural data on the testicular spermatozoa of Dactylopodolidae, integrating those available on the penetrated sperm (Fisher 1996), will be of basic importance in supporting this view of the evolution of gastrotrich sperm.

Two species of Turbanellidae have been spermatologically examined so far, *T. cornuta* (see Teuchert 1976) and *T. ambronensis* (see Ferraguti and Balsamo 1995). With respect to the *Turbanella* model, the spermatozoon of *P. teissieri* species has a longer acrosome, with an anterior rectilinear portion containing a peculiar paracrystalline structure; moreover, the sheath surrounding the acrosome is twisted towards its base whereas it is rectilinear in *Turbanella* species. The internal structures of the twisted acrosomal portion, as well as the nucleus involving a single mitochondrion and the structure of the tail are similar in all the examined species, thus showing a common ultrastructural plan of the spermatozoon of the Turbanellidae species, even if fine details allow to distinguish that of the species of *Turbanella* and *Paraturbanella*, respectively.

Both the structure of the reproductive apparatus and the fine morphology of the spermatozoa of Turbanellidae species agree with the evolutionary view, recently supported by morphological and molecular data, which puts this taxon in a separate clade, early divergent from the stem of Macrodasyida (Hochberg and Litvaitis 2000, 2001; Balsamo et al. 2001).

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