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Ontogeny of the Complex Sperm in the Macrostomid Flatworm *Macrostomum lignano* (Macrostomorpha, Rhabditophora)

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ABSTRACT Spermiogenesis in *Macrostomum lignano* (Macrostomorpha, Rhabditophora) is described using light- and electron microscopy of the successive stages in sperm development. Ovoid spermatids develop to highly complex, elongated sperm possessing an undulating distal (anterior) process (or "feeler"), bristles, and a proximal (posterior) brush. In particular, we present a detailed account of the morphology and ontogeny of the bristles, describing for the first time the formation of a highly specialized bristle complex consisting of several parts. This complex is ultimately reduced when sperm are mature. The implications of the development of this bristle complex on both sperm maturation and the evolution and function of the bristles are discussed. The assumed homology between bristles and flagellae questioned. J. Morphol. 270:162–174, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: spermiogenesis; flatworm; *Macrostomum lignano*; bristles; ultrastructure

Spermiogenesis and sperm morphology are often considered important characters in studies on the phylogenetic relationships within the taxon Platyhelminthes (flatworms) (Hendelberg, 1965, 1969a,b, 1974, 1977, 1983, 1986; Euzet et al., 1981; Ehlers, 1985; Justine, 1991, 1995, 2001; Bâ and Marchand, 1995; Watson and Rohde, 1995; Watson, 1999, 2001; Rohde, 2001; Miquel et al., 2005; Levron et al., 2006; Liana and Litvaitis, 2007). In flatworms, sperm (the germ line), as well as the rest of the male somatic germ line (i.e., testes, copulatory stylet), are formed by neoblasts (stem cells) during postembryonic development through epigenesis (Zayas et al., 2005). It is still poorly understood how the testes are formed de novo, and how sperm develop from primordial germ cells within them.

Macrostomid flatworms (Macrostomorpha, Rhabditophora) have sperm with an unusual, complex morphology (Hendelberg, 1969a,b; Rieger, 1971; further references in Rohde and Watson, 1991; Rohde and Faubel, 1997, 1998). Some species have sperm with two well-developed lateral bristles (e.g., *Macrostomum tuba*; see Rhode and Watson, 1995), whereas in other species these bristles are rudimentary (e.g., *Macrostomum pusillum*; see Rhode and Faubel, 1997) or even absent (*Paramalostomum fusculum*, *M. rubrocinctum*; see Hendelberg, 1969a). Remarkably, although bristles are considered an autapomorphy for the Macrostomida (Watson and Rhode, 1995), their ultrastructure and ontogeny were never adequately described, and it is still unclear whether these bristles are modified flagellae or not and what their exact function may be. To better understand spermiogenesis and sperm ultrastructure in the taxon Macrostomida, we studied these features in *Macrostomum lignano* (Ladurner et al., 2000, 2005; habitus in Fig. 1A,B, sperm in Fig. 1C), a species having sperm with bristles. We scrutinized the process of spermiogenesis by studying the successive developmental stages within the testes and in the vesicula seminalis.

MATERIALS AND METHODS

Culture

M. lignano (for a comprehensive description see Ladurner et al., 2005; see habitus in Fig. 1A,B) is a marine flatworm from the Adriatic (Lignano, Italy). The animals were cultured as described by Rieger et al. (1988).

Light Microscopy

Adult sperm were studied from animals placed on a slide with a few drops of artificial seawater (ASW), cut transversally through the false seminal vesicle, and then covered with a cover slip and excess water removed with filter paper. Finally, the slides were sealed using vaseline. For the study of earlier devel-

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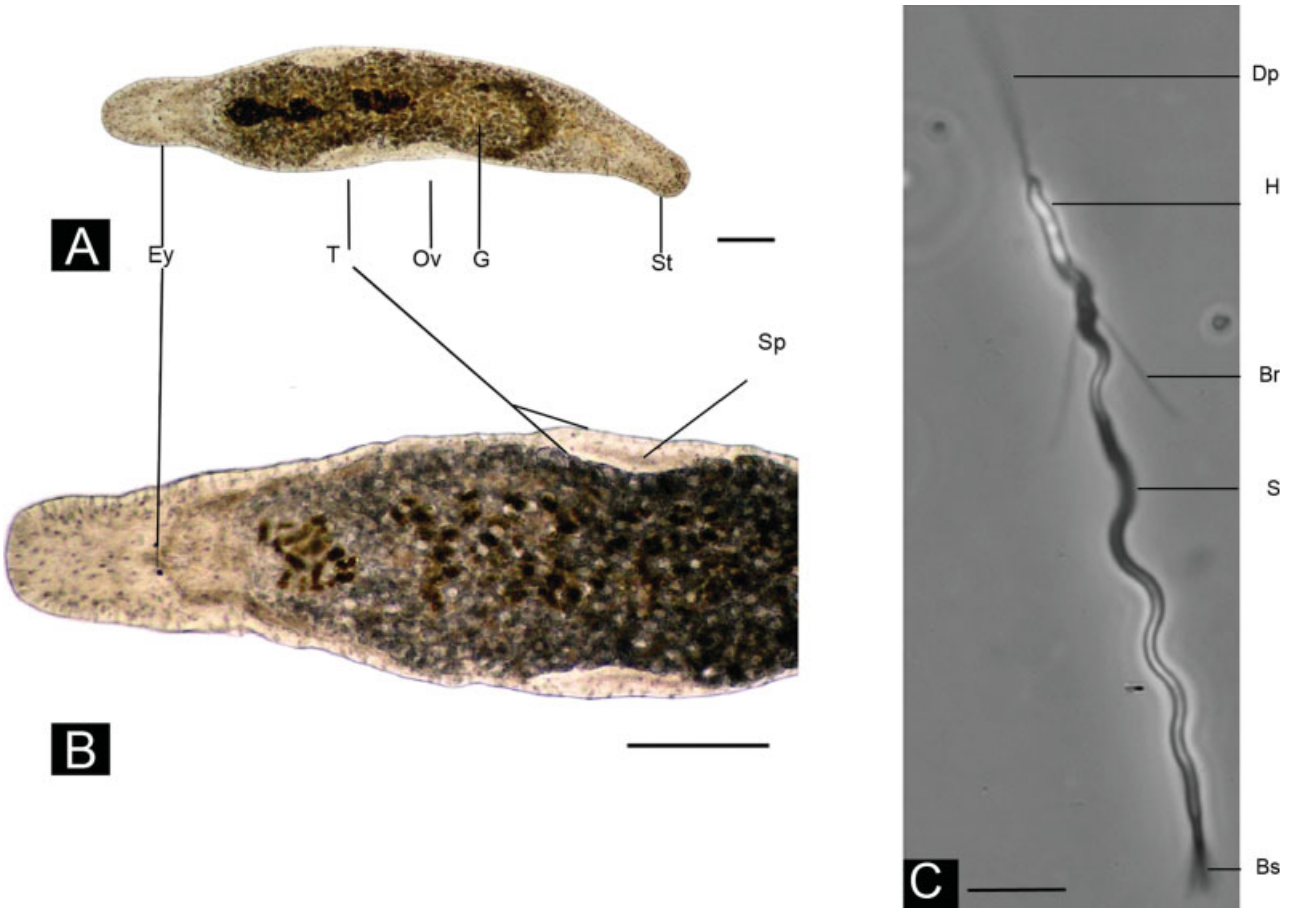

Fig. 1. *Macrostomum lignano*. **A**: Habitus. Anterior is to the left. **B**: Habitus with detail of testes region. Note the darker region in the middle of the testes representing undulating sperm. Anterior is to the left. **C**: Adult sperm. Top is anterior (A), bottom is posterior (P). Br, bristle; Bs, brush; Dp, distal process (feeler); Ey, eyes; G, gut; H, head; Ov, ovarium; S, shaft; St, position of copulatory stylet; T, testes. Scale bar A, B: 200 μm; C: 20 μm.

opmental stages, animals were treated in the same way, but were cut transversally through the testes. The slides were studied using an Olympus BX 51 microscope (equipped with an Olympus C5060 digital camera), using interference contrast. In some cases (e.g., study of the bristles), phase contrast proved to give better results, for which we used an Olympus BH2 microscope (equipped with a Canon EOS 350D Camera).

Transmission Electron Microscopy

Adult worms were anesthetized using a 1/1 ASW-$MgCl_2$ (7.14%) solution and fixed in Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde) in an 0.2 M Na-cacodylate buffer (pH 7.4), containing 21 mg/ml NaCl. After overnight incubation at 4°C, the specimens were rinsed for 8 h in Na-cacodylate buffer. Worms were then postfixed in 2% osmium tetroxide with a Na-cacodylate/NaCl buffer. After overnight incubation, animals were rinsed again and dehydrated in a standard ethanol series and finally embedded in Spurr's low viscosity resin (Spurr, 1969).

Areas of interest were located using semithin sections (0.5 μm), made with a Reichert-Jung ultracut E ultramicrotome, stained with a double staining (5% methylene blue and 5% azur II in 5% borax), and mounted in DePeX (Gurr, BDH laboratory, UK). Serial ultrathin (70 nm) sections of testes and seminal vesicle were made using a Leica-Ultracut-S-ultramicrotome (Leica, Vienna, Austria), mounted on formvar-coated single-slot copper grids (Agar Scientific, Stansted, UK) and contrasted with lead citrate and uranyl acetate (EMstain, Leica). Observations were made with a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan) operating at 60 kV, and pictures were digitized using a DITABIS system (Pforzheim, GERMANY).

RESULTS

Light Microscopic Observations: Morphology of Adult Sperm

The adult sperm is elongated (Fig. 1C). Anteriorly, the head is somewhat swollen, with a long, narrow, rapidly undulating extension (termed "distal process" by Newton, 1980 and "feeler" by Ferguson, 1940a,b). At the junction between head and shaft a pair of bristles projects posteriorly. At the posterior end, a terminal brush occurs.

Light Microscopic Observations: Developmental Stages

Spermatids develop in clusters of four cells. Several of these clusters often lie very near to each

other, giving the impression that some clusters consist of more than four cells (see two clusters of four in Fig. 2A). The four cells are connected to each other by cytoplasmic bridges (cytophore) and differentiate synchronously (Fig. 2B).

Early spermatids form a conical projection at the distal end (Fig. 2B), which gradually elongates. The lateral bristles appear at the base of the conical projection (Fig. 2B′). During further development, the conical projection grows somewhat but elongation of the sperm is mainly due to the elongation of its proximal part, forming the shaft and the head of the sperm.

Mid spermatids are characterized by further elongation of the shaft (Fig. 2C) and the formation of a notch proximal to the bristles and a lateral invagination distal from the bristles (Fig. 2G).

Late spermatids remain connected to each other by a residual body (Fig. 2D,D′) and undulating movements of the shaft and distal process are visible.

At the proximal end, where sperm detach from the cytoplasmic residues (Fig. 2D), a terminal brush consisting of short (±3 μm) pointy extensions develops (Fig. 2E; magnification of brush in Fig. 2F). From the moment of detachment spermatozoa move with the distal end forward. Therefore, in mature sperm, we refer to the distal end as anterior and the proximal end as posterior. In the testes, the bristles are always oriented anteriorly (detail of bristle area in Fig. 2G) or have started to rotate posteriorly (Fig. 2E). In the seminal vesicle, however, the bristles are always oriented posteriorly (Fig. 2H) (Fig. 2p and Fig. 4j in Ladurner et al., 2005 were labeled as mature sperm but probably depicted immature sperm from the testes).

Ultrastructural Changes During Spermiogenesis

General observations. As described earlier from light microscopy, spermatids are always organized in clusters of four cells, which are connected to each other by cytoplasmic bridges (asterisk in Fig. 3A). When the distal end of the conical projection starts to elongate, the nucleus also elongates and migrates into the elongating conical projection, preceded by the dense bodies and followed by mitochondria (Fig. 3B,C).

The plasma membrane of the conical projection is supported by two sets of cortical microtubules. Cortical microtubules occur in two groups, separated by a part of the membrane devoid of tubules, and extend longitudinally throughout the entire length of the spermatid. Each group consists of a maximum of 42 microtubules (Fig. 3D) with a decreasing number toward both ends of the spermatid. At both sides of the spermatid in the mid region, a longitudinal cytoplasmic ridge or wing occurs, supported by the two outermost microtubules of each set with some electron-dense material underneath (Fig. 3D′), the so-called dense fiber (term used by Newton, 1980). Connections between the cortical microtubules and the cell membrane were not observed.

In the distal process, there is a continuous sheath of 8–10 microtubules, no "wings" and no dense fiber. In mature sperm, these microtubules extend into the posterior tapering structures, which together form the terminal brush. These tapering structures have a diameter of about 60 nm and are up to 3 μm long. Their exact number could not be determined (Fig. 3E).

The nucleus of the early spermatid is spherical (Figs. 2A and 3A), but it becomes more and more elongated in later stages during distal migration (Figs. 2C and 3B). In mature sperm, it is long and narrow. The karyoplasm in early spermatids consists of fine granules, uniformly distributed within the nucleus. During nuclear elongation, the chromatin condenses into a number of discrete bodies, which stay connected to each other by small bridges. This gives the nucleus a "sausage link" appearance (Fig. 3F,F′). This chromatin condensation occurs in a very late phase of spermiogenesis, just before the sperm become mature. In mature sperm, the nucleus is located proximally from the bristles, and at one side of the sperm when observed on longitudinal sections (no nucleus present anterior from the bristle complex in Fig. 3G).

Most mitochondria are closely associated with the migrating nucleus and the head region (Fig. 3A,C) although some are present throughout the entire sperm except at the very proximal end.

There are three types of dense bodies in the fully mature sperm (Fig. 3G): T1, T2, and T3, all associated to some extent with the plasma membrane. T1 are very electron dense, elongated to kidney-shaped, 50 nm in diameter and maximally 300 nm long. They are most distal in the conical projection when it elongates. T2, moderately electron-dense, have a diameter of ±200–300 nm, and migrate into the conical projection behind type 1. T3 have a diameter of 30 nm, have a tubular nature and appear in the early spermatid shortly after the other two types. In mature sperm, these tubules bend and are associated both with the plasma membrane of the notch (posterior of the base of the bristles) as well as the plasma membrane of the lateral invagination (anterior of the base of the bristles).

In mature sperm T1, T2, and most of T3 dense bodies are found in the head region of the sperm anterior to the bristles, each type within a specific region, T1 most anterior, T2 behind T1, and T3 behind T2, closest to the base of the bristles (Fig. 3G).

Ontogeny and Structure of the Bristles

In the early spermatid, the bristles originate at the base of the conical projection as short (±3 μm

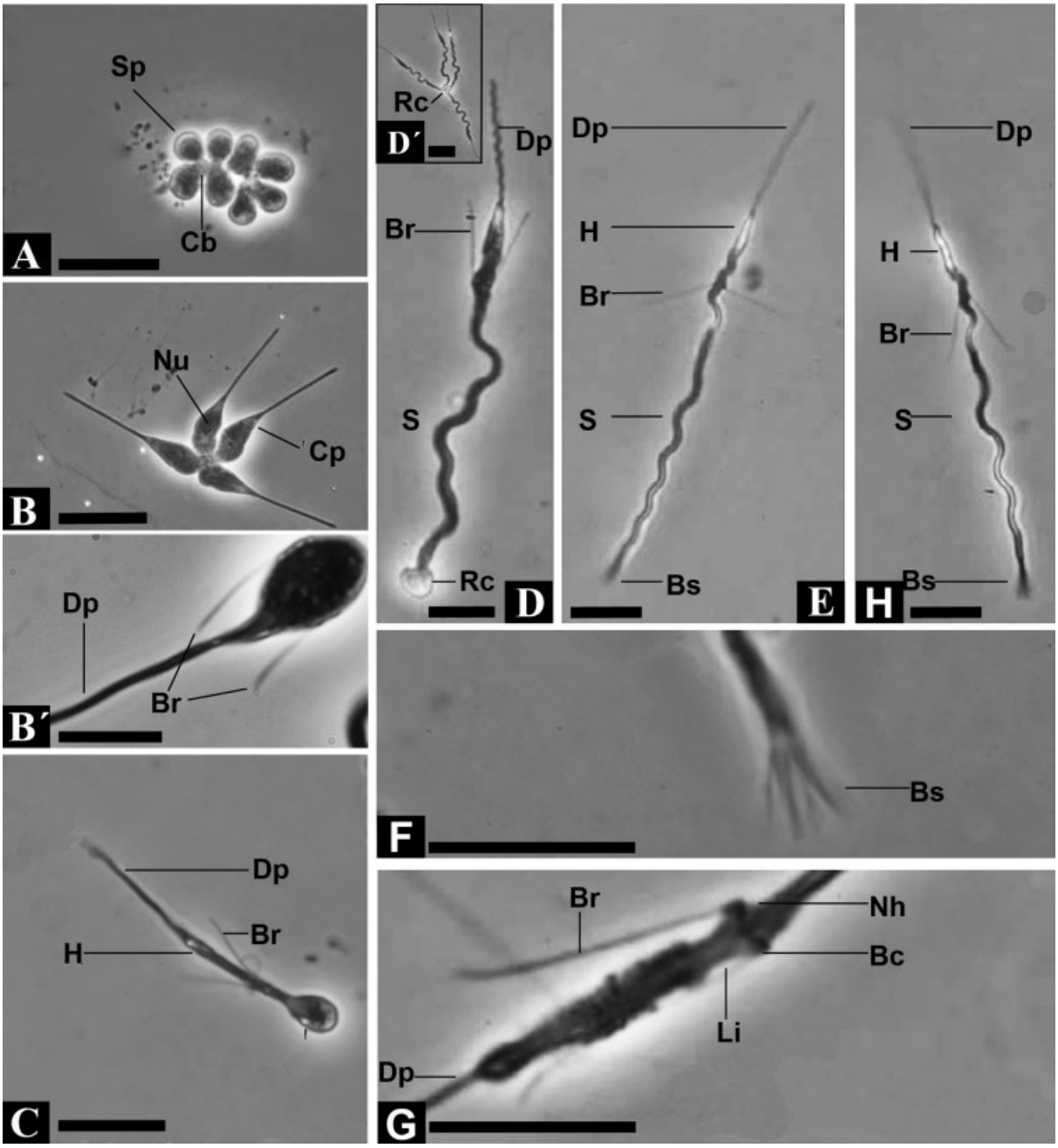

Fig. 2. *Macrostomum lignano*. Developmental stages during spermiogenesis in the testes (A–G), and of mature sperm from the vesicula seminalis (H). LM. **A**: Two adjacent clusters, each of four spermatids joined by a cytoplasmic bridge (cytophore). **B**: Each spermatid, still in quartet, forms an elongating conical projection with the nucleus at its base. In the isolated spermatid (**B′**), i.e., removed from its cluster, developing bristles can be seen at either side of the conical projection. **C**: Mid spermatid. Elongation carries the bristles distally from the base of the conical projection. Subsequent elongation will only take place at the proximal side of the bristles. The basal part of the conical projection will form the future head of the sperm, distally from the bristles. **D**, **D′**: Late spermatid, detached from the residual cytoplasm. Normally at this stage spermatids are still connected in a cluster of 4 as shown in D′. The distal process, the head and the shaft are fully developed. The bristles are still oriented distally. **E**: Late spermatid. The rotation of the bristles is the last step in developing sperm. Sperm is composed of a distal process, a head with two bristles, a shaft, and a terminal brush. Note that the bristles are positioned halfway, whereas in H the bristles are oriented posteriorly. **F**, **G**: High power magnification of a mid spermatid showing details of the proximal brush (F) and the head region with the bristle complex (G). The brush is composed of several tapering structures. Anterior is up in F and to the left in G. **H**: Mature sperm from the vesicula seminalis. Bristles are oriented posteriorly. Anterior is to the left. Br, bristle; Bs, brush; Cb, cytoplasmic bridge; Cp, conical projection; Dp, distal process; H, head; Li, lateral invagination; Nh, notch; Nu, nucleus; Rc, residual cytoplasm; S, shaft; Sp1, primary spermatocyte; Sp, spermatid; Scale bar A–E and H: 20 μm; F and G: 10 μm.

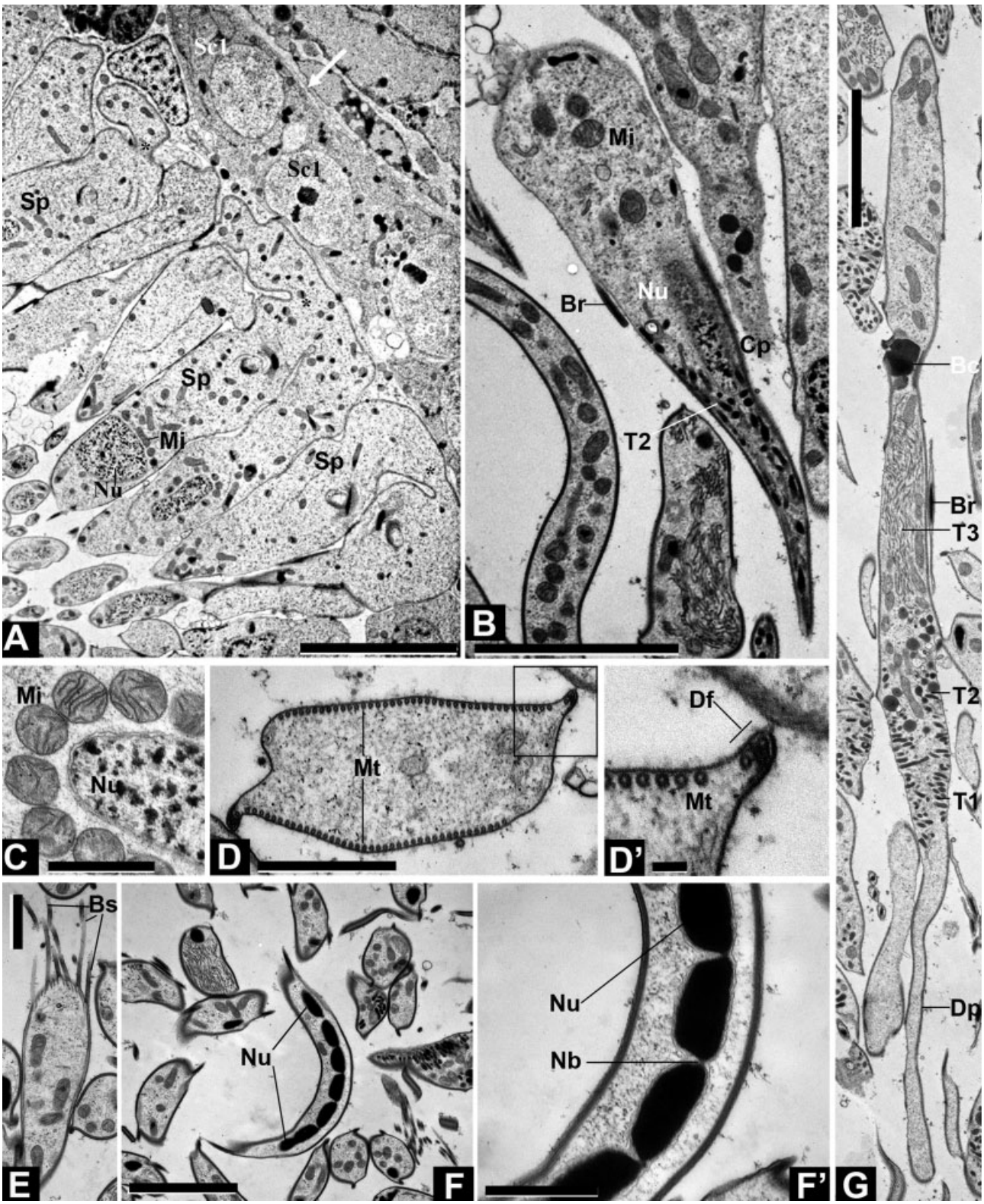

Fig. 3. *Macrostomum lignano*. Developing sperm from the testes. TEM. **A**: Longitudinal section through early spermatids at the margin of the testis. Spermatids are connected to each other by cytoplasmic bridges (asterisks; compare with Fig. 2A,B). Arrow shows the margin of the testis. **B**: Development of the conical projection (compare with Fig. 2B) in the early spermatid. Type 2 dense bodies are located distally from the nucleus. Distal is down. The bristles are already visible. **C**: The same developmental stage as in A. Note the close association of mitochondria with the nucleus. **D**, **D′**: Cross section through the shaft of a mid spermatid. Two sets of cortical microtubules are situated immediately below the plasma membrane. Two microtubules of each set extend into a ledge forming the so-called dense fiber. **E**: Detail of the structures composing the proximal brush (compare with Fig. 2F). **F**, **F′**: Longitudinal section through the nucleus of a fully differentiated sperm. Detail of the condensed nucleus. Note that the nuclear granules remain connected to each other by nuclear bridges. **G**: Longitudinal section through head region of a late spermatid. Distribution of the three types of dense bodies in the head of a late spermatid. Distal is down. Br, bristle; Bc, basal complex of the bristles; Cp, conical projection; Df, dense fiber; Dp, distal process; Mi, mitochondria; Nb, nuclear bridge; Nu, nucleus; SC1, primary spermatocytes; Sp, spermatids; T1/T2/T3, different types of dense bodies. Scale bar A: 10 μm; B: 3 μm; C: 800 nm; D: 800 nm; D′: 200 nm; E: 1 μm; F: 3 μm; F′: 1 μm; G: 3 μm.

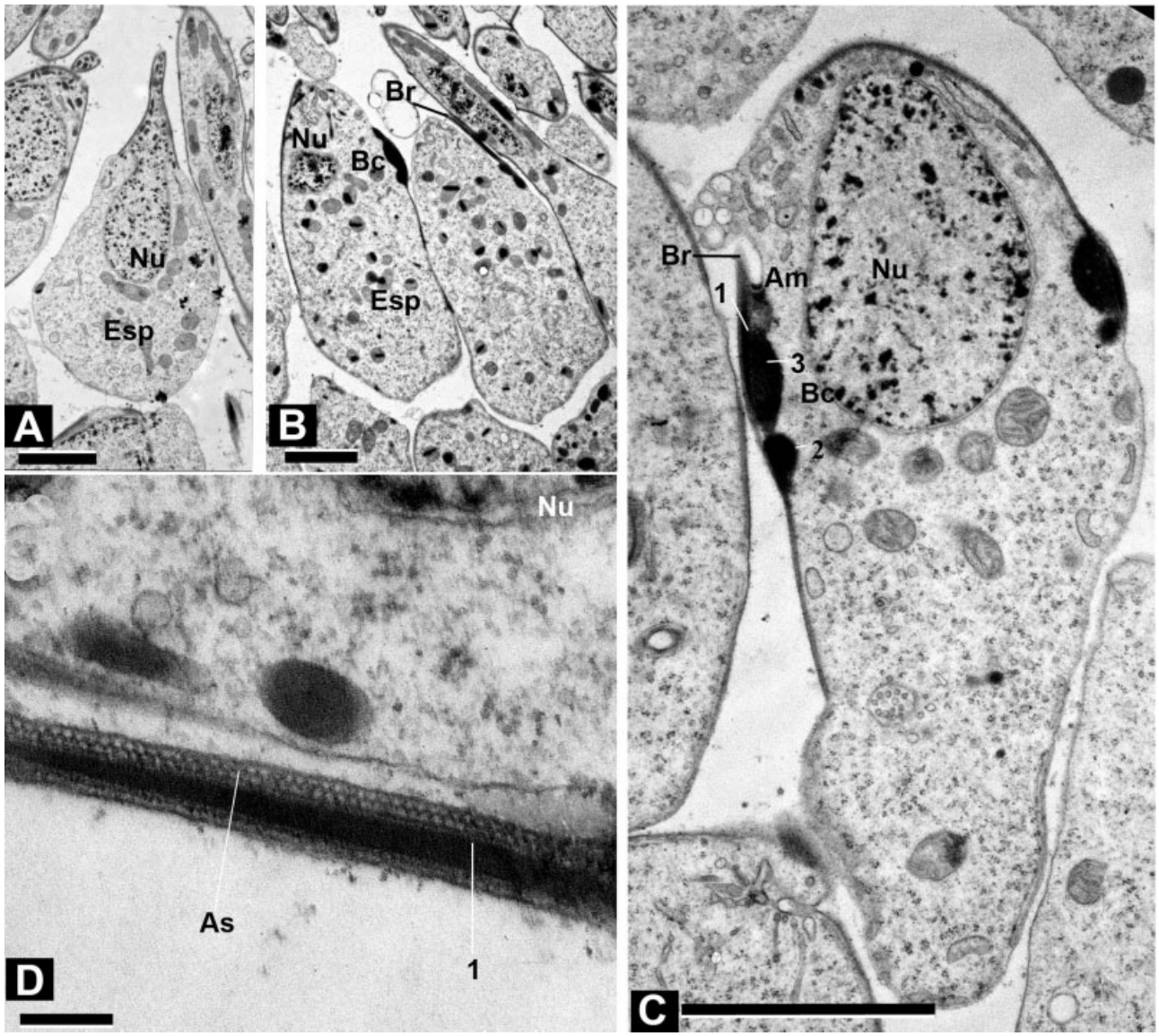

Fig. 4. *Macrostomum lignano*. Early spermatid (longitudinal section). TEM. **A**, **B**: Same spermatid, subsequent sections to observe the position of the bristles. **C**: Magnification of the early spermatid shown in (A) and (B). The bristle complex is composed of three dense structures. Part 1 of the bristle complex represents the axis of the bristle. The most proximal dense structure is named part 2. Distal from part 2 is part 3. The arching membrane is formed where the bristle leaves the cytoplasm. **D**: Detail of the ultrastructure of the bristle, longitudinal section through the axis. Note the annular structure (two rows) around the bristle. As, annular structure; Am, arching membrane; Bc, bristle complex; Br, bristle; Esp, early spermatid; Nu, nucleus; 1, 2, 3, parts of the bristle complex. Scale bar A, B: 2 μm; C: 3 μm; D: 400 nm.

long), distally-oriented extensions, situated at both sides of the spermatid (Fig. 4A–C).

Base and axis of the bristles consist of a complex electron-dense structure: the bristle complex. It consists of three parts with equal electron density, which we have called 1–3 (Fig. 4C). Part 1 is the axis of the bristles, directed distally, the two other parts are located at the base of the bristle: part 2 most proximal, part 3 most distal. The bristle complexes of the two bristles are not connected to each other. Where the bristle leaves the spermatid body, a semicircular invagination of the spermatid body occurs: the arching membrane (Fig. 4C).

Just beneath the membrane of the bristle, two rows of small annular structures occur. They can be noticed in longitudinal sections of the bristles (Fig. 4D).

In the mid spermatid, a triangular notch appears proximally of the bristle complex (Fig. 5A–D; arrow in Fig. 5C). This notch is possibly formed by a process of local autophagy of the cell membrane and cytoplasm, as several vesicles (phagosomes?) can be observed near to the notch,

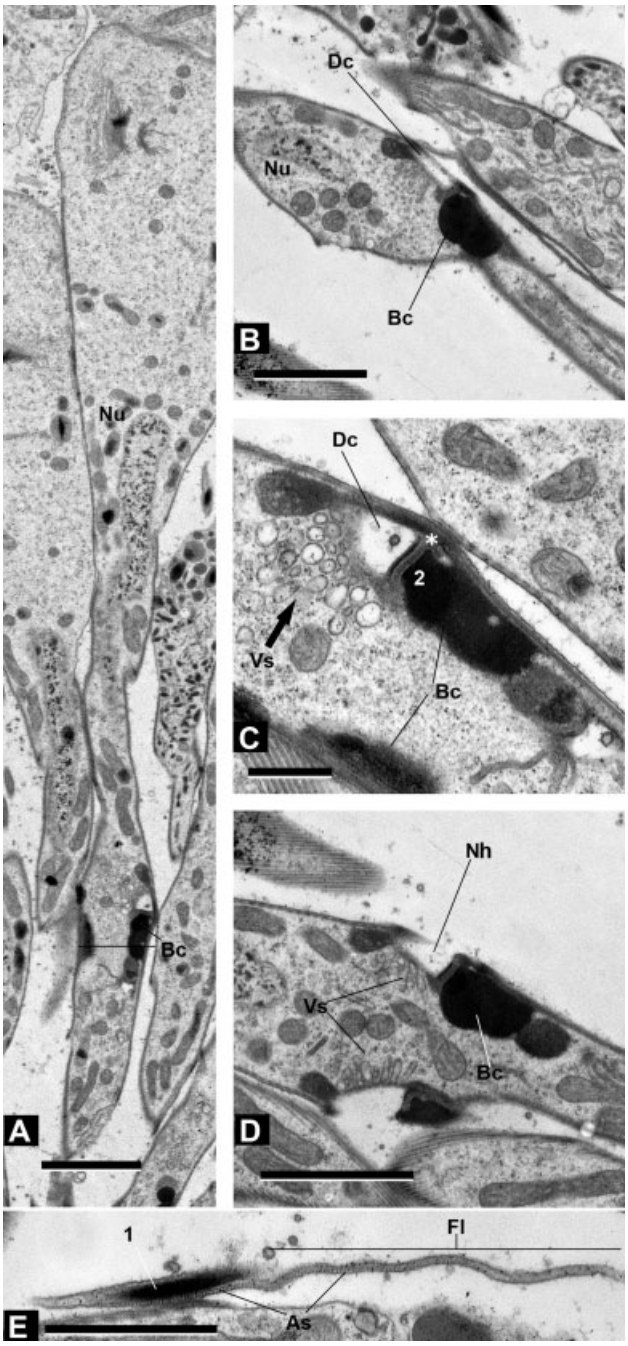

Fig. 5. *Macrostomum lignano*. Mid spermatid and notch formation. TEM. **A**: Longitudinal section through mid spermatid. Top is proximal. **B**, **C**, **D**: Detail of bristle complex of spermatid in A–D represent subsequent sections of the same spermatid. Right is distal. A transverse membrane, perpendicular to the cell membrane, is formed at the proximal side of part 2 of the bristle complex [asterisk in (C)]. C, D: A triangular shaped portion of the cytoplasm disappears. Note the presence of vesicles (arrow). **E**: Ultrastructure of the bristle, longitudinal section. The cytoplasmic extension, or flag, extends away from the axis, parallel with the cell membrane. Note that the annular structure runs from the axis of the bristle along the length of the flag. As, annular structure; Bc, bristle complex; Dc, disappearing cytoplasm; Fl, flag; Nh, notch; Nu, nucleus; Vs, vesicle; 2, part 2 of bristle complex. Scale bar A: 2 μm; B: 1, 5 μm; C: 500 nm; D: 1, 5 μm; E: 1 μm.

prior to and during its formation. Distally from the notch, and perpendicular to the cell membrane, an intracellular, electron-dense membrane-like structure can be seen (indicated with an asterisk in Fig. 5C).

It becomes clear that the bristles are flattened structures (Fig. 5E), with the axis located at one side. The other side forms a "flag," which points away from the cell membrane, with the flags of both bristles pointing in opposite directions. The annular structures can be found along the entire length of the flag (Fig. 5E).

In the late spermatid, parts 2 and 3 of the bristle complexes each show small processes (Fig. 6A,B; asterisk in Fig. 6B). Later on, the processes of part 2 of both bristle are connected to each other by a connective structure (median connective structure α, Mc α), which is less electron dense than part 2 itself and runs over a series of serial sections. In the same way, both parts 3 are connected to each other by a second median connective structure (median connective structure β, Mc β), also less electron dense. At this time, parts 2 and 3 are still connected to each other and to the axis of the bristle. As a result, both bristles are firmly connected to each other.

Mature sperm are only found in the seminal vesicle and can be recognized very easily by the fact that the bristles now point posteriorly. Mc α, Mc β, and part 3 of the bristle complexes have disappeared, leaving only part 2 at the base of the bristle and part 1 as the axis. Part 2 now appears as a tapering structure, triangular in shape in transverse sections (the "anchor"). Between axis and anchor there is a small, curved, T-shaped dense structure (Fig. 7A,A′). The annular structures that supported the flag of the bristles have disappeared (Fig. 7B). A diagrammatic view of the mature spermatozoon with detail of the bristle complex is shown in Figure 8.

DISCUSSION

Up to now, ultrastructural data on spermiogenesis in the taxon *Macrostomum* were only available for *M. tuba* and *M. pusillum*, both species having sperm with bristles (Rohde and Watson, 1991; Rohde and Faubel, 1997). Moreover, in *M. pusillum* the bristles cannot be seen in the light microscope.

Our observations, however, went into more detail as to the ultrastructural morphology of developing sperm and more specifically into the ontogeny of all the complex structures associated with the bristles.

Cytomorphological Changes During Spermiogenesis

Dense bodies. Although the occurrence of several types of dense bodies has been described in

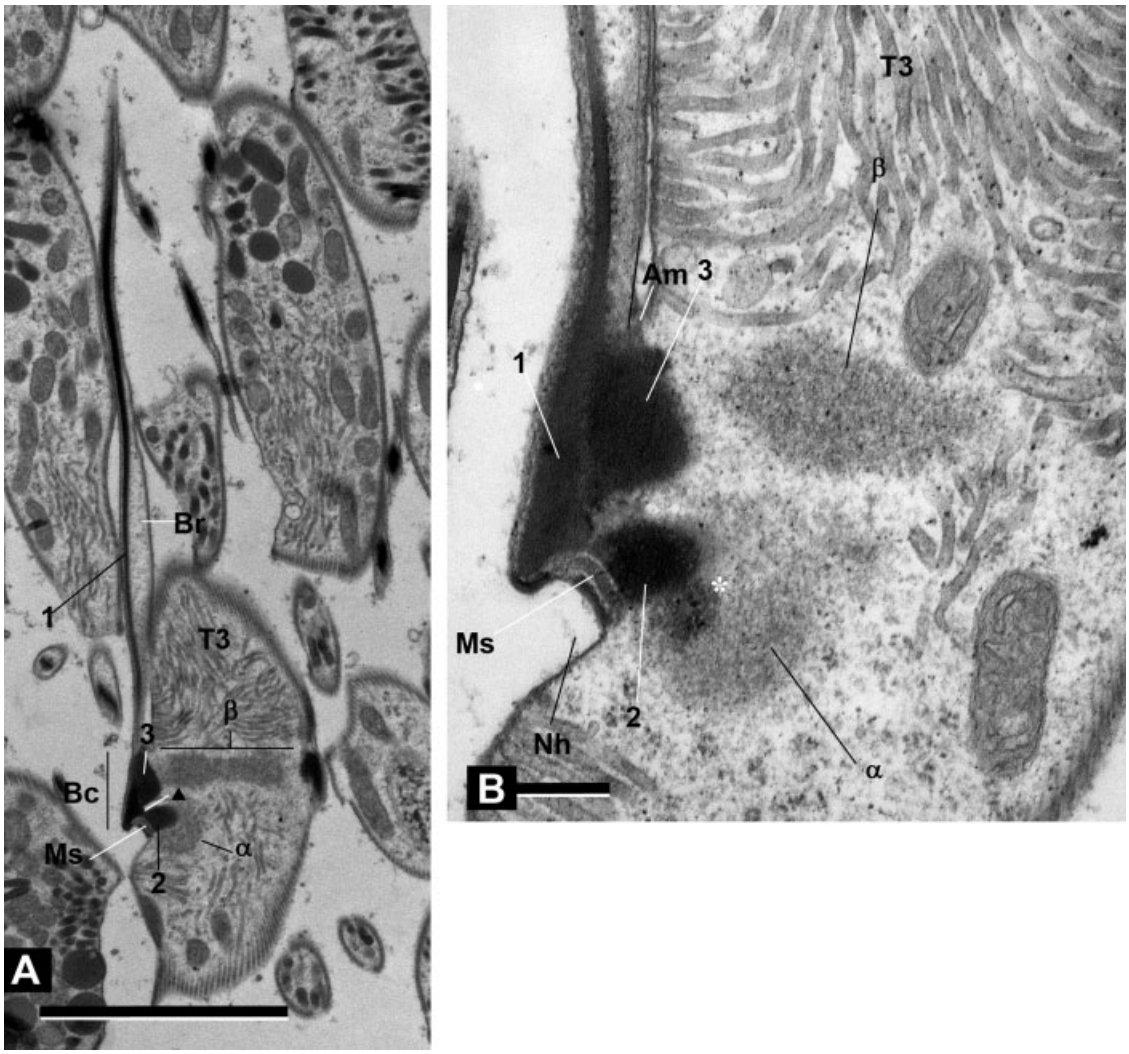

Fig. 6. *Macrostomum lignano*. Late spermatid. TEM. **A**: Longitudinal section through the basal complex of both bristles. Top is distal. **B**: Detail of the bristle complex and associated structures. Part 2 of the bristle complex (future anchor) is positioned between the transverse membrane, the axis of the bristle, and the notch. Part 2 of the bristle complex is also connected to the basal part of the axis. Median structure α initiates at part 2 (note the process; arrowhead) of one complex and runs to part 2 of the opposite as seen on subsequent serial sections. The median structure β is only connected to part 3 of both complexes. Based on the position of dense bodies of type 3, it is clear that the bristles are still directed distally. Note that no annular structure was observed along the axis of the bristle. Bc, bristle complex; Br, bristle; Ms, transverse membrane; T3, type 3 dense bodies; 1, 2, 3, part 1, 2, 3 of bristle complex; α, β, median connective structure Mc α and Mc β. Scale bar: A 2 μm; B: 200 nm.

the past for macrostomid sperm, it was always thought that these types were distributed randomly in the sperm cell, without any specific order (see Sopott-Ehlers and Ehlers, 1999 and references therein). In *M. lignano*, however, the three types of dense bodies always occur in a very specific pattern, as described in the Results section.

Nothing is known about the possible function of dense bodies in turbellarians (the parasitic Neodermata have sperm without dense bodies; Noury-Sraïri et al., 1989). Supposedly, they play a role in the penetration of the oocyte because in some taxa they were absent after fertilization (Watson, 1999). Alternatively, because sperm in *M. lignano* are stored in the female receptaculum (Ladurner et al., 2005), a nutritive function of one of these dense bodies is also conceivable.

Possibly, T2 dense bodies of *M. lignano* are analogous to the acrosomal vesicles (Parvinen, pers. comm.). In *M. lignano*, we also observed such vesicles in the proximal part of primary spermatocytes (own unpublished data). These vesicles and T2 dense bodies have a comparable structure. Moreover, in the female antrum allosperm is positioned in such a way that the cell region contain-

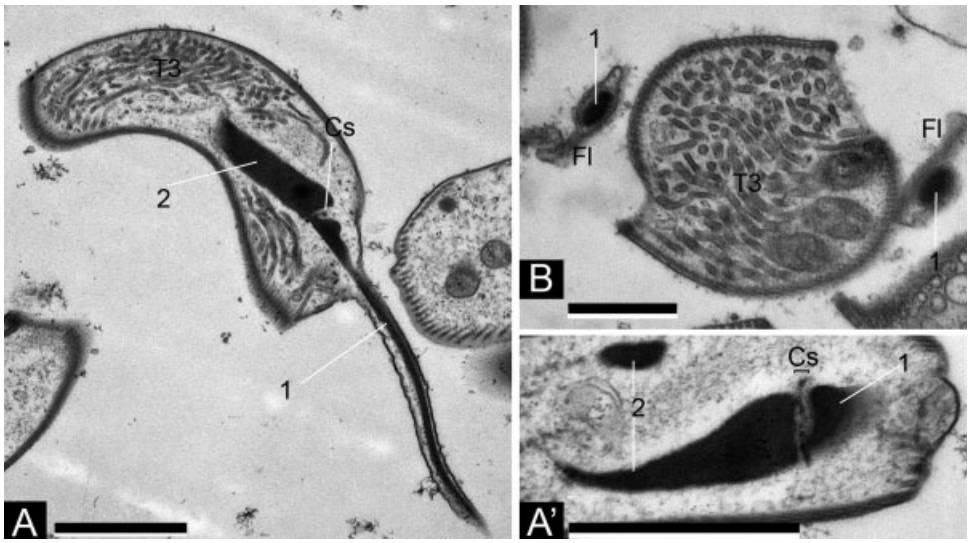

Fig. 7. *Macrostomum lignano*. Sperm in the false vesicula seminalis. TEM. **A**: Longitudinal section through bristle and bristle complex. The anteroposterior rotation of the bristles has occurred. The bristle is finally composed of an anchor and an axis. Based on the position of type 3 dense bodies, it is clear that the bristles are directed posteriorly. The anchor is a cone-shaped tapering structure. Note the absence of the annular structure around the axis. Left is distal. **A′**: Detailed view of the T-shaped connective structure between anchor and axis. Note the connective structure, a differentiated part of the basis of the axis, between anchor and axis. Top is proximal. **B**: Transverse section through an adult sperm at the level of the bristles. The bristles are oriented contralaterally. The flag of each pair of bristles point in opposite directions. 1, 2, part 1 (axis) and 2 (anchor) of bristle complex; Fl, flags; Cs, connective structure; T3, type 3 dense bodies. Scale bar: A, A′: 1 μm; B: 500 nm.

ing T2 dense bodies is closest to the oocyte (see Ladurner et al., 2005: Fig. 7b).

T3 dense bodies are also very peculiar. The reasons for their highly tubular nature and association with the plasma membrane of the notch and the lateral invagination (as seen in Fig. 6A,B) are still puzzling.

The bristles. Newton (1980) and Rohde and Watson (1991) mentioned that the bristles have a dense column and dense base in young spermatids of species of *Macrostomum*. However, in this contribution, we have presented a detailed account of the morphology and ontogeny of the bristles, showing that they have a very complex morphology. For the first time, we show that the basal region consists of two parts, which in a late stage of spermatogenesis become connected to each other. Moreover, we have shown that these bristles are in fact flat, paddle-like structures, with the axis positioned eccentrically.

In a mid-stage spermatid, a transverse notch arises proximally from part 2 of the bristle complex. Such a notch is noted for the first time in the genus *Macrostomum*. This notch will delineate the base of part 1 of the bristle. Probably, this notch has a function in the anterior–posterior rotation of the bristles, acting as a socket wherein the bristle can rotate and push against the formed anchor (part 2 of bristle complex) (Figs. 5A–D and 6B).

The occurrence of two median connective structures (Mc α and Mc β) is noted for the first time. It is not clear how these median structures originate. It is possible that they are formed starting from the processes observed on parts 2 and 3 of the complexes, left and right then growing toward each other, or they are formed completely de novo.

The function of these median structures remains elusive. Possibly together with the bristle complexes, they form a sort of firm intracellular skeleton. This skeleton, however, has partly disappeared in mature sperm in the seminal vesicle, leaving only the axis and the anchor. At that stage, the bristles have a posterior orientation. As the skeleton originates before the rotation of the bristles occurs, and disappears after the rotation has occurred, a possible function in this process is conceivable. This hypothesis, however, needs to be tested in kinematic studies, which might also elucidate the exact way in which the rotation occurs (i.e., movies of 3D rendered bristle complex simulating the possible rotations, unpublished data). The function of the T-shaped structure, which is the modified end of the axis in mature sperm, may be to absorb shocks or facilitate the contact between anchor and axis.

Newton (1980) hypothesized that inactive sperm have anteriorly oriented bristles, whereas in active sperm these bristles would be oriented posteriorly. Our data clearly show that the orientation of the bristles is dependent on the developmental stage of spermatogenesis and not linked to the activity of the sperm. Probably, the anterior–posterior rota-

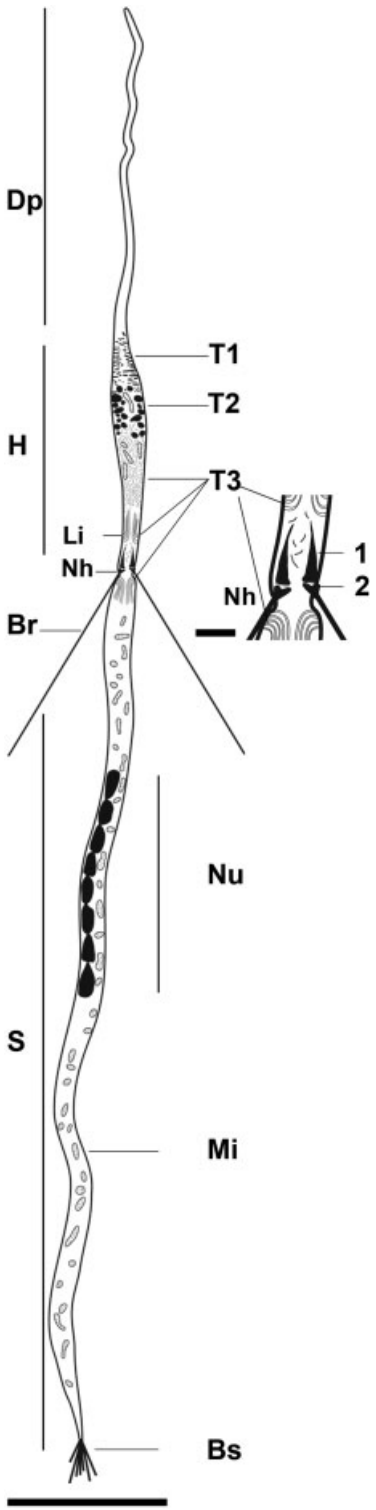

Fig. 8. *Macrostomum lignano*. Drawing of mature sperm and magnification of mature bristle complex. Anterior is to the top. Exact number of nuclear granules and tapering structures of brush could not be quantified. Note the close association of the tubular T3 dense bodies with the lateral invagination and the notch (cfr. Fig. 6A,B). 1, axis of bristle; 2, anchor of bristle; Br, bristle; Bs, brush; Dp, distal process; H, head; Li, lateral invagination; Mi, mitochondria; Nh, notch; Nu, nucleus; S, shaft; T3, type dense body. Scale bar: 10 μm; inset: 2. 5 μm.

tion of the bristles occurs before the sperm enter the vas deferens. In the testes, sperm are found with the bristles oriented anteriorly or perpendicular to the long axis of the sperm, whereas in the seminal vesicle bristles are always oriented posteriorly. As mature sperm pass through the very narrow vas deferens and stylet headfirst, posteriorly oriented bristles facilitate this passage. We never observed that the bristles of live mature sperm were actively moving, nor did we find any proof that they facilitate the active movements of the sperm, as was suggested by Hendelberg (1969a,b).

The function of the bristles is not clear, but the extraordinary postcopulatory behavior of *M. lignano* (see Schärer et al., 2004 for a detailed description) may offer a clue. After copulation, the worm often folds its mouth toward its own female gonopore exhibits a sucking behavior. After this action, the posterior parts of the allosperm partly protrude from the female gonopore, all having the same length. This could be explained by the sperm remaining internally attached (Schärer et al., 2004). Measuring the protruding parts indicates that the bristles can function as flukes to keep the sperm internally attached in the ciliary tuft that delineates the female pore (Schärer L. and Vizoso D, pers. comm.).

The Annular Structure and the Cytoplasmatic Extension. The annular structure, we observed in the bristles of the spermatozoa of *M. lignano*, was also described in the bristles of the spermatozoa of *M. retortum* (Bedini and Papi, 1970; in Rohde and Watson, 1991). These authors described it as a regular row of transversally-sectioned microtubules, which they interpreted as closely packed microtubular spirals surrounding the axis. However, these structures are much smaller than regular cortical microtubules (ca. 13 nm vs. ca. 22 nm diameter). Moreover, although cortical microtubules running spirally beneath the plasma membrane are found in many flatworm species (see refs. in Justine, 2001), the fact that they run spirally around the bristle axis and the fact that they are only temporarily present is also very uncommon for microtubules.

We, therefore, doubt that this annular structure is indeed composed of microtubules.

In their study of *M. tuba*, Rohde and Watson (1991) failed to find these annular structures, but on transverse section they did find dense rods that surround the axis. They concluded that the annular structure of *M. retortum* and the dense rods of *M. tuba* may be corresponding structures, although the way the latter run around the axis was unclear. Note that, in *M. lignano* we found the transversally-sectioned microtubules running spirally around the axis in longitudinally cut bristles (Fig. 4D) not in cross-sectioned bristles as observed in *M. retortum* and *M. tuba*.

In *M. lignano*, the annular structure is also found in the cytoplasmic flag of the bristle. Its

close association with the axis and the flag suggests that it provides support for the bristles.

The annular structure disappears before the complete intracellular skeleton is formed in late spermatids. The reason this annular structure is only temporally associated with the axis of the bristle is puzzling but it could be responsible for bristle formation.

Bristles as Modified Flagellae?

Because the bristles are associated with tapering dense structures and have a dense axis surrounded by dense rods, Rohde and Watson (1991) interpreted the bristles of the species of *Macrostomum* as modified flagellae. This view was apparently further supported by the presence of striated plates between the anchor and the axis in *M. tuba* (see Fig. 14 in Rohde and Watson, 1991), which were interpreted by those authors as being homologous to the intercentriolar plates. In flatworms, these plates are one of the components of the intercentriolar body (ICB), a structure essential for the formation of flagellae (Watson, 1999). The presence of a T-shaped structure, actually the modified proximal end of the axis, between part 1 and 2 of the basal complex in *M. lignano* (Fig. 7A′), however, casts some doubt upon this interpretation. The striated plates observed in *M. tuba* could as well be homologous to this T-shaped structure, and both structures would then not be homologous at all to the intercentriolar plates of the ICB. By consequence, the bristles would not be flagellae. This interpretation is supported by several observations: 1) Formation of a flagellum in spermatogenesis of non-neodermatan flatworms begins with the formation of a zone of differentiation in the apical region of the cell (Watson, 1999). This is not the case for the bristles, which originate at the base of the conical projection. 2) In flagellate spermatozoids two centrioles will develop in the zone of differentiation (Watson, 1999), which will later become the basal bodies of the flagella. Such centrioles were never observed in spermatids of *M. lignano* nor in those of *M. tuba*. 3) An ICB, which consists of a central, dense plate, surrounded by smaller, less dense striated plates normally develops between the two centrioles of developing flagellae. This we did not observe in *M. lignano*. As said earlier, the striated plate found between anchor and axis of the bristle in *M. tuba* was considered homologous to the striated plates of the ICB by Rohde and Watson (1991). However, they did not report any of the other structures typically associated with an ICB. 4) The centrioles will develop into basal bodies, which act as nucleation sites for the growth of the axonemal microtubules and anchor the flagella in the cell (Watson and Rohde, 1995). The bristles in *M. lignano*, however, originate from two dense structures (parts 2 and 3 of the bristle complex), located at the plasma membrane. 5) In the spermatozoids of most non-neodermatan flatworms, striated rootlets are associated with the basal bodies of the flagella. Such structures were never observed in *M. lignano* nor in *M. tuba* (Rohde and Watson, 1991).

The differences summed up above clearly indicate that there is no reason to assume that the bristles in species of *Macrostomum* are modified flagellae. They have a completely different ultrastructural morphology, serve a different function, and arise from a different location on the spermatozoid.

Fully-developed bristles only occur in a number of species of *Macrostomum* and *Promacrostomum* (An der Lan, 1939), whereas a few species have vestigial bristles (e.g., *M. pusillum*; Ax, 1951). Such bristles are unknown for any other turbellarian taxon, even within the Macrostomida. Rhode and Faubel (1998) described "bristles" from the macrostomid *Haplopharynx rostratus* (Meixner, 1938), but here the "bristles" are modified granules and never protrude from the cell surface. Basically, the bristles of *M. lignano* are also elaborate granules, but it is at the moment impossible to say whether they can be derived from, and thus are homologous with the "bristles" of *H. rostratus*. Also, the vestigial bristles of *M. pusillum* are closely associated with the plasma membrane and originate from dense granules.

Sopott-Ehlers and Ehlers (1999) described a pair of ledges that occur during spermiogenesis in the macrostomid species *Bradynectes sterreri* (Rieger, 1971) and *Psammomacrostomum turbanelloides* (Karling, 1974). These ledges are surrounded by a membrane, lie underneath the cortical microtubules, and do not protrude from the sperm body. According to Sopott-Ehlers and Ehlers (1999), these ledges originate from dense material that is associated with the surface of the plasma membrane. Therefore, these authors suggest that the real bristles, as observed in many species of *Macrostomum*, are derived from these ledges.

The complete lack of flagellar structures is almost unique within the "turbellaria" (Hendelberg, 1969a,b). In some other species of macrostomids, the flagella of the spermatozoids are very much reduced, but centrioles (*B. sterreri*; see Sopott-Ehlers and Ehlers, 1999) or centrioles and striated rootlets (*P. fusculum*; see Rohde and Faubel, 1997) are found during spermatogenesis. The occurrence of these centrioles and striated rootlets brought Rohde and Faubel (1997) to the conclusion that the bristles of *P. fusculum* are derived flagella, whereas Sopott-Ehlers and Ehlers (1999) concluded the opposite based upon the concurrent presence of centrioles and ledges in *B. sterreri*. As was discussed earlier, the wealth of data now available shows that the bristles are indeed not derived from flagellae.

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LITERATURE CITED

An der Lan H. 1939. Zur rhabdocoelen turbellarienfauna des ochrida-sees (Balkan). Sitz-ber Akad Wiss Wien Math-Naturw Kl 148:195–204.

Ax P. 1951. Die turbellarien des eulitorals der kieler bucht. Zool Jahrb Abt Syst Oekol Geogr Tiere 80:277–378.

Bâ CT, Marchand B. 1995. Spermiogenesis, spermatozoa and phyletic affinities in the Cestoda. In: Jamieson BG, Ausio J, Justine J-L, editors. Advances in Spermatozoal Phylogeny and Taxonomy. Paris: Museum National D'Histories Naturelle. pp 265-283. Mem Mus Natn Hist Nat 166:87–95.

Bedini C, Papi F. 1970. Peculiar patterns of microtubular organisation in spermatozoa of lower Turbellaria. In: Baccetti B, editor. Comparative Spermatology. New York: Academic Press. pp 363–368.

Euzet L, Świderski Z, Mokhtar-Maamouri F. 1981. Ultrastructure comparée du spermatozoide des cestodes. Relations avec la phylogénèse. Ann Parasitol (Paris) 56:247–259.

Ehlers U. 1985. Das Phylogenetisches System Der Platyhelminthes. Stuttgart: Fischer.

Ferguson FF. 1940a. A monograph of the genus *Macrostomum O.* Schmidt, 1848. VI. Zool Anz 129:21–48.

Ferguson FF. 1940b. A monograph of the genus *Macrostomum O.* Schmidt, 1848. VII. Zool Anz 129:120–146.

Hendelberg J. 1965. On different types of spermatozoa in Polycladida, Turbellaria. Ark Zool 18:267–304.

Hendelberg J. 1969a. On the number and ultrastructure of the flagella of flatworm spermatozoa. In: Baccetti B, editor. Comparative Spermatology. New York: Academic Press. pp 367–374.

Hendelberg J. 1969b. On the development of different types of spermatozoa from spermatids with two flagella in the Turbellaria with remarks on the ultrastructure of the flagella. Zool Bidrag Fran Uppsala 38:1–50.

Hendelberg J. 1974. Spermiogenesis, sperm morphology and biology of fertilization in the Turbellaria. In: Riser NW, Morse MP, editors. Biology of the Turbellaria. New York: McGraw-Hill. pp 148–164.

Hendelberg J. 1977. Comparative morphology of turbellarian spermatozoa studied by electron microscopy. Acta Zool Fennica 154:149–162.

Hendelberg J. 1983. Platyhelminthes-Turbellaria. In: Adiyodi KG, Adiyodi RG, editors. Reproductive Biology of Invertebrates, Vol. 2: Spermatogenesis and Sperm Function. New York: Wiley. pp 75–104.

Hendelberg J. 1986. The phylogenetic significance of sperm morphology in the platyhelminyhes. Hydrobiologia 132:53–58.

Justine J-L. 1991. Phylogeny of parasitic Platyhelminthes: A critical study of synapomorphies proposed on the basis of the ultrastructure of spermiogenesis and spermatozoa. Can J Zool 69:1421–1440.

Justine J-L. 1995. Spermatozoal ultrastructure and phylogeny in the parasitic Platyhelminthes. In: Jamieson BG, Ausio J, Justine J-L, editors. Advances in Spermatozoal Phylogeny and Taxonomy. Paris: Museum National D'Histories Naturelle. Mem Mus Natn Hist Nat 166:55–86.

Justine J-L. 2001. Spermatozoa as phylogenetic characters for the Platyhelminthes. In: Littlewood DTJ, Bray RA, editors. Interrelationships of the Platyhelminthes. London: Taylor and Francis. pp 231–238.

Karling T. 1974. On the anatomy of the turbellarian orders. In: Riser NW, Morse MP, editors. Biology of the Turbellaria. New York: McGraw-Hill. pp 1–16.

Ladurner P, Rieger R, Baguña J. 2000. Spatial distribution and differentiation potential of stem cells in hatchlings and adults in the marine platyhelminth *Macrostomum sp.*: A bromodeoxyuridine analysis. Dev Biol 226:231–241.

Ladurner P, Schrärer L, Salvenmoser W, Rieger RM. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). J Zool Syst Evol Res 43:114–126.

Levron C, Brunanska M, Poddubnaya LG. 2006. Spermatological characters in *Diphyllobothrium latum* (Cestoda, Pseudophyllidea). J Morphol 267:1110–1119.

Liana M, Litvaitis M. 2007. Comparative spermatology of selected polyclad flatworms (Platyhelminthes). J Morphol 268: 891–897.

Meixner J. 1938. Turbellaria (Strudelwuermer). I. D. Tier. d. Nord-u. Oostsee. Allgemeiner Teil. In: Grimpe G, Wagler E, Remane A, editors. Die Tierwelt der Nord_und Ostsee 33 (IVb):1–146.

Miquel J, Swiderski Z, Mtocicki D, Eira C, Marchand B. 2005. Ultrastructure of spermatogenesis of the anoplocephalid cestode *Gallegoides arfaai* (Mobedi et Ghadirian, 1977; Tenora et Mas-Coma, 1978). Acta Parasitol 50:132–144.

Newton WD. 1980. Ultrastructural analysis of the motile apparatus of the aflagellate spermatozoon of *Macrostomum tubum*. J Ultrastr Res 73:318–330.

Noury-Sraïri N, Justine J-L, Euzet L. 1989. Ultrastructure compare de la spermiogenèse et du spermatozoïde de trais espèces de Paravortex (Rhabdocoela, "Dalyellioida". Graffillidae) Turbellariés parasites intestinaux de Mollusques. Zool Scr 18:161–174.

Rieger R. 1971. Die Turbellarienfamilie Dolichomacrostomidae nov. fam. (Macrostomida). I. Teil. Vorbemerkungen und Karlingiinae nov. subfam. Zool Jb Syst. 98:236–314.

Rieger R, Gehlen M, Hazpruhnar G, Homlund M, Legnitit A, Salvenmoser W, Tyler S. 1988. Laboratory cultures of marine Macrostomida (Turbellaria). Forts Zool 36:525.

Rohde K. 2001. Protonephridia as phylogenetic characters. In: Littlewood DTJ, Bray RA, editors. Interrelationships of Platyhelminthes. London: Taylor & Francis. pp 203–216.

Rohde K, Faubel A. 1997. Spermatogenesis of *Macrostomum pusillum* (Platyhelminthes, Macrostomida). Invert Reprod Dev 32:209–215.

Rohde K, Faubel A. 1998. Spermatogenesis of *Haplopharinx rostratus* (Platyhelminthes). Belgian J Zool 128:177–188.

Rohde K, Watson N. 1991. Ultrastructure of spermatogenesis and sperm of *Macrostomum tuba*. J Submicrosc Cytol Pathol 23:23–32.

Rohde K, Watson N. 1995. Ultrastructure of spermatogenesis and sperm of two species of the Tricladida (Platyhelminthes), Romankenkius libidenosus (Paludicola) and an unidentified species of the Maricola. Invert Reprod Dev 27:181–186.

Schärer L, Joss G, Sandner O. 2004. Mating behaviour of the marine turbellarian *Macrostomum sp.*: These worms suck. Marine Biol 145:373–380.

Sopott-Ehlers B, Ehlers U. 1999. Ultrastructure of spermiogenesis and spermatozoa in *Bradynectes sterreri* and remarks on sperm cells in *Haplopharynx rostratus* and *Paramalostomum fusculum*: Phylogenetic implications for the Macrostomorpha (Platyhelminthes). Zoomorphol 119:105–115.

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43.

Watson N. 2001. Comparative spermatology in the "Turbellarian" Rhabdocoela. In: Littlewood DTJ, Bray RA, editors.

Interrelationships of Platyhelminthes. London: Taylor & Francis. pp 217–230.
Watson NA. 1999. Platyhelminthes. In: Adiyodi KG, Adiyodi RG, Jamieson BGM, editors. Reproductive Biology of Invertebrates, Vol. 9A: Progress in Male Gamete Ultrastructure and Phylogeny. New Delhi: Oxford and IBH Publishing. pp 97–142.
Watson NA, Rohde K. 1995. Sperm and spermiogenesis of the "turbellaria" and implications for the phylogeny of the phylum Platyherlminthes. In: Jamieson BGM, Ausio J, Justine J-L, editors. Advances in Spermatozoal Phylogeny and Taxonomy. Paris: Museum National D'Histories Naturelle. Mem Mus Natn Hist nat 166:37–54.
Zayas RM, Hernández A. Habermann B, Wang Y, Stary JM, Newmark PA. 2005. The planarian *Schmidtea mediterranea* as a model for epigenetic germ cell specification: Analysis of ESTs from the hermaphroditic strain. Proc Natl Acad Sci USA 102:18491–18496.