

Sperm and Spermiogenesis of the "Turbellaria" and Implications for the Phylogeny of the Phylum Platyhelminthes

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ABSTRACT

This chapter reviews recent ultrastructural investigations of sperm and spermiogenesis in turbellarian platyhelminths (Catenulida, Nemertodermatida, Acoela, Macrostomida and Trepaxonemata including Polycladida, Lecithoepitheliata, Prolecithophora, Proseriata, Tricladida, and Rhabdocoela, including Typhloplanida, Kalyptrorhynchia, Dalyelliida, Temnocephalida and Fecampiida). Some distinctive characteristics of the differentiating spermatid especially in the zone of differentiation may be useful for phylogenetic considerations. Spermiogenetic and mature sperm features including axonemal or flagellar characteristics, dense bodies, nuclear components, mitochondria and microtubule arrangements are documented for each of the major taxa. It is concluded that more investigations are needed and they must be comprehensive to build a solid data base for a cladistic analysis of the phylum based on sperm and spermiogenetic characteristics.

RÉSUMÉ

Spermatozoïdes et spermiogénèse des "Turbellaria" et implications pour la phylogénie de l'Embranchement des Plathelminthes

Les résultats récents sur les spermatozoïdes et la spermiogénèse des Plathelminthes Turbellariés (Catenulida, Nemertodermatida, Acoela, Macrostomida et Trepaxonemata y compris Polycladida, Lecithoepitheliata, Prolecithophora, Proseriata, Tricladida et Rhabdocoela, y compris Typhloplanida, Kalyptrorhynchia, Dalyelliida, Temnocephalida et Fecampiida) sont synthétisés. Certaines caractéristiques distinctives de la spermatide en cours d'évolution, spécialement dans la zone de différenciation, peuvent être utiles pour des considérations phylogénétiques. Les caractères suivants de la spermiogénèse et du spermatozoïde sont décrits pour chacun des taxons majeurs: caractéristiques des axonèmes et des flagelles, corps denses, composants du noyau, mitochondries et arrangement des microtubules. La conclusion est que des recherches supplémentaires exhaustives sont nécessaires pour construire une base de données destinée à une analyse cladistique de l'embranchement basée sur les caractères des spermatozoïdes et de la spermiogénèse.

"Turbellaria" is used throughout this review as a convenient term for all the non-neodermatan platyhelminths, i.e. the groups other than Trematoda, Monogenea and Cestoda; it does not imply monophyly. Early studies of spermatogenesis and spermatozoa in Platyhelminthes, based on light microscopy (LM), contained only limited phylogenetic information. Electron microscopy (EM) has proved of greater use. For a recent bibliography of EM of turbellarians, see [74] (also [47, 51]). The last comprehensive review of turbellarian

spermiogenesis and spermatozoa was published by HENDELBERG in 1983 [25] with further discussion of their phylogenetic significance in 1986 [26]. Turbellarian spermatozoa are considered aberrant since they differ markedly from the supposedly primitive and modified forms found in many other animal groups in association with the primitive mode of sperm transfer, viz. external fertilisation [1, 20, 25, references therein]. They are generally elongate, without distinct head, middle piece and tail, and may be without axonemes, have free flagella (usually two) or axonemes incorporated in the sperm body. In this review we summarise the information currently available from each of the major turbellarian taxa and discuss implications for the phylogeny of the phylum. The classification follows CANNON, 1986 [10], except for the Fecampiida (removed from Rhabdocoela on the basis of ultrastructural and DNA evidence [53]).

OBSERVATIONS

Within each taxon, data on sperm and spermiogenesis will be summarised with reference to the following characteristics:

— Sperm: presence, number, location of axonemes or flagella; presence, arrangement of longitudinal microtubules; nature of nucleus; nature, arrangement of mitochondria; presence, nature of dense bodies.

— Spermiogenesis of flagellate or axonemal sperm: structures in the zone of differentiation (microtubules, rootlets, intercentriolar body, dense plates); changes during elongation (movement of basal bodies, rotation of flagella, fusion of flagella with shaft, etc).

Catenulida

SCHUCHERT & RIEGER [61] described spermiogenesis in *Retronectes atypica* by EM [see also 50]. Ciliary rudiments are present during maturation of sperm (also in *R. sterreri* [15]), associated with conspicuous lamellar bodies. Specialised cell protrusions, interpreted as intercellular bridges, were seen during a restricted early period of spermatogenesis, and at a slightly later stage groups of microvillar protrusions occur along the plasma membrane of the spermatocyte. The spermatozoon has a more condensed nucleus and numerous lamellar bodies, no longer with ciliary rudiments. There are frequent infoldings from the cell membrane and thin branched mitochondria. There appear to be no dense bodies of any kind resembling those in other turbellarian orders.

Nemertodermatida

Only mature sperm of two genera have been examined by EM, *Meara* and *Nemertoderma* [24, 72, 73]. Although modified into an elongate form, the spermatozoa resemble the primitive metazoan type with a distinct head containing the nucleus, middle region where a single axoneme is surrounded by a coiled mitochondrial derivative, and tail where the axoneme extends as a free flagellum. These attributes clearly distinguish Nemertodermatida from all other platyhelminth taxa including the Acoela. The axoneme has the 9+2 microtubule arrangement as in normal body cilia and in the axonemes of many acoel sperm.

