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Anatomy and ultrastructure of the reproductive organs in *Dactylopodola typhle* (Gastrotricha: Macrodasyida) and their possible functions in sperm transfer

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Abstract. The reproductive anatomy of gastrotrichs is well known for several species, especially for the marine taxon Macrodasyida. However, there is little information on the reproductive organs and the modes of mating and sperm transfer in putative basal taxa, which is necessary for accurate reconstruction of the ground pattern of the Gastrotricha. We present the first detailed morphological investigation of the reproductive system of a putative basal gastrotrich, *Dactylopodola typhle,* using transmission and scanning electron microscopy, histology, and microscopic observations of living specimens. *Dactylopodola typhle* is a hermaphrodite that possesses paired female and male gonads, an unpaired uterus with an outlet channel that we call the cervix, and an additional accessory reproductive organ, the so-called caudal organ. We hypothesize that the hollow, secretory caudal organ serves for picking up autospermatozoa (self-sperm), for spermatophore formation, and finally for transferring the autospermatophore to a mating partner. The allospermatophore (foreign spermatophore) is stored within the uterus where fertilization occurs. We think that the mature and fertilized egg is released through the cervix and the dorsolateral female gonopore, and not by rupture of the body wall. Based on the morphology, we provide a plausible hypothesis for spermatophore formation and transfer in *D. typhle*. Preliminary phylogenetic considerations indicate that the stem species of Macrodasyida, perhaps that of all Gastrotricha, had paired ovaries and paired testes, an unpaired uterus, and only one accessory reproductive organ.

Additional key words: mating, reproductive biology, phylogenetic implications

Marine Gastrotricha of the subtaxon Macrodasyida are hermaphroditic organisms that inhabit a wide range of littoral and sublittoral sediments. Most species of Macrodasyida possess one or two female gonads, one or two testes, and a set of two peculiar accessory reproductive organs. These organs are (1) the caudal organ that functions for picking up and transferring the autospermatozoa to a mating partner and (2) the frontal organ that serves for storing foreign spermatozoa before fertilization (Ruppert 1991). These accessory organs show a high diversity in structure and therefore, in all probability, in function as well.

The whole mating and sperm transfer process, and the function and interaction of the gonads and accessory reproductive organs, are understood for at least one gastrotrich species: *Macrodasys* sp. (Ruppert 1978a). There exist, however, further studies on the reproductive system of several other species, allowing us to make inferences on their mating process (Ruppert & Shaw 1977; Ruppert 1978b). What is lacking up to now is a hypothesis on the mode of sperm transfer and oviposition in the stem species of Gastrotricha, because morphological and behavioral data are minimal. Dactylopodolidae is a putative basal taxon within Macrodasyida (Hochberg & Litvaitis 2000, 2001a), or even within Gastrotricha as a whole (Zrzavý 2003). Accordingly, our current study of the reproductive system in *Dactylopodola typhle* Remane 1927 will provide important new details on the morphology of the reproductive organs within this putative sister group of all remaining gastrotrichs. We establish a hypothesis on the process of mating and sperm transfer in *D. typhle* and compare it with hypotheses on this topic for *Dactylopodola baltica* (Teuchert 1968) and *Dactylopodola* sp. (Ruppert 1991).

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Methods

All specimens of *Dactylopodola typhle* investigated were collected during a meiobenthos expedition to the southern North Sea (German Bight) with RV "Heincke," May 2–4, 2006 (HE 248). Specimens of *D. typhle* were found in coarse sands at a depth of 23 m north-west of the island of Helgoland (54°46′N, 7°30′E) and in coarse sands mixed with shell gravel at a depth of 24 m at "Borkum Riffgrund," north-west of the island of Borkum (53°60′N, 6°28′E). Samples were taken with a multiple corer equipped with 12 tubes (Ø 9.5 cm) and a Van Veen grab sampler (see Fleeger et al. 1988). Extraction of animals from the sediment sample was carried out either by the seawater–ice treatment (Uhlig 1964; Uhlig et al. 1973), or by narcotization with $MgCl_2$ solution (7% w/v), stirring the sample, and decanting the resulting supernatant through a plankton gauze (40-μm mesh size) (cf. Pfannkuche & Thiel 1988). Retained meiofaunal organisms were rinsed with fresh seawater into a culture dish and separated under a dissecting microscope.

For transmission electron microscopy (TEM) and histological observations, the extracted animals were narcotized in a 7% $MgCl_2$ solution for a few minutes. After relaxation of the specimens, they were fixed in 2.5% glutaraldehyde (with a small amount of ruthenium-red added) in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.2) for 45 min to 1 h on ice. All specimens were postfixed for 60 min at 4°C in 1% OsO_4 (buffered in 0.1 mol L^{-1} sodium cacodylate solution, pH 7.2), dehydrated in an increasing acetone series, and embedded in Araldite. Serial ultrathin sections (70 nm, cross sections) and serial semithin sections (0.5 μm, cross and horizontal sections) were carried out on a Reichert Ultracut E microtome (Leica Biosystems, Wetzlar, Germany).

Serial histological sections were dried on a microscope slide, stained with toluidine blue without previous deresination (see Böck 1984), mounted under coverslips, and recorded with a digital camera (Color View I, Olympus Imaging, Hamburg, Germany) mounted on a Leica DMLB compound microscope (Leica Microsystems, Wetzlar, Germany).

Ultrathin sections for TEM observations were stained automatically with uranyl acetate and lead citrate (Leica EM Stain) and were viewed on a Zeiss EM 902 (Carl Zeiss, Oberkochen, Germany) at 80 kV. The images of sections were captured with a Dual Scan CCD camera (Proscan Electronic Systems, Lagerlechfeld, Germany). Owing to the small size of the CCD chip, many micrographs had to be assembled digitally with the multiple image alignment tool of the iTEM® software (soft imaging systems). Owing to the decreasing accuracy of the software with an increasing number of images to be assembled, some of the TEM micrographs presented here may show slight misalignment.

Reconstruction of the reproductive anatomy in *D. typhle* was performed on the basis of low-magnification TEM micrographs, histological sections, and observations on living specimens (conducted on a Leica DMLB microscope with differential interference contrast). Most drawings were performed digitally with the Adobe® Illustrator software (Adobe Systems Inc., San Jose, CA, USA) (for instructions see Coleman 2003).

Some of the fixed specimens were prepared for scanning electron microscopy (SEM). After postfixation with OsO_4, specimens were dehydrated in an increasing ethanol series, critical point dried, and mounted on a round coverslip coated with a thin layer of special wax (TempFix, Plano, Wetzlar, Germany). The coverslip was transferred to an SEM stub and specimens were coated with gold. Observation and documentation was conducted with a Zeiss DSM 940 scanning electron microscope.

Results

General reproductive anatomy

Mature individuals of *Dactylopodola typhle* are hermaphroditic animals that have a well-developed female and male genital tract (Figs. 1–4). There is a pair of bilateral ovaries lying alongside the intestine within the caudal third of the trunk region (Figs. 1A,B, 3B). The female gonads expand frontally where they fuse in the mid-trunk region (Figs. 1A, 3A). Here, the mature oocyte is enveloped by the uterus wall. When present, a foreign spermatophore, the so-called allospermatophore, lies next to the mature oocyte within the uterus (Figs. 1A, 2A,B, 4C). Frontally, the thin uterus wall extends to a solid, epithelial, outlet duct, the cervix (Figs. 1A, 2A). The cervix leads to the unpaired, dorsolateral female gonopore (Fig. 1A). The paired, bilateral testes lie at both sides of the midgut and expand from the end of the first fourth of the trunk to the region where both ovaries fuse (Figs. 1A,B, 2A,B, 3A, 4B). Each testis has a separate, ventral male gonopore that lies at the level of the caudal fourth of the male gonad (Fig. 1B). In the caudal third of the trunk region, there is an additional, glandular accessory sex organ, the caudal organ. It is situated dorsolateral to the intestine and between both ovaries (Figs. 1A,B, 3B, 4D). The caudal organ opens to the outside via a ventral, unpaired pore (Fig. 1A). An external feature that is suspected to play an important role in gamete transfer is a V-shaped groove, which we call the anal pit, at the ventral surface of the caudal end of the trunk region (Fig. 4E).

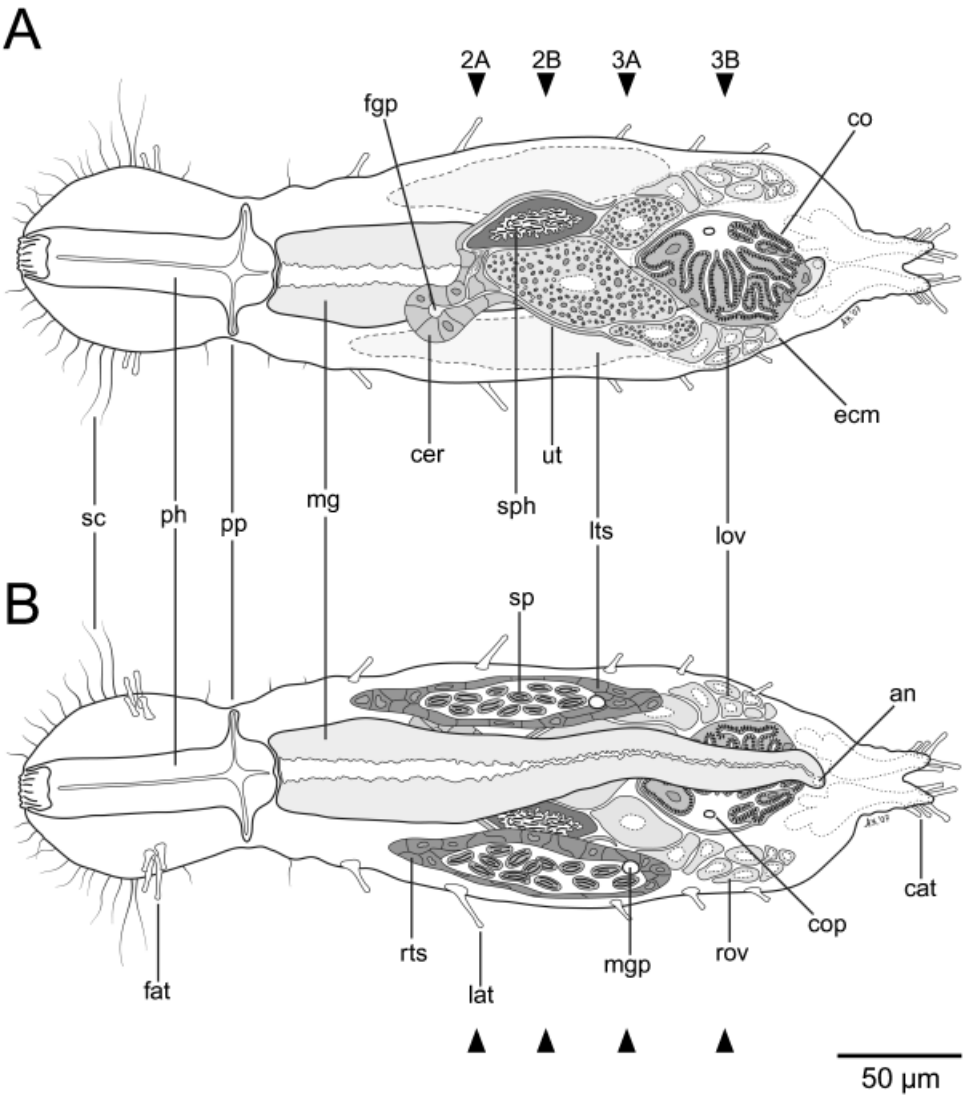

Fig. 1. Schematic horizontal views of the whole animal with special focus on the reproductive organs. Habitus drawn as animal was slightly squeezed. **A.** View from dorsal (female and accessory reproductive organs). **B.** View from ventral (male reproductive organs). Black triangles (2A, 2B, 3A, 3B) indicate sectional planes of the schematic cross sections in Figs. 2 and 3. an, anus; cat, caudal adhesive tubes; cer, cervix; co, caudal organ; cop, caudal organ pore; ecm, extracellular matrix; fat, frontal adhesive tubes; fgp, female genital pore; lat, lateral adhesive tubes; lov, left ovary; lts, left testis; mg, midgut; mgp, male genital pore (proposed); ph, pharynx; pp, pharyngeal pore; rov, right ovary; rts, right testis; sc, sensory cilia; sp, bundles of spermatozoa; sph, foreign spermatophore; ut, uterus.

Ovaries

Within each of the two ovaries, the germ cell stages (oogonia to vitellogenic oocytes) mature in a frontal direction from the caudal region toward the unpaired uterus, where both ovaries fuse (Figs. 1A, 3A,B). There are ~15 germ cells per ovary. Early stages are densely packed and have diameters of 5–10 µm (Fig. 5A,B), whereas advanced (mature) stages have dimensions of 10–15 µm in diameter (Fig. 7D). There is neither an ovarial wall tissue enveloping the oocytes nor any kind of germ blasteme at the posterior pole of the gonads. Circular or horseshoe-shaped muscles of the trunk surround the ovaries (Fig. 5A,B). These muscles, as well as bodies of parenchyma cells, are separated by a very thin layer of extracellular matrix (ECM) lining the organs (Fig. 5C).

Early oogenetic cells have an electron-lucent cytoplasm containing many mitochondria, membrane-bound, electron-lucent vesicles, and a high density of electron-dense granules (glycogene) (Fig. 5A–C). The nuclei appear with medium electron-dense, granulated chromatin, and well-organized nucleoli (Fig. 5A,B). Later oogenic cells contain different types of yolk granules and vesicles (in the most dorsal lying cell in Fig. 5B, vitellogenesis has started). There are electron-dense lipid droplets (diameter: 0.5 µm), membrane-bound vesicles with a medium electron-dense core, and a more lucent cortex (diameter: 1–3 µm); a third type of granule (diameter: 750 nm) contains a characteristic pattern of concentric rings consisting of electron-dense content (Fig. 7B,D). Inclusive of the mature oocyte, there are approximately five vitellogenic oocytes in the female genital tract (Fig. 7D).

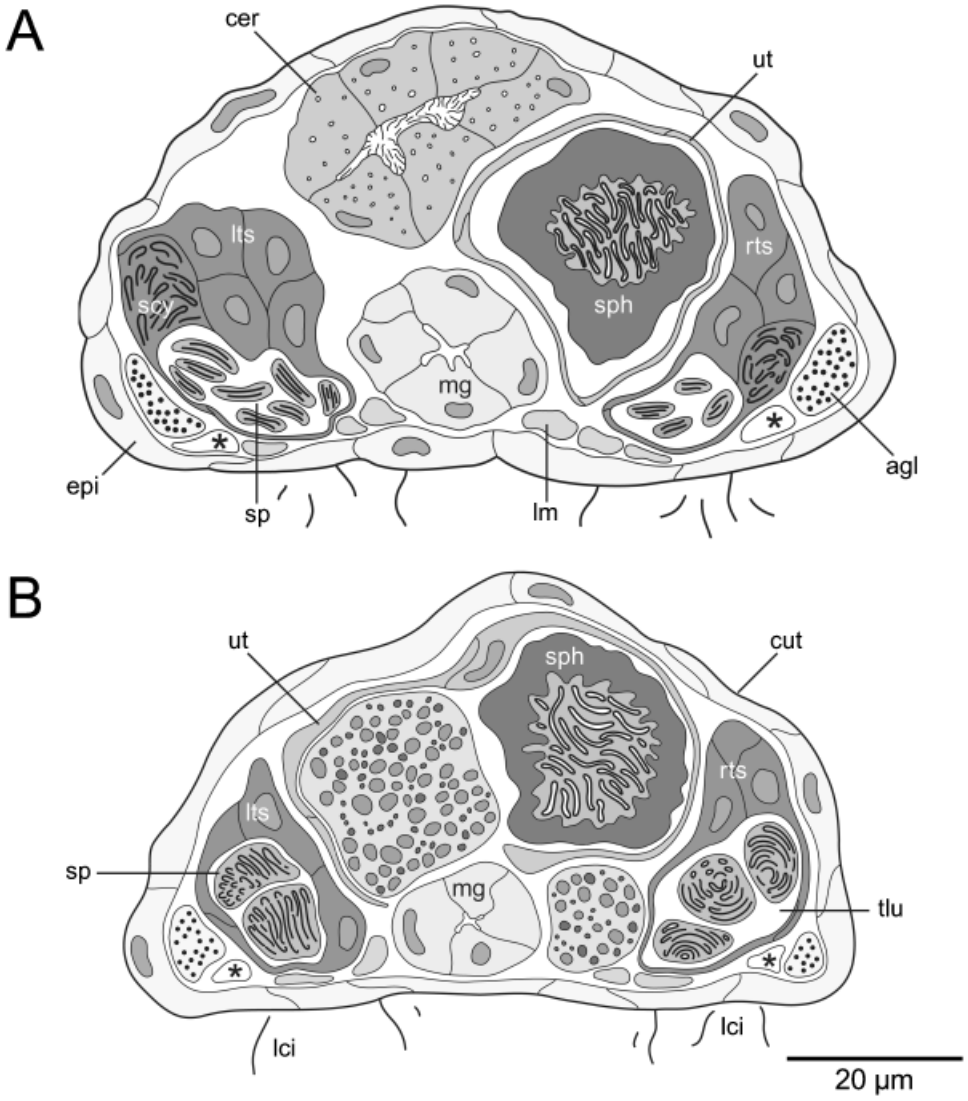

Fig. 2. Schematic cross sections of the trunk. **A.** Section of the cervix region. **B.** Section of the uterus region. Levels of sectional planes are indicated in the horizontal views (black triangles in Fig. 1A). The white area surrounding all organs is occupied mainly by body muscles and parenchyma cells (not drawn). agl, adhesive gland; cer, cervix; cut, cuticle; epi, epidermis; lci, locomotory cilia; lm, longitudinal muscles; lts, left testis; mg, midgut; rts, right testis; scy, spermatogenic cyst; sp, bundles of spermatozoa; sph, spermatophore; tlu, testicular lumen; ut, uterus; asterisks, longitudinal nerve cords.

Uterus and cervix

A few micrometers frontal to the fusion of the bilateral ovaries is the mid-dorsal, mature oocyte, which is enveloped by a thin cellular wall (proximal region of the unpaired uterus). The uterus wall also envelopes the allospermatophore, so that the mature oocyte and the foreign spermatophore are situated together inside the uterus chamber (Figs. 1A, 2A,B). The proximal region of the uterus wall epithelium is 50–100 nm thick (Fig. 7C). In more distal sections of the uterus, the wall cells have a thickness of $\leq$3 µm (Fig. 7A,E). The lumen of the uterus chamber is completely filled with the mature oocyte and the allospermatophore (Fig. 7A,C,E). Basally, the uterus wall is lined by longitudinal and circular (or horseshoe-shaped) muscle strands of the trunk musculature (Figs. 7A,C,E, 10C,D). A thin layer of ECM separates these muscles from the cytoplasmic membrane of the uterus wall cells. The ECM is present as a fine gray matter between the thin uterus wall and the muscle cells (Fig. 7C). The gap between the uterus wall and circular muscles (see Fig. 7A) is an artifact caused by tissue shrinkage during dehydration of the specimens.

Distally, the uterus continues as a solid, epithelial outlet duct (cervix; see above) that consists of prismatic epithelial cells that enclose a central lumen (Figs. 1A, 2A, 8A,B). The whole cervix is $\leq$20 µm in diameter (Figs. 2A, 8A). The reconstruction revealed that the short, proximal cervix lumen is branched (see Fig. 1A). The branch that leads directly to the allospermatophore is sealed, presumably with a secretion product (Fig. 8C). A few micrometers distally, the branches fuse to form a common cervix lumen with a diameter of >10 µm (Figs. 2A,

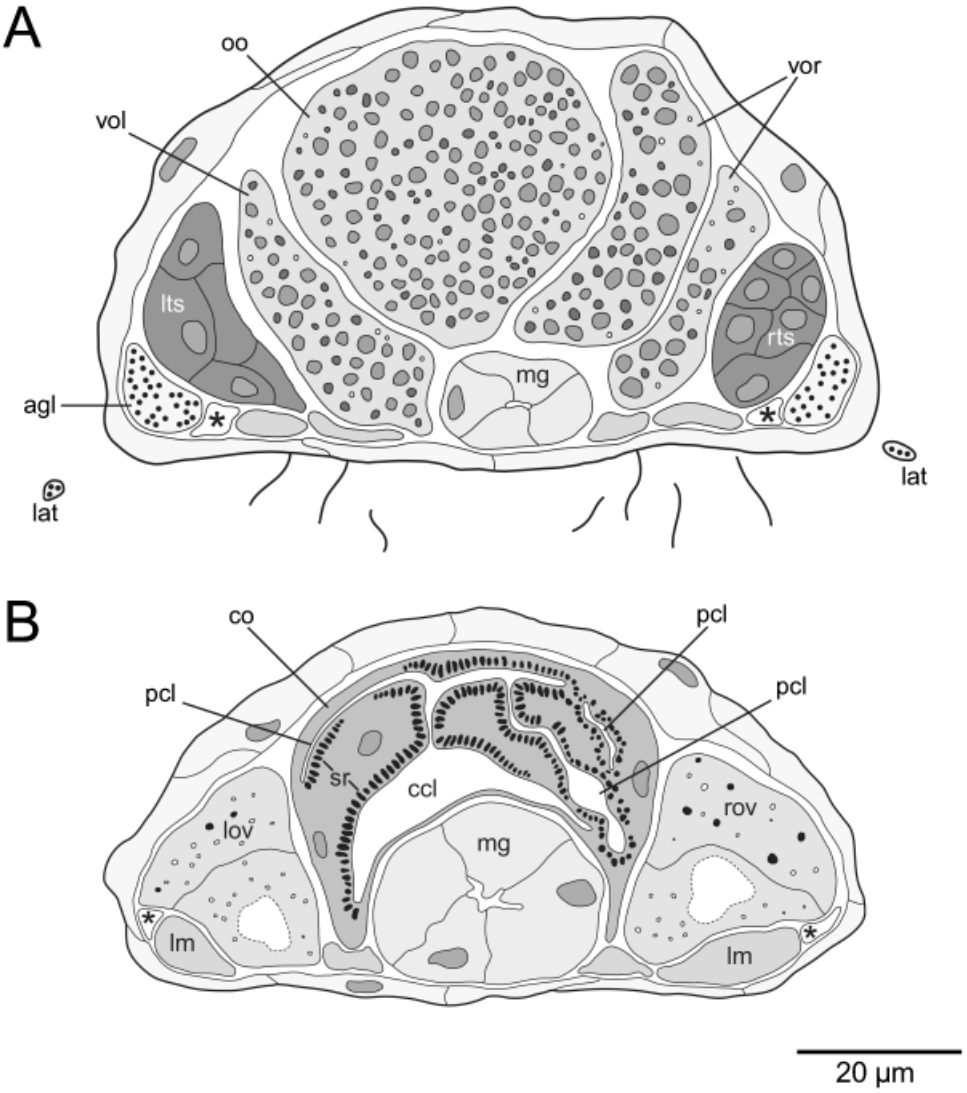

Fig. 3. Schematic cross sections of the trunk. **A.** Section of the region where the two ovaries fuse. **B.** Section of the caudal organ region. Levels of sectional planes are indicated in the horizontal views (black triangles in Fig. 1A). The white area surrounding all organs is occupied mainly by body muscles and parenchyma cells (not drawn). agl, adhesive gland; ccl, central caudal organ lumen; co, caudal organ; lat, lateral adhesive tubes; lm, longitudinal muscles; lov, left ovary; lts, left testis; mg, midgut; oo, mature oocyte; pcl, peripheral caudal organ lumen; rov, right ovary; rts, right testis; sr, secretion rods; vol, vitellogenic oocyte of the left ovary; vor, vitellogenic oocyte of the right ovary; asterisks, longitudinal nerve cords.

8A). The apical membrane of the cervix epithelial cells generates numerous microvilli that fill the cervix lumen nearly completely (Fig. 8B). The cervix (and with it the whole uterus) opens to the outside via a narrow pore that is situated dorsolaterally on the left side of the animal (Figs. 1A, 8D, 10E, 11A). The cervix epithelial cells have an electron-lucent cytoplasm that contains many electron-dense vesicles (diameter: $<0.5\,\mu m$). The cells seem to be vacuolated (Fig. 8A–D). The polymorphic nuclei are situated peripherally within the cervical cells (Fig. 8A). Basally, there is a well-developed ECM (Fig. 8A,C) and the cervix is lined by subepithelial, somatic muscles (Fig. 8A).

The position of the allospermatophore and the mature oocyte within the lumen of the uterus can vary, presumably due to protraction or contraction of the whole body. Figure 1A reflects the situation of an animal that was fixed and sectioned for TEM. In live animals, the allospermatophore can be positioned caudal to the mature oocyte (Fig. 4A,C). One animal sectioned for histological study had the spermatophore in front of the egg (Fig. 10C,D). However, in all the cases mentioned above, the allospermatophore was in proximity to the most mature oocyte.

Allospermatophore

The allospermatophore is easily detectable in intact animals observed under a compound light microscope. It is characterized by its position close to the mature oocyte and its content of rod-like bodies, which are foreign spermatozoa (Figs. 1A, 4C). This cluster of rod-like sperm is encapsulated within a common sheath of moderate electron density (Figs. 2A,B, 7A,C). Overall, the spermatophore is nearly as

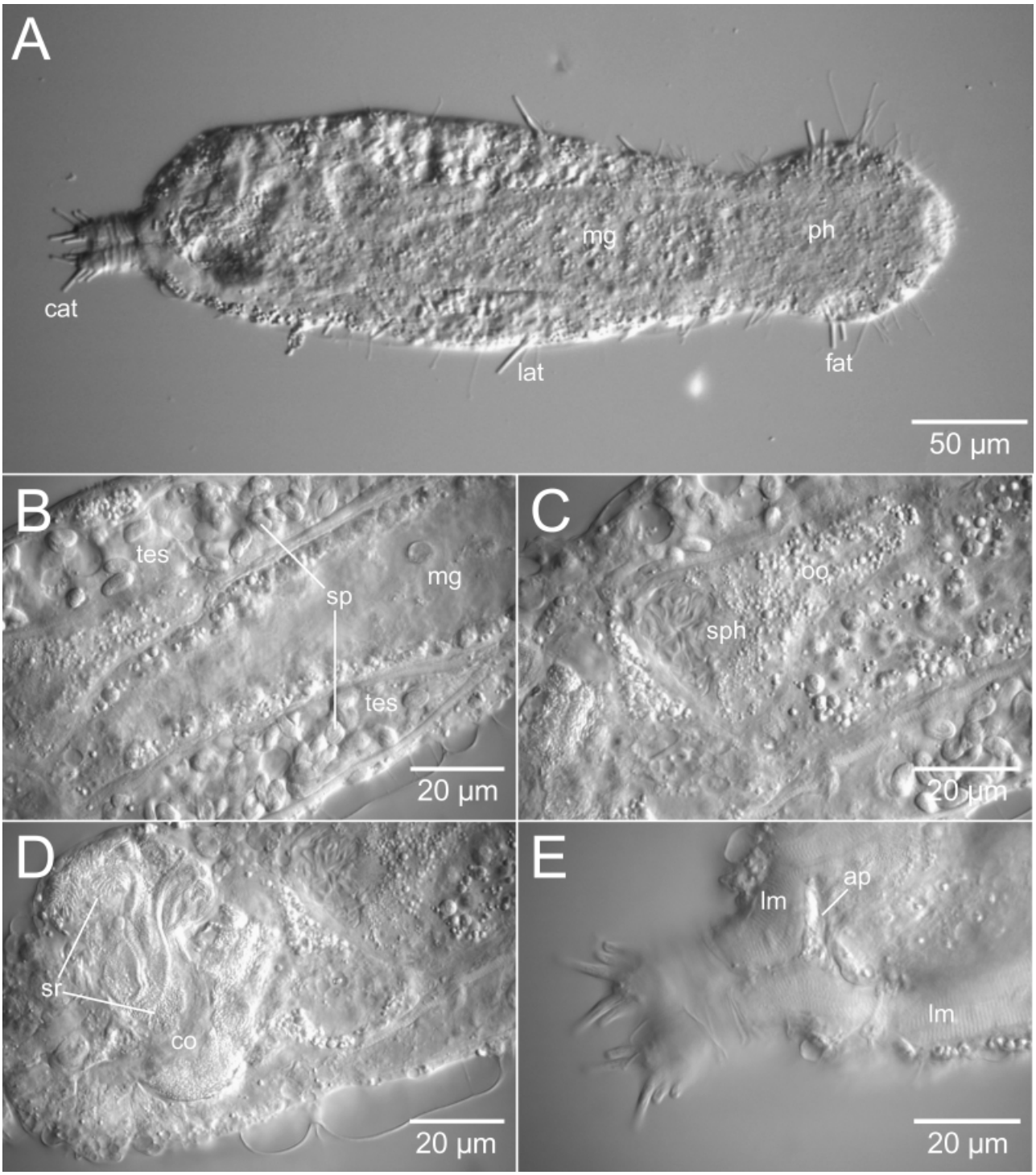

Fig. 4. Living specimen examined by differential interference contrast microscopy. **A.** View from dorsal side; animal slightly compressed. **B.** Mid trunk region with midgut (mg) and testes (tes); note the longitudinal muscles lining the testes. **C.** Uterus region with mature oocyte (oo) and spermatophore (sph). **D.** Caudal organ (co) with numerous secretion rods (sr). **E.** Ventral view of the caudal adhesive feet and the anal pit (ap); note the strong longitudinal musculature (lm) within this region. cat, caudal adhesive tubes; fat, frontal adhesive tubes; lat, lateral adhesive tubes; ph, pharynx; sp, bundles of spermatozoa.

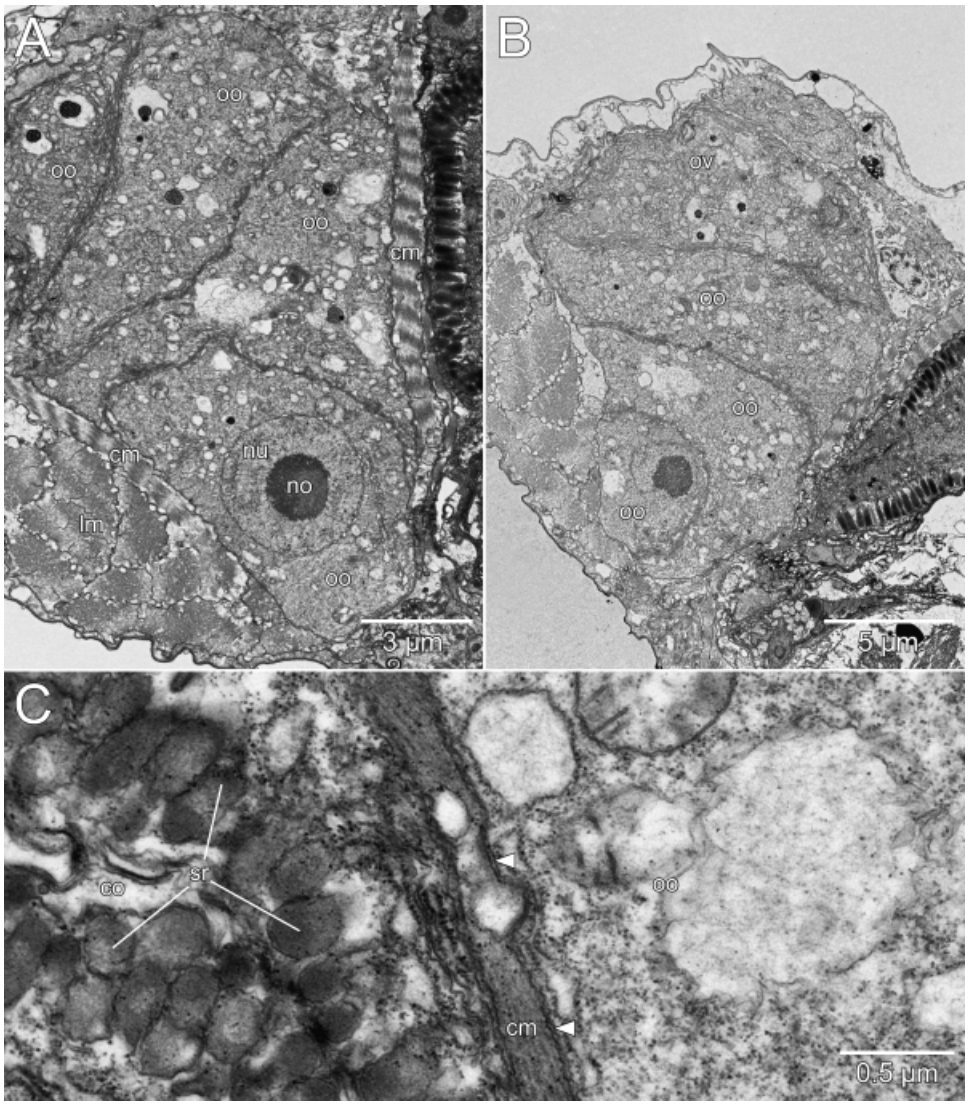

Fig. 5. Ultrastructure (transmission electron microscopy) of the ovaries. **A, B.** Cross sections of the left ovary with four oogonia (oo). Within the dorsal oogonium in B, vitellogenesis has begun. **C.** Detail of the right ovary and the neighboring caudal organ (co). The circular muscle (cm) between both organs is separated from each through a thin extracellular matrix (arrowheads). lm, longitudinal muscles; nu, nucleus of an oogonium; no, nucleolus; ov, oogonium with initial vitellogenesis; sr, secretion rods of the caudal organ.

wide as the mature oocyte and reaches a diameter of 10–15 µm (Fig. 7A). Within the putative cytoplasmic sheath, there are granules that resemble the large yolk granules of vitellogenic oocytes, only much smaller. The diameter of the granules of the spermatophore is <0.5 µm (Fig. 7A). In no sections were fertilized eggs observed, nor were spermatozoa from the allospermatophore seen in direct contact with a mature oocyte.

Testes

The conspicuous, cigar-, or spindle-shaped testes consist of numerous germ cells (spermatogonia) that form solid caps at the anterior and posterior poles of each testis of the specimens investigated. In the middle region of each testis, the germ cells also function as wall cells surrounding the testicular lumen (Figs. 1B, 2A,B, 3A). In the dorsal and dorsomedian sector, the testicular wall has a thickness of ≤ 5 µm and consists of two or more rows of cells (Fig. 6A,C). The median sector of the testicular wall consists of one row of germ cells (≤ 2 µm thickness), whereas the lateral and the ventral sectors consist of a thin cytoplasmic wall (≤ 50 nm thickness) formed by protrusions of certain male germ cells (Fig. 6B,E).

The prospective spermatogonia of the dorsal, dorsomedian, and median wall of the testes enter spermatogenesis and develop into spermatogenic cysts, in which numerous gametes at different stages of spermatogenesis are enveloped within a common cytoplasmic sheath (Figs. 2A, 6A,B,D). Later in spermatogenesis, the cytoplasmic sheath degenerates and numerous bundles of densely packed mature spermatozoa are stored within the testicular lumen (Figs. 2, 4B, 6A–C, 10D–F).

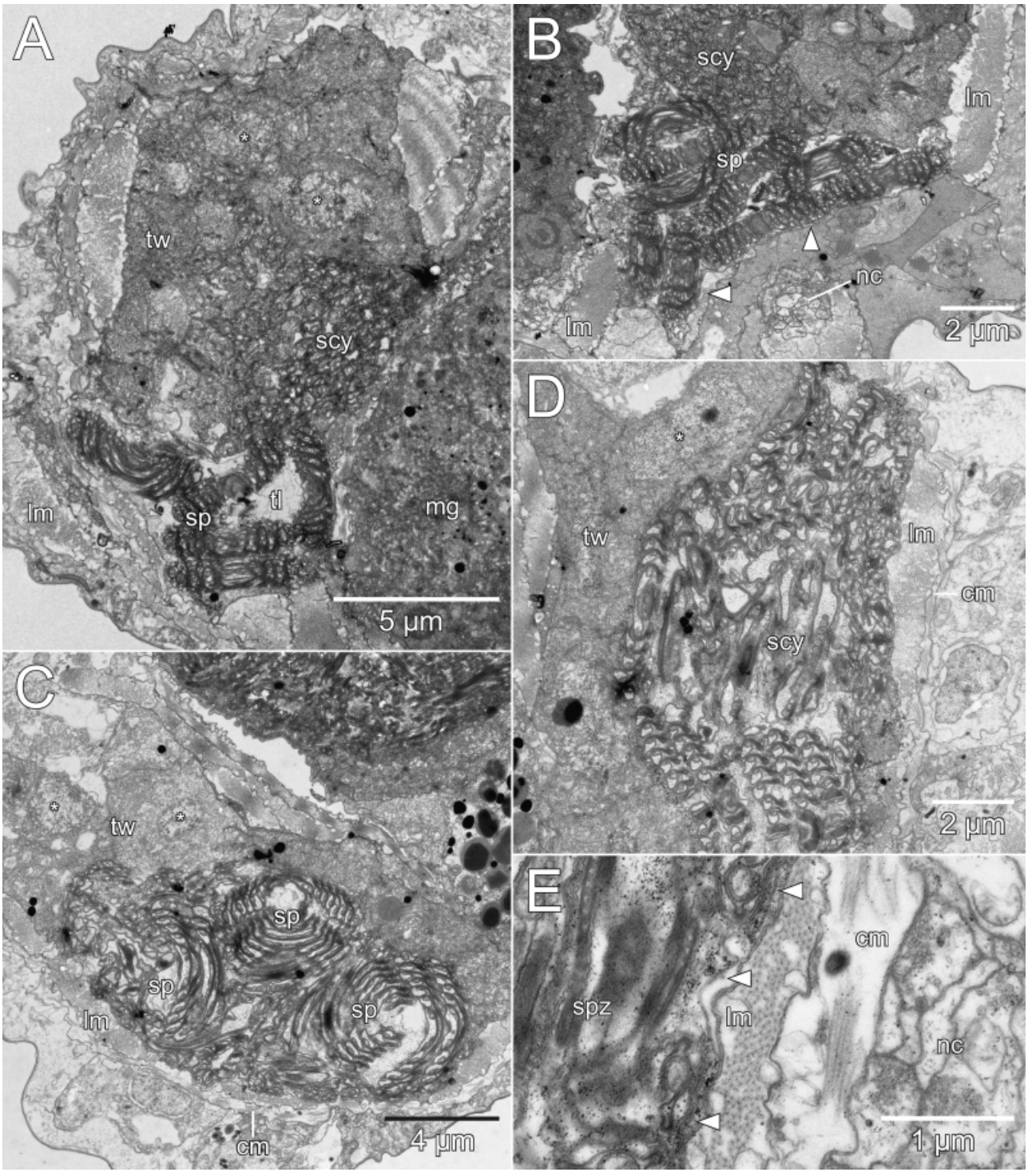

Fig. 6. Ultrastructure (transmission electron microscopy) of the testes. **A.** Cross section of the left testis at different stages of spermatogenesis. **B.** Cross section of the right testis with bundles of spermatozoa (sp). **C.** Detail of the right testis with three bundles of spermatozoa (sp). **D.** Spermatogenic cyst (scy). **E.** Detail of the thin ventrolateral testicular wall (arrowheads) of the right testis. cm, circular muscles; lm, longitudinal muscles; mg, midgut; nc, ventrolateral nerve cord; scy, spermatogenic cysts; sp, bundles of mature spermatozoa; spz, spermatozoa; tl, testicular lumen; tw, testicular wall consisting of spermatogonia; arrowheads, testicular wall; asterisks, nuclei of spermatogonia.

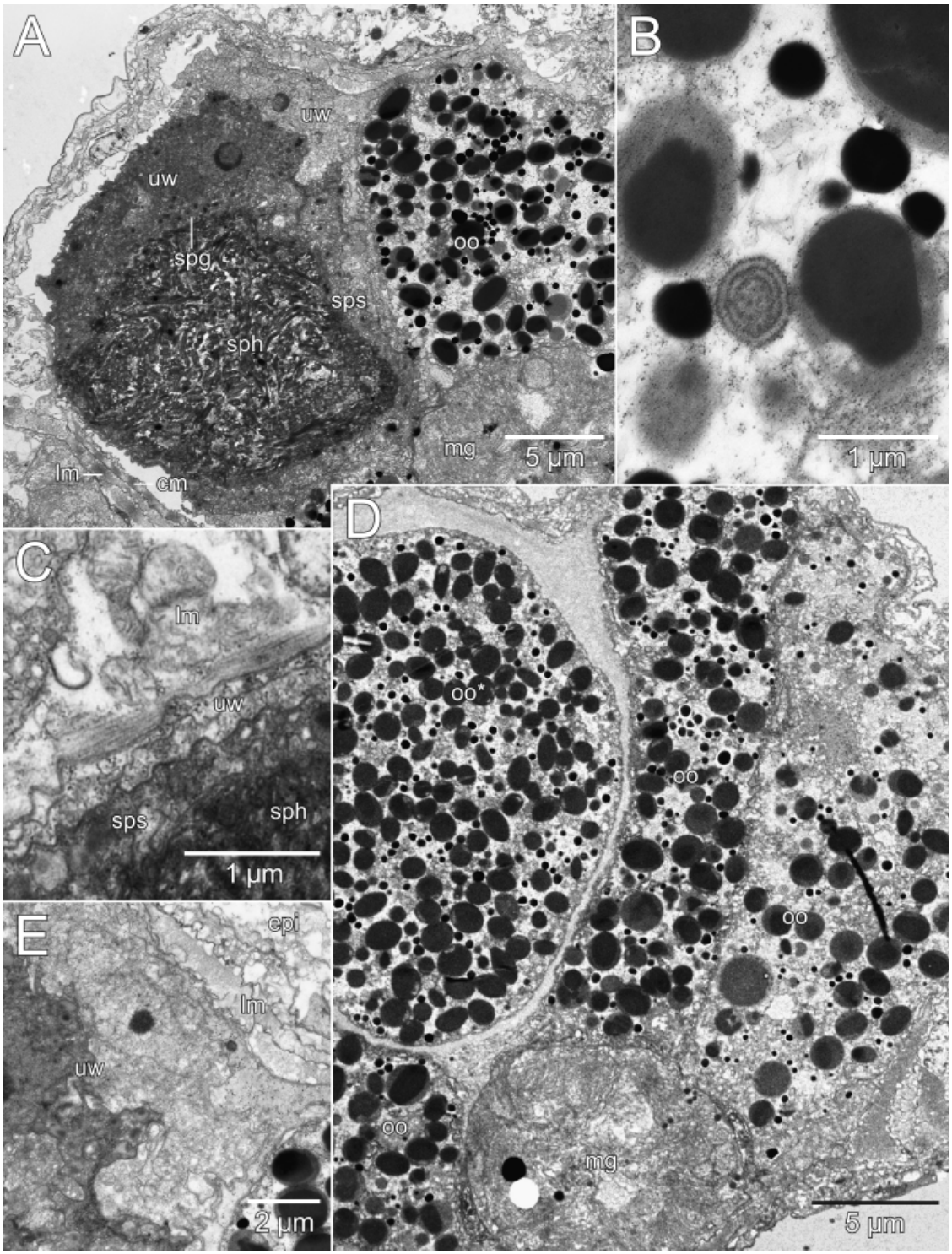

Fig. 7. Ultrastructure (transmission electron microscopy) of the uterus, vitellogenic oocytes and the allospermatophore. **A.** Cross section of the uterus with mature oocyte (oo) and adjacent allospermatophore (sph). **B.** Detail of yolk granules and lipid droplets of the mature oocyte. **C.** Uterus wall (uw) and the accompanying body musculature. **D.** Region of fusion of both ovaries. The middorsal mature oocyte is marked with an asterisk (oo*). **E.** Close up of the contact zone of two uterus wall cells. cm, circular muscle; epi, epidermal cell; lm, longitudinal muscle; mg, midgut; oo, oocytes at different stages; spg, granules of the spermatophore; sph, allospermatophore (cluster of spermatozoa); sps, sheath of spermatophore; uw, uterus wall.

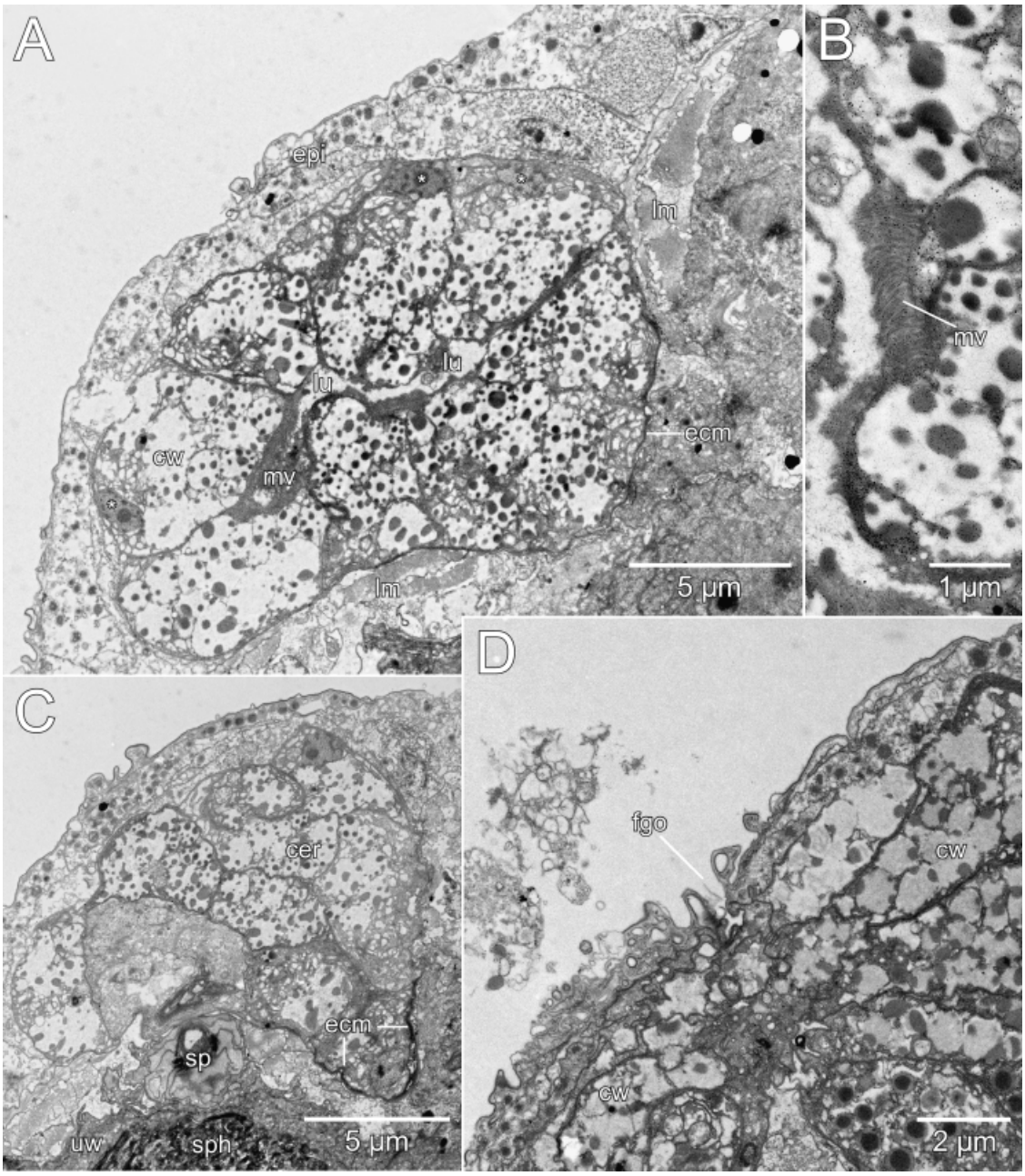

Fig. 8. Ultrastructure (transmission electron microscopy) of the cervix. **A.** Cross section of the mid region of the cervix. Nuclei of the cervical wall cells (cw) are indicated (asterisks). **B.** Close-up of the cervix lumen with luminal microvilli (mv). **C.** Proximal region of the cervix with the secretory product (sp) sealing one shank of the proximally branched lumen. **D.** Detail of the aperture region of the cervix. cer, cervix; cw, epithelial wall cells of the cervix; ecm, extracellular matrix; epi, epidermis; fgo, female gonopore; fgp, female genital pore; lm, longitudinal muscles; mv, microvilli; sp, secretory product; sph, spermatophore; uw, uterus wall.

The testicular lumen opens to the exterior via a ventral pore that is situated in the caudal fourth of each testis (Fig. 1B). We could detect the pore of the right testis in one specimen prepared for SEM (Fig. 11B,D). The pore of the left testis of that specimen might have been covered by cilia of the ventral locomotory epidermal cells or it might not have developed yet (Fig. 11D). In fact, the testicular pores are likely to appear just before spermatozoa are released. Even after intensive scrutiny of the whole section series (ultrathin as well as semithin sections), we could not find additional testicular pores. Nonetheless, it is

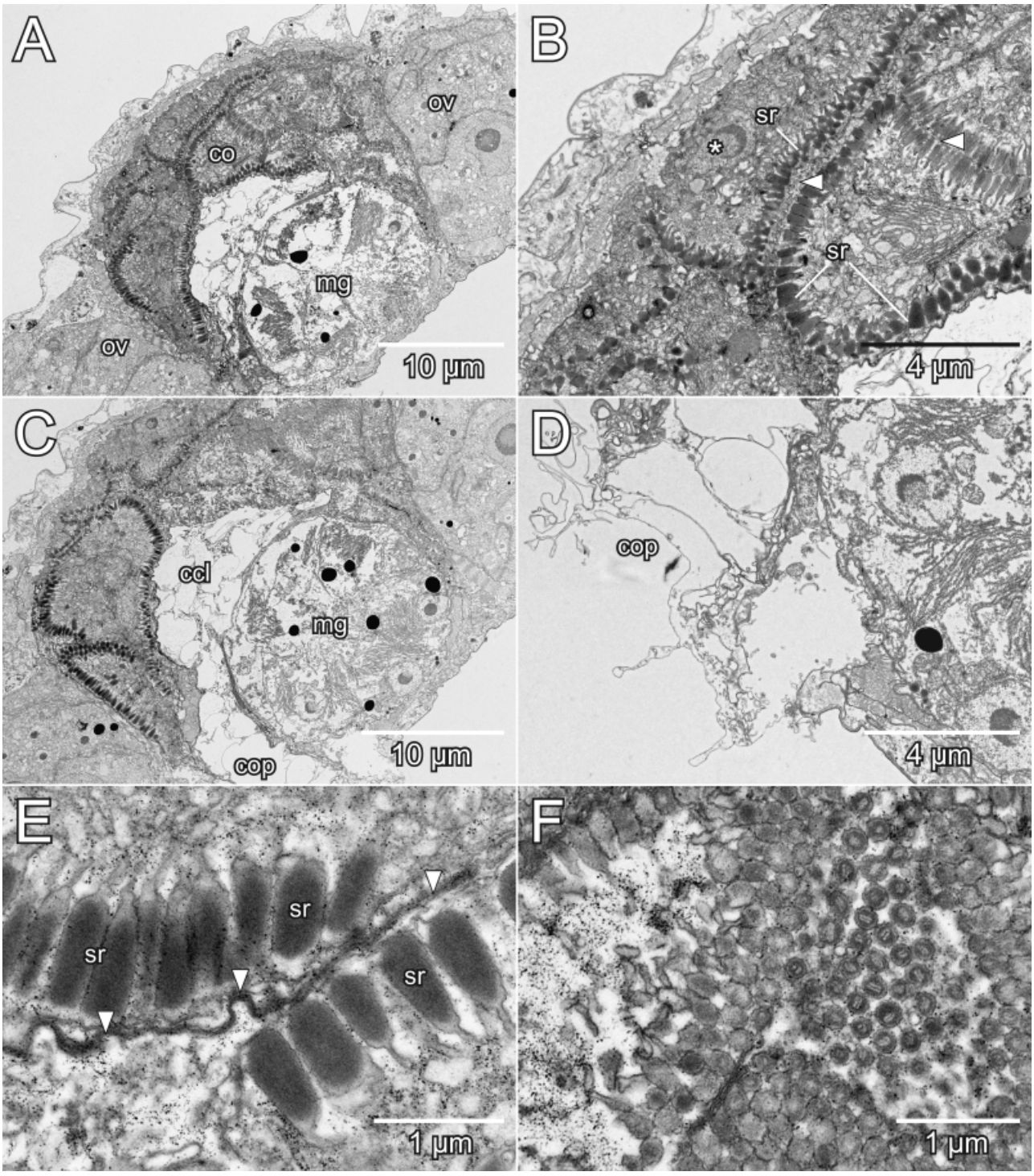

Fig. 9. Ultrastructure (transmission electron microscopy) of the caudal organ. **A.** Cross section of the caudal organ. **B.** Enlargement from (A) showing the branched peripheral caudal organ lumen (arrowheads) lined with numerous secretion rods (sr). **C.** Cross section of the caudal organ at the level of its pore; note the voluminous central caudal organ lumen (ccl). **D.** Detail of the caudal organ pore (cop). **E.** Secretion rods and the narrow peripheral caudal organ lumen (arrowheads). **F.** Tangential section of the secretion rods. ccl, central caudal organ lumen; co, caudal organ; cop, caudal organ pore; mg, midgut; ov, ovary; sr, secretion rods; asterisk, nucleus of the caudal organ.

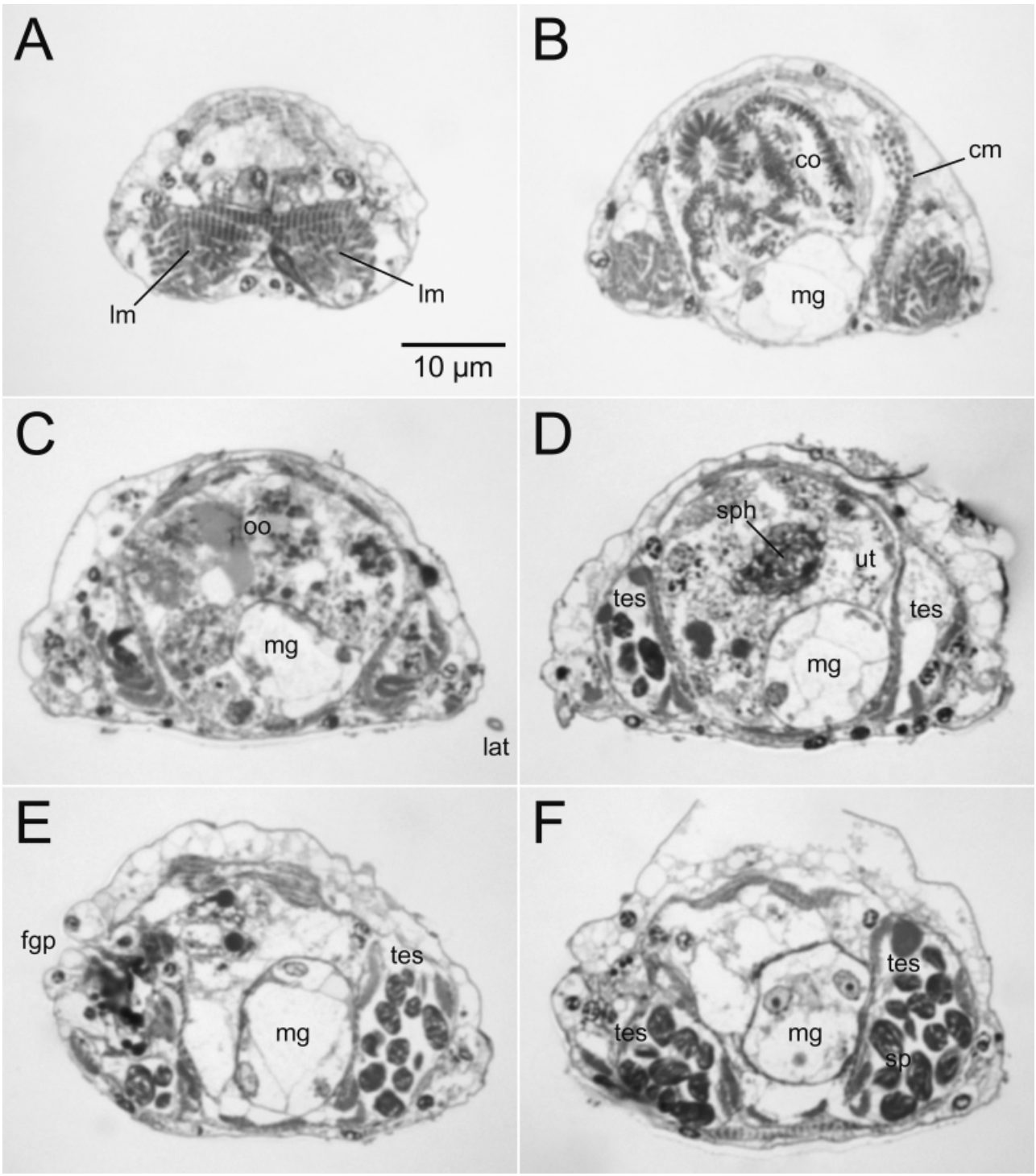

Fig. 10. Microscopic images (BF) of selected semithin cross sections at different levels of the reproductive system. Sections ordered from caudal to frontal, all images in scale. **A.** Rear trunk region with strong longitudinal muscles (lm). **B.** Caudal organ. **C.** Middorsal mature oocyte (oo). **D.** Uterus with a spermatophore (sph) containing allosperms. **E.** Female gonopore (fgp). **F.** Bilateral testes (tes) and bundles of spermatozoa (sp). cm, circular muscle; co, caudal organ; fgp, female gonopore; lat, lateral adhesive tube; lm, longitudinal muscle strands; mg, midgut; oo, oocyte; sp, bundles of spermatozoa; sph, spermatophore; tes, testis; ut, uterus.

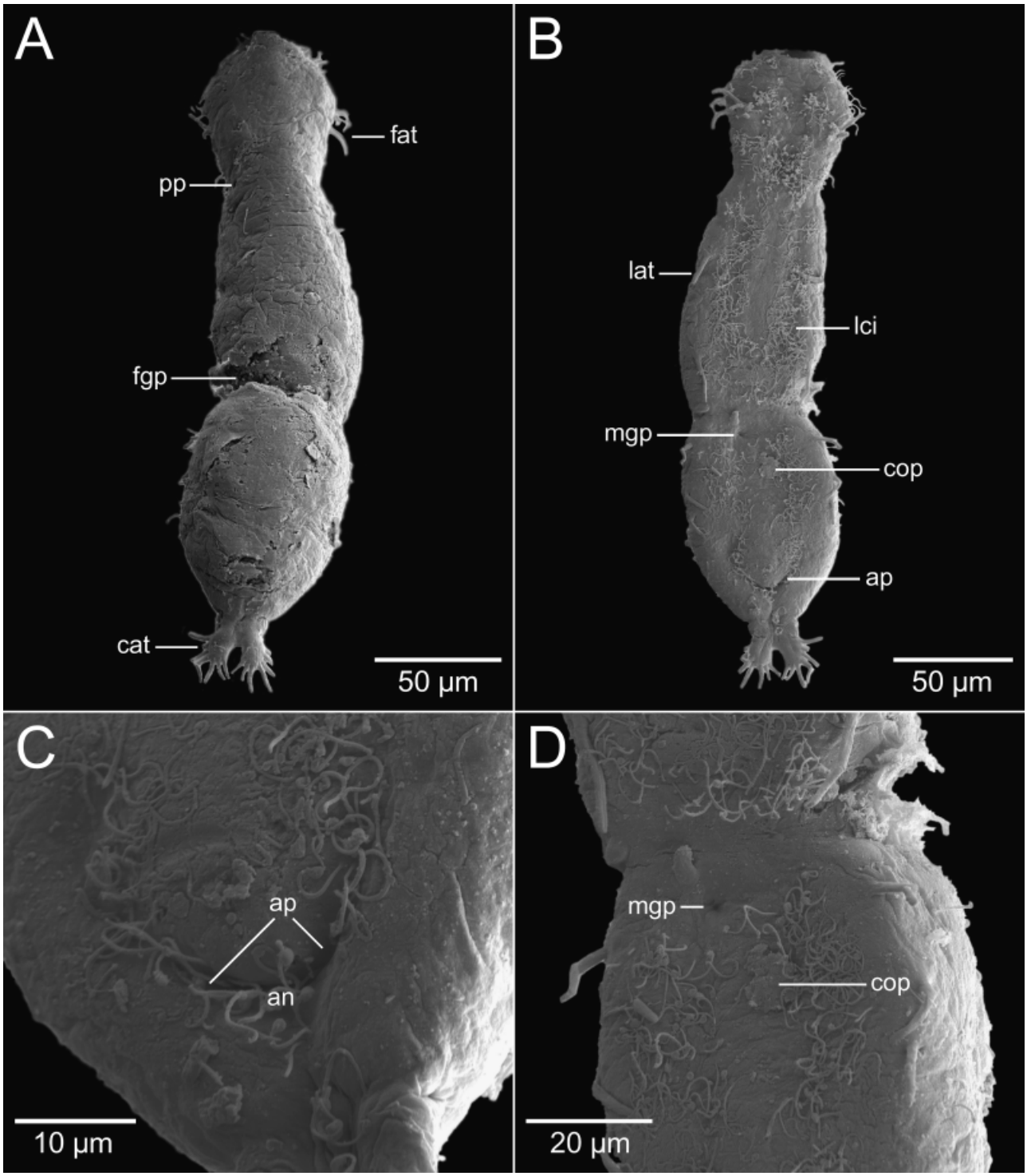

Fig. 11. Scanning electron microscopy. **A.** Dorsal view. **B.** Ventral view. **C.** Detail of the anal region, ventral view. **D.** Detail of the mid trunk region, ventral view. an, anus; ap, anal pit; cat, caudal adhesive tubes; cop, caudal organ pore, covered with mucous material; fat, frontal adhesive tubes; fgp, region of the female genital pore; lat, lateral adhesive tubes; lci, locomotory cilia; mgp, male genital pore (of the right testis); pp, pharyngeal pore.

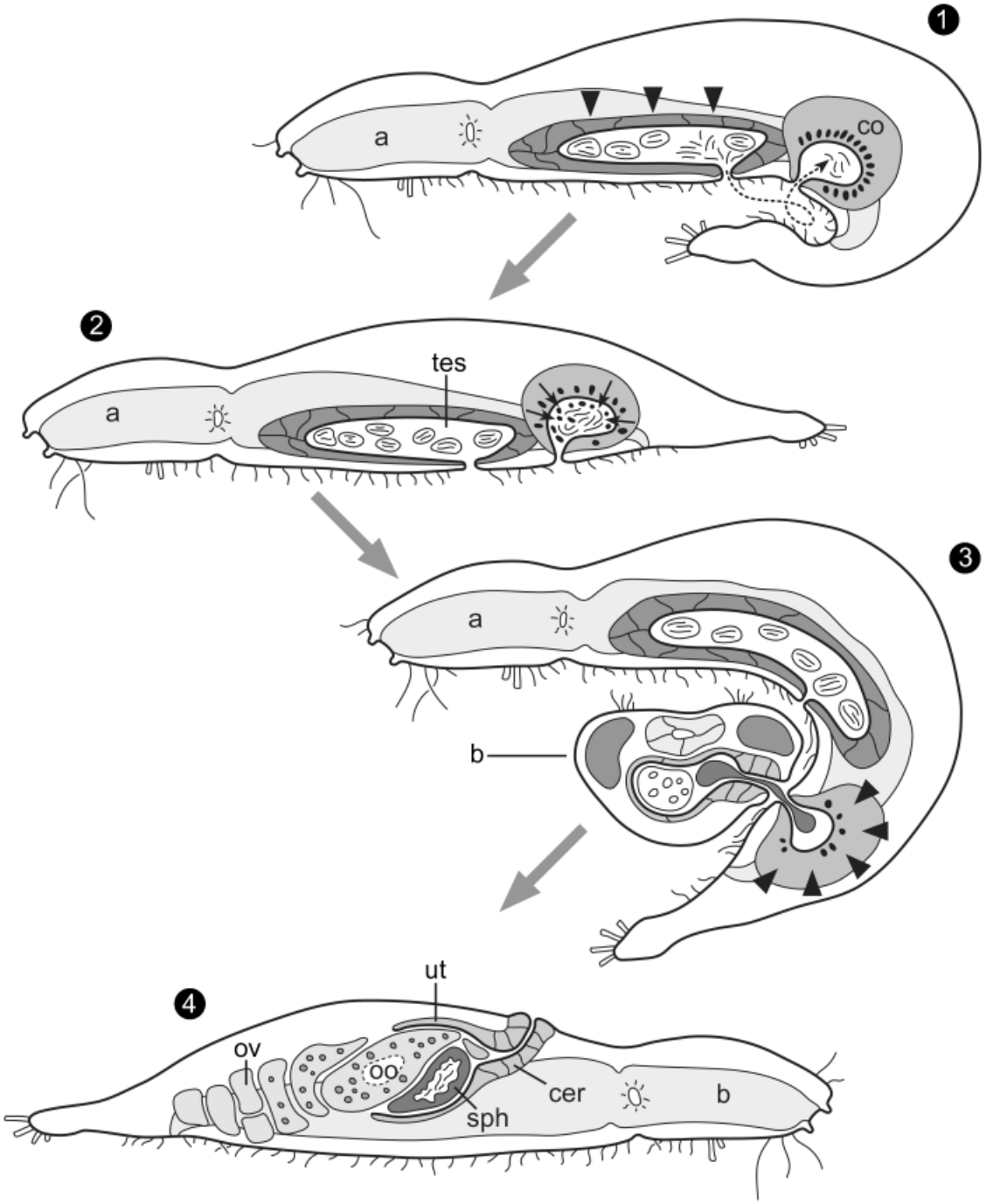

Fig. 12. Hypothetical mode of spermatophore formation and transfer in *Dactylopodola typhle*. (1) Spermatozoa are released through the male gonopores and transported toward the caudal organ (broken line) by beating of the ventral cilia. (2) Within the caudal organ, the secretion rods are discharged (black arrows) and envelope the autosperms thus forming the spermatophore. (3) The spermatophore is extruded from the caudal organ into the cervix of the mating partner. (4) In inseminated animals, the foreign spermatophore lies within the uterus beneath the mature oocyte. a, specimen a; b, specimen b; cer, cervix; co, caudal organ; oo, mature oocyte; ov, ovary; sph, spermatophore; tes, testis; ut, uterus; black triangles, pressure arising from contracting body muscles (especially ring muscles).

certain that there are no special or elongated seminal ducts. The lumen opens directly via the aforementioned pores.

Each testis is accompanied by longitudinal muscles and circular or C-shaped muscles (Fig. 6). These muscles are ''subtesticular'' and belong to the system

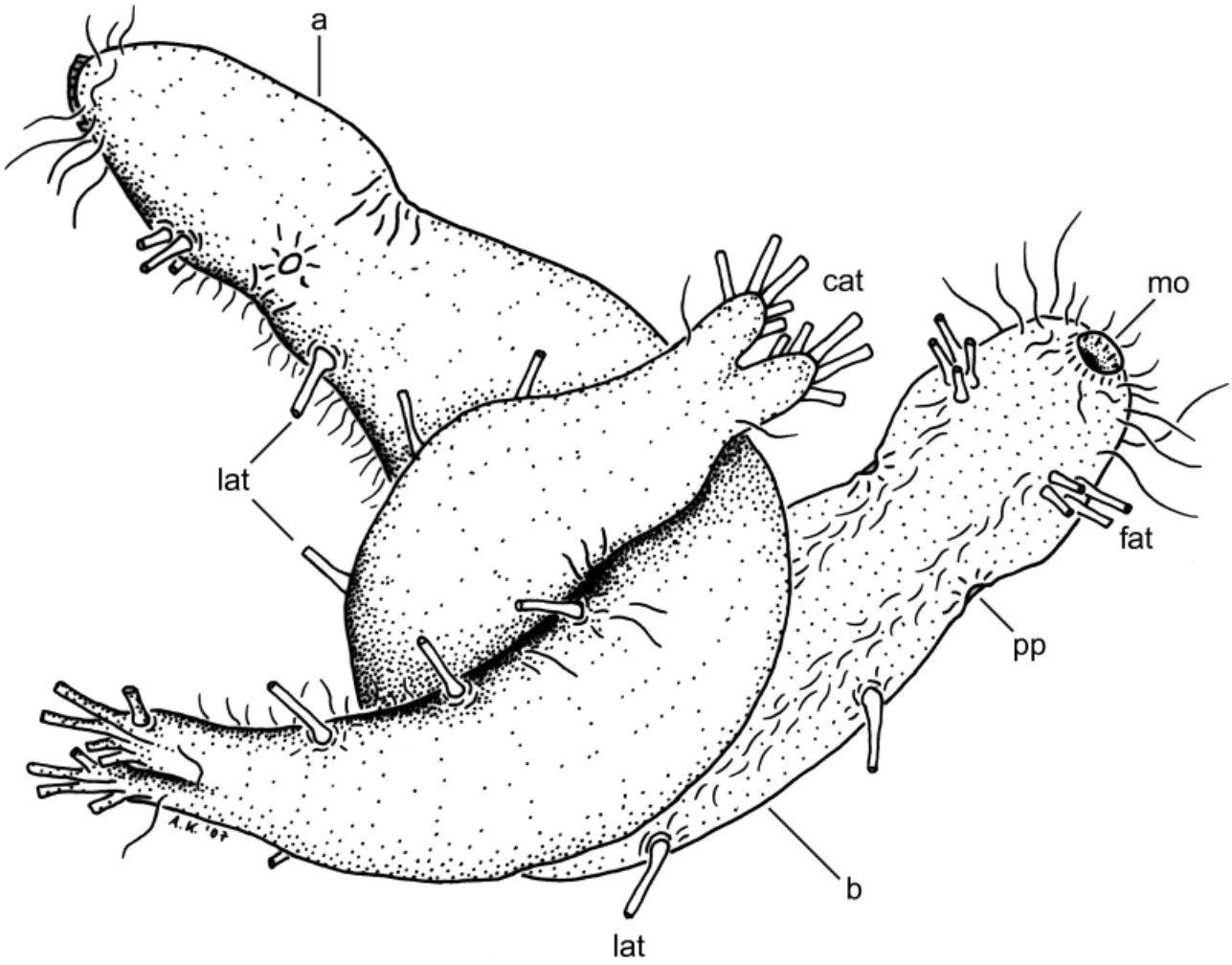

Fig. 13. Scheme of the hypothesized copula in *Dactylopodola typhle*. Two animals curl their caudal trunk section around one another. By doing so, the ventral caudal organ pore of one animal is adjacent to the dorsolateral female gonopore of the other one, facilitating transfer of the spermatophore. a, specimen a; b, specimen b; cat, caudal adhesive tubes; fat, frontal adhesive tubes; lat, lateral adhesive tubes; mo, mouth opening; pp, pharyngeal pore.

of trunk musculature. There is no close muscular sheath but rather distinct muscular strands. A definite peritesticular ECM is difficult to detect because the intercellular gap between testis and muscle cells is very narrow (Fig. 6E).

A detailed reconstruction of spermatogenesis and sperm ultrastructure is not the subject of this study and shall be given in another contribution.

Caudal organ

Within living, mature specimens of *D. typhle,* a glandular organ fills nearly the whole caudal fourth of the trunk region. Characteristic for this caudal organ is a pattern of numerous small refractive granules that are arranged in loops or regular clusters (Fig. 4D). The large, globular caudal organ is $\sim 50 \times 40\,\mu m$ in fronto-caudal dimension and width, and is 30 μm in height. It is situated dorsal to the intestine and sends two ventral lobes to each side of the gut (Figs. 1A,B, 3B, 9A,C, 10B). Within the caudal organ, there is a wide central lumen and many compressed peripheral caudal organ lumina. The peripheral lumina are connected with the central lumen and represent branched invaginations of the latter (Figs. 3B, 9A–C). The central caudal organ lumen opens to the outside via an unpaired ventral pore, the caudal organ pore (Figs. 1B, 9C,D, 11B,D). This pore is situated just a few micrometers beside the median line of the ventral surface of the animal and may be covered with mucous material (Figs. 9C,D, 11B,D).

Beneath the membrane of the central and peripheral lumen, there are a large number of secretion rods that are highly ordered and accompany the

margins of the lumen like rows of palisades (Figs. 1A, 3B, 9A–C). These rods have a conspicuous ultrastructure: they are membrane-bound granules with an apical ''trunk'' and a basal pedicle (dimension: 300 nm × 1 μm). Within the secretion rods, there is an electron-dense core and a more lucent cortex (Fig. 9E). Cross sections of such secretion rods revealed that the dense core is not of homogeneous content (Fig. 9F).

The cytoplasm of the caudal organ is filled with globular mitochondria and many membrane stacks (endoplasmic reticulum and dictyosomes), so that it has a general electron-dense appearance (Fig. 9A–C). Therefore, we could not determine whether the epithelial caudal organ is organized as a syncytium or is cellular. However, the caudal organ possesses many peripheral and active nuclei (e.g., Fig. 9B).

Anal pit and rear trunk muscles

During light microscopic and SEM observations, we discovered two interesting structures that we believe play an important role in the transfer and processing of the auto-spermatozoa: (1) a very strong longitudinal musculature in the rear trunk region and (2) a V-shaped groove along the ventral surface at the end of the trunk, i.e., the anal pit. Both these features can be resolved when observing live animals with differential interference contrast optics (Fig. 4E).

SEM reveals that two ventral bands of cilia come together and fuse into a single band around the anal pit (Fig. 11B,C). In cross sections of that region, there are several distinct packets of longitudinal muscles that nearly fill half of the trunk (Fig. 10A). The observation of living specimens under a dissecting microscope revealed that the animals are able to bend their tail and rear trunk section toward the ventral surface of the mid trunk.

Discussion

Reproductive anatomy within Macrodasyida

During the past decades, there has been considerable research activity dealing with reproduction biology (e.g., Hummon & Hummon 1992), gametogenesis (e.g., Hummon & Hummon 1983a,b), eggshell formation (Rieger & Rieger 1980), and sperm ultrastructure (see Marotta et al. 2005 and references therein) of gastrotrichs. Other contributions give a comprehensive overview of the different reproductive modes and organs that can be found in gastrotrichs (e.g., Hummon & Hummon 1988, 1989, 1993; Ruppert 1991; Balsamo et al. 1999).

Up to now, the reproductive system in eight species of Macrodasyida have been fully reconstructed or partly so on the basis of serial sections and ultrastructure: *Dolichodasys carolinensis* (Ruppert & Shaw 1977), *Macrodasys* sp. I and II (Ruppert 1978a), *Acanthodasys thrinax, Diplodasys ankeli, Platydasys* cf. *ocellatus, Tetranchyroderma bunti,* and *Thaumastoderma heideri* (Ruppert 1978b). For a congener of *Dactylopodola typhle, Dactylopodola baltica,* there is a detailed study on the reproductive behavior and life-cycle (Teuchert 1968) as well as ultrastructural data on the ovaries, oocytes, and spermatozoa (Fischer 1996). Nine additional species have been the subject of detailed investigations using conventional light microscopy, with further work on the ultrastructure of their sperm: *Acanthodasys aculeatus* (Guidi et al. 2003a), *Cephalodasys maximus* (Fischer 1994), *Lepidodasys unicarenatus* and *Lepidodasys* sp. (Guidi et al. 2004), *Mesodasys adenotubulatus* (Fregni et al. 1999), *Mesodasys laticaudatus* (Ferraguti & Balsamo 1994), *Neodasys ciritus* and *Musellifer delamarei* (Guidi et al. 2003b), and *Paraturbanella teissieri* (Balsamo et al. 2002). One species, the chaetonotid gastrotrich *Lepidodermella squamata,* has received the majority of attention concerning reproductive biology and behavior (Weiss & Levy 1979; Hummon 1984a,b,c), but for the purposes of reconstructing the ground pattern of the Gastrotricha, this chaetonotid and many of the macrodasyidans are highly derived and therefore unsuitable for understanding the plesiomorphic condition within the phylum. We therefore focus on the putatively basal species, *D. typhle,* and make comparisons only with the more derived taxa that have been the subject of detailed ultrastructural investigations, for the purposes of elucidating general patterns of reproductive biology in the Gastrotricha.

Gonads

Members of *D. typhle* are hermaphrodites with separate female and male gonads. Hermaphroditism is known for most of the species of Gastrotricha, but a hermaphroditic gonad that produces both male and female germ cells has only been verified for *T. bunti* and *T. heideri* (Ruppert 1978b). The paired ovaries of *D. typhle* fuse mid-dorsally in the trunk region and the gametes mature in a caudo-frontal direction. Such states of ovarial anatomy are also present in *D. baltica* (Fischer 1996), *P. teissieri* (Balsamo et al. 2002), and *P.* cf. *ocellatus* (Ruppert 1978b). An unpaired ovary, however, seems to be more common in Macrodasyida species, including *D. carolinensis* (Ruppert & Shaw 1977), *A. thrinax, D. ankeli,*

T. bunti, T. heideri (Ruppert 1978b), *Macrodasys* sp. (Ruppert 1978a), and *Mesodasys* sp. (Ferraguti & Balsamo 1994; Fregni et al. 1999).

Thin epithelial ovarial walls are reported for most species mentioned above. The presence of such wall cells is not reported for *M. adenotubulatus* (Fregni et al. 1999), *M. laticaudatus* (Ferraguti & Balsamo 1994), and *P. teissieri* (Balsamo et al. 2002), but the ovaries of these species were not investigated at the ultrastructural level. Moreover, it remains unclear in which regions of the ovary or ovaries an epithelium exists, when it is in fact reported. In some cases, the ovarian wall might be mistaken to be a uterine wall. *D. typhle* definitely lacks an ovarial epithelium. Here, early oogenic germ cells are clearly enveloped by ECM. In *D. baltica,* however, there is an "incomplete covering of wall cells" (Fischer 1996). We think these "wall cells" could be misinterpreted cell bodies of, for example, parenchyma cells of undetermined function.

Oviducts are often reported as solid cellular bands or masses that connect the ovaries to a genital pore (e.g., see Ruppert 1978b); these have been described in *A. thrinax, D. ankeli, P.* cf. *ocellatus, T. bunti,* and *T. heideri* (Ruppert 1978b). A direct connection of the uterus (the region of the ovary surrounding the mature oocyte) to a permanent or preformed female gonopore, as was found in *D. typhle,* is likely at least in *A. thrinax* and *D. ankeli* (Ruppert 1978b). The "rosette organ" of these species has been interpreted as the female gonopore (Ruppert 1978b). At this point, we have to comment on the term "oviduct." The use of this term, as defined for gastrotrichs by Ruppert (1978b), is confusing because it refers to both the compact bands or masses of cells (as mentioned above) and to an ovarian epithelium. In this study, we have chosen to differentiate the ovarian epithelium (as a uterus) from the compact mass of cells (as a cervix and not an oviduct). This terminology helps to distinguish these two systems based on ultrastructure and function. We develop a hypothesis on the function of the cervix below.

Paired bilateral testes are very common in macrodasyid gastrotrichs. Within the Thaumastodermatidae, there are species with a single testis, a clearly derived condition in the Macrodasyida. This testis is disposed to the left or the right side of the body in *P.* cf. *ocellatus, T. bunti,* and *T. heideri* (Ruppert 1978b). Testicular walls consisting of the germ cells themselves—a germinal epithelium—as is present in *D. typhle,* are known from several species. In most cases, these germinal epithelia are restricted to a certain region of each testis. Other regions of the testes are covered with somatic wall cells (Ruppert 1991). For example, strongly flattened epithelial cells are reported for *A. thrinax* and *T. heideri* (Ruppert 1978b). In *D. typhle,* however, such flattened areas of the testicular wall are protrusions provided by the germ cells. In Macrodasyida, maturation of male germ cells may proceed from caudal to frontal but also in the opposite direction. In *D. typhle,* spermatogenesis occurs along the whole length of each testis, from the dorsal and medial walls toward the testicular lumen. During spermatogenesis, spermatogenic cysts are formed. Later, these cysts develop into clusters of curled spermatozoa. Such a mode of spermatogenesis may also be present in *Dactylopodola mesotyphle* (Hummon et al. 1998), *Dactylopodola* sp. (Ruppert 1991), and in the recently described chaetonotid *Diuronotus rupperti* (Todaro et al. 2005), because all of these species develop putative spermatogenic cysts.

All species mentioned here, with the exception of *D. baltica* (where no data on the testicular morphology exist), possess distinct seminal ducts (vasa deferentia). These ducts may fuse like in *M. adenotubulatus* (Fregni et al. 1999), *P. teissieri* (Balsamo et al. 2002), and in all of the Turbanellidae. Seminal ducts open separately into one or two pores on the ventral surface of the animal like in *D. carolinensis* (Ruppert & Shaw 1977), *Macrodasys* sp. (Ruppert 1978a), *T. bunti,* and *T. heideri* (Ruppert 1978b), or discharge internally into the caudal organ like in *A. thrinax, D. ankeli, P.* cf. *ocellatus* (Ruppert 1978b), *M. adenotubulatus* (Fregni et al. 1999), and *M. laticaudatus* (Ferraguti & Balsamo 1994). Additionally, we know from most species descriptions that the single seminal duct discharges directly into the caudal organ in all of the Thaumastodermatinae. In *D. typhle,* there are no seminal ducts; testes open directly to the outside via preformed gonopores.

Accessory sex organs

In addition to the gonads, species within the Macrodasyida and those of the taxon *Neodasys* possess a set of so-called accessory reproductive organs, which play an important role in sperm processing, transfer, storage, and fertilization. These organs are (1) the caudal organ, which can function as a penis, copulatory, or spermatophoral organ and (2) the frontal organ, which may function as a bursa and/or a seminal receptacle (Ruppert 1991). However, a caudal organ was not detected in *P. teissieri* (Balsamo et al. 2002) or in *N. ciritus* (Guidi et al. 2003b). Although the caudal organs show considerable structural variation among Gastrotricha, the actual function and role of these organs in the life cycle often remain unclear (Ruppert 1991).

According to several studies (Ruppert & Shaw 1977; Ruppert 1978a,b, 1991), the caudal organ is a hollow epithelial, secretory organ, often equipped with a muscular sheath. The caudal organ lumen can be complex and subdivided as in *Macrodasys* sp. (Ruppert 1978a) or it is a simple, spheric chamber as in *Mesodasys* sp. (Ferraguti & Balsamo 1994; Fregni et al. 1999). There are at least as many different expressions of the caudal organ as genera or even species of Macrodasyida. In some genera, the caudal organ provides protrusible copulatory tubes that are equipped with cuticularized hard structures (*Macrodasys* sp., Ruppert 1978a) or stylets (*Urodasys* spp., Schoepfer-Sterrer 1974). The function of the caudal organ for picking up the autosperms, sperm transfer, and insemination of a sexual partner is well documented for at least two undetermined *Macrodasys* species (Ruppert 1978a). It is suspected that this type of coupling of multiple functions for the caudal organ is widespread within the Macrodasyida, with the exception of the aberrant conditions in Thaumastodermatidae, in which self-fertilization is likely (Ruppert 1991). For *D. typhle,* we suggest the caudal organ is a spermatophore-building and -transferring feature. Despite the fact that we have never observed spermatozoa or a ready-to-release spermatophore in the caudal organ of *D. typhle,* our structural data of the whole reproductive system, especially the occurrence of spermatophores, give strong support for our assumption. Micrographs of a recently described species, *Dactylopodola australiensis,* give evidence for a caudal organ that contains secretion rods, as does the caudal organ of *D. typhle* (Hochberg 2003; Fig. 3B). Additionally, we suggest that the ''spindle-shaped structures'' (Hochberg 2003; Fig. 3B) are an autospermatophore of *D. australiensis*. For *D. baltica* and an undescribed *Dactylopodola* species from South Carolina (USA), there is the hypothesis that the whole seminal receptacle (frontal organ), into which the seminal ducts discharge, is released *in toto* as a spermatophore (Teuchert 1968; Ruppert 1991).

Members of *D. typhle* do not possess a distinct frontal organ as present in almost all Macrodasyida (Ruppert & Shaw 1977; Ruppert 1978a,b, 1991). Species of *Mesodasys* also appear to lack this organ (Ferraguti & Balsamo 1994; Fregni et al. 1999). However, individuals of *D. typhle* have a well-developed cervix and uterus that functions in place of a formal frontal organ, or ''seminal receptacle.'' Based on our structural observations of the cervix and the position of allospermatophores, we suggest that the cervix directs allospermatophores from the female gonopore toward the mature oocyte. The uterus is the presumed site of fertilization, as observations indicate that both allospermtophore and mature oocyte are in proximity to one another and are always surrounded by the uterine wall. The opening for this ''seminal receptacle'' is provided by the cervix lumen and the female gonopore.

Distinct frontal organs in other species of Macrodasyida that function as seminal receptacles often lie frontally or caudally to the mature oocyte, and provide an internal pore that is directed toward the egg, e.g., *Macrodasys* sp. (Ruppert 1978a), *A. thrinax, D. ankeli, T. bunti,* and *T. heideri* (Ruppert 1978b). This close relation of the frontal organ and the uterus could indicate a continuity of both organs. We suggest that, within Gastrotricha, a distinct frontal organ is a specialized compartment of the uterus. In this context, one has to reconsider the question of homology of the frontal organ of different gastrotrich taxa. Here, it might be important which region or pole of the uterus or oviduct (or cervix) is exactly involved in frontal organ formation.

Body musculature

Gonads and the caudal organ in *D. typhle* are accompanied by longitudinal and circular muscles of the trunk but do not likely possess a dedicated musculature. While the muscular system in *D. typhle* has not been analyzed, there is a detailed study on the musculature of *D. baltica* using fluorescence microscopy (Hochberg & Litvaitis 2001b). As we suspect no fundamental differences between these two congeneric species, we will compare the muscular pattern of *D. baltica* with our findings on the reproductive system of *D. typhle*.

The ventrolateral longitudinal muscle strands of *D. baltica* have a prominent splitting in the rear trunk region. According to Hochberg & Litvaitis (2001b), this division correlates with the position of the paired testes. We suggest that each testis is accompanied by both longitudinal muscle portions and the correlated somatic circular muscles. Pressure of the contracting muscles may discharge the testicular lumen. Furthermore, we presume that the uterus and the caudal organ, together with the intestine (the diameter of the gut in the region of the reproductive organs is only a third of that in more frontal regions), lie inside the splanchnic musculature (helicoidal, circular, and longitudinal muscles; see Hochberg & Litvaitis 2001b). These observations are consistent with our ultrastructural findings.

Spermatophore transfer in *D. typhle*

Detailed studies on mating behavior and gamete transfer exist for at least three species of Macrodasyida.

While for *D. baltica* and *Turbanella cornuta* these studies were based exclusively on light microscopic observations (Teuchert 1968), a combination of studies using living specimens, TEM, and SEM have been applied to discover the entire mating process of *Macrodasys* sp. (Ruppert 1978a). This study currently provides the most comprehensive information on the mating process of a macrodasyidan gastrotrich (see Ruppert 1991 for summary). In our study, we could not make extensive observations on the mating behavior because we did not have a stable laboratory culture of *D. typhle*. However, we can suggest a hypothesis on gamete processing, transfer, and mating on the basis of our structural results. This hypothesis has to be verified subsequently by studying the "natural communities" of *D. typhle*.

We suggest that individuals of *D. typhle* charge their own caudal organ with autosperms by curling the rear trunk region toward the ventral surface of the trunk. When the immobile sperms are released through the male gonopores, they are transported by the beating of the ventral cilia toward the anal pit. As the apex of the anal pit is now aligned with the caudal organ pore, the spermatozoa can be placed directly into the caudal organ lumen (Fig. 12: 1). Here, the spermatophore is formed by fusion of the secretion products of the released secretion rods (Fig. 12: 2). Cross-insemination might be realized when two individuals of *D. typhle* curl their caudal ends around one another (Fig. 13). Consequently, each animal brings its own caudal organ pore in contact with the female gonopore of the mating partner (Fig. 12: 3). When the splanchnic muscular rings and strands that accompany the caudal organ are contracted, the spermatophore is pressed into the cervix and further into the uterus of the mating partner (Fig. 12: 4). Additional secretion products of the caudal organ may provide a seal that closes one branch of the cervix lumen. This kind of sperm transfer corresponds to the conditions in *Macrodasys* sp. (Ruppert 1978a, 1991). Fertilization has to take place within the uterus although we have not yet found evidence for this. However, fertilization within the uterus and ovaries has been verified in *D. baltica* (Fischer 1996). The mature and fertilized egg of *D. typhle* is released via the cervix lumen and the female gonopore, and not by rupture of the body wall as is reported for *D. baltica* (Teuchert 1968). Here again, contracting body muscles could squeeze the mature oocyte out of the uterus.

Members of *D. baltica* are protandric hermaphrodites that pass several alternating male and female phases during an individual life (Teuchert 1968). For *D. typhle,* we are not able to give information on a possible protandric or a protogynous life cycle. The sectioned specimens had fully developed female gonads and eggs ready to release. The testes were still in the state of spermatogenesis but with no male gonopores formed. This may give evidence for a protogynous life cycle, but if the fixed animals resided in a phase between both sexes, our data would not disagree with a protandric life cycle for *D. typhle*.

Compared with previous data and hypotheses on mating behavior in *D. baltica* (Teuchert 1968) and *Dactylopodola* sp. (Ruppert 1991), our observations indicate that *D. typhle* engages in a more direct form of copulation. For example, both of the previous species engage in a form of indirect copulation, wherein they place their spermatophores externally on their mating partners; the recipient must then, via an undetermined process, transfer the spermatophore to the female gonopore and into the uterus. This hypothesis by Teuchert (1968) contrasts with our observations on *D. typhle,* which indicate that the spermatophore is injected directly into the mating partner's female gonopore. While these two processes are quite different, they emphasize the fact that even closely related species in the Gastrotricha may show drastically different forms of mating.

Phylogenetic prospects

Despite a large number of morphological studies on the whole reproductive system in certain gastrotrich species, there are few ultrastructural investigations and reconstructions of the reproductive system of the important (putative) basal taxa. As Dactylopodolidae is suspected to occupy the most basal position within Macrodasyida (Hochberg & Litvaitis 2000, 2001a) or even within Gastrotricha (Zrzavý 2003), our study on *D. typhle* is the first step toward elucidating the reproductive anatomy and reproductive mechanisms of the stem species of Gastrotricha. We provide a brief outlook on the major effects that the results of this study will have on the reconstruction of the ground pattern of Gastrotricha.

Paired gonads. Within Gastrotricha, there are species, or supra-specific taxa, that are characterized by an unpaired, medial ovary, and others that have bilateral ovaries like *D. typhle*. Among Gastrotricha, testes are predominantly paired organs. Taxa that possess a single, unilateral testis, such as species of Thaumastodermatinae, represent the derived condition in the Macrodasyida (Hochberg & Litvaitis 2000, 2001a; Todaro et al. 2006). Considering the basal position of *Dactylopodola,* the stem species of Gastrotricha or at least the stem species of

Macrodasyida would have paired testes and paired ovaries. A fusion of the latter is probably obligatory.

Existence of the frontal organ. If we consider that members of *D. typhle* do not have a distinct frontal organ, it is possible that this structure, perhaps as a special formation of the uterus wall or cervix, evolved later during phylogenesis of Gastrotricha. This issue has to be handled with care because the morphological situation in other *Dactylopodola* species is very uncertain and a distinct frontal organ may exist in these species.

Spermatophore transfer. For members of *Dactylopodola,* this mode of mating is unquestionable. It is likely that spermatophore formation and transfer is present in other species of Gastrotricha (e.g., *Neodasys,* Ruppert 1991), and may even be part of the ground pattern of Gastrotricha, since *Neodasys* occupies a basal position within Chaetonotida, the sister taxon of Macrodasyida. But as the occurrence of spermatophores is often coupled with aberrant and immobile sperms (Fischer 1996), we think spermatophore formation within Gastrotricha reflects the derived condition. This is consistent with the phylogenetic analysis of Todaro et al. (2006). Here, *Neodasys* and *Dactylopodola,* together with a few other macrodasyidan taxa are positioned in a common monophyletic group for which spermatophore formation might be synapomorphic.

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