Acoela

RAIKOVA & JUSTINE [48] recently summarised data on sperm ultrastructure of 30 species from 11 families, including three investigated in that paper. There are two incorporated axonemes, extending from the proximal end up to, and sometimes into the nuclear region, and a considerable volume of cytoplasm. Sometimes each axoneme lies along the outer edge of a cytoplasmic extension, forming an undulating membrane along each side of the sperm. There are possibly four different arrangements of microtubules in the axonemes of acoel sperm: 9+2, 9+0, 9+1 and 9+"1". The most common configuration is 9+2/9+0, i.e. part of the axoneme has the normal 9+2, with some region(s) lacking the central pair of microtubules (i.e. 9+0) (Fig. 1). Some species

appear to have 9+0 throughout the axonemes. In some species, an axonemal central element consisting of a hollow tube (microtubule?) surrounded by an electron-dense halo has been described, denoted 9+1 [24, 57]. It does not have the double helical arrangement found in the dense central element of Trepaxonemata (see below). A dense central element possibly identical with Trepaxonemata sperm axonemes (9+"1"), was described in *Childia groenlandica* [24] and illustrated in *Mecynostomum auritum* [5]; it is not known whether this element has a double helical structure. RAIKOVA & JUSTINE [48] suggested that a misidentification cannot be excluded for the report of "*Mecynostomum*" with a 9+"1" structure. We have recently found a central dense element with a hollow centre and without double helical structure in *M. auritum* (personal observation).

Individual mitochondria, membrane-bound electron dense bodies of one or two kinds, and cortical and/or central microtubules are present in most species. In *Amphiscolops* [57] spermatids have some peripheral but no internal microtubules, while in the maturing sperm only internal ones were seen (Fig. 1). RAIKOVA & JUSTINE [48] suggest that these two groups of microtubules may be mutually exclusive, and that the internal ones derive from the external ones by an infolding of a longitudinal groove. Several species have been described without any microtubule arrays in the sperm. A fringe of terminal filaments has been observed by LM in two species [22, 27].

Spermiogenesis has been studied by EM in only a few acoel species [e.g. 6, 8, 48, 57]. ROHDE *et al.* [57] observed two centrioles, initially at right angles to each other in the early spermatid. Two free axonemes grow from the spermatid and fuse with the elongating shaft in a distal to proximal direction. The basal body region thus becomes distal. Nucleus, dense bodies and mitochondria migrate into the shaft, the elongated nucleus remaining in the proximal region. There is no intercentriolar body (see below) nor rootlets associated with flagellar basal bodies.

Macrostomida

Most species examined are from Macrostomidae, one from Dolichomacrostomidae (*Paramacrostomum tricladoides*) [22] and two from Microstomidae (*Microstomum* spp. [22]). No appendages are reported in the sperm of the last two families, nor of some species of *Macrostomum* [22] and *Bradynectes sterreri* [49] (both Macrostomidae). A pair of stiff bristles has been described from several species of *Macrostomum* [e.g. 6, 44, 55], and from *Promacrostomum gieysztori* [6]. They are anchored in tapering dense structures in the mid-region of the sperm with a dense, banded region between the base of the rod and the anchor [55]. In cross section, bristles interpreted as modified flagella, consist of a large central dense rod, surrounded by irregular smaller dense rods [55] (Fig. 2). The proximal end of *M. tubum* sperm is split into a fringe of microvilli [44, 55] and there are from 3-6 dense chromatin granules in a row just behind this proximal region (Fig. 3). The remainder of the sperm body is rich in mitochondria and elongate dense bodies with fewer, large dense bodies in close contact with the cell membrane. Two symmetrical, contralateral rows of peripheral microtubules extend along most of the shaft (Fig. 2), each bristle arising from the edge of one of these rows.

Spermatogenesis has been studied by EM only in *M. tubum* [55]. Cells containing numerous sections through chromatin were interpreted as spermatocytes. These gave rise, without further division, to bristles and the lateral rows of microtubules, thus spermatozoa formed directly from spermatocytes. The presence of up to six discrete chromatin granules in the sperm may indicate belated nuclear division or nuclear fragmentation.

Trepaxonemata

The remaining platyhelminth taxa form a monophylum, distinguished by an autapomorphic feature of the sperm axonemes (when present). Instead of the 9+2 arrangement of microtubules found in primitive sperm, the 9 pairs of peripheral microtubules surround a unique central complex (Fig. 6), made up of an electron dense core, a lighter zone around the core and an outer

electron dense zone which, in longitudinal sections, has a double helical structure (Fig. 4). This is known as the 9+"1" arrangement and is unique to this monophylum of the platyhelminths. Many of these taxa exhibit common structures and events during spermiogenesis, resulting in similarities in mature sperm. We will thus describe these basic events, present individual taxa data in tabulated form (Table 1), and note variations or noteworthy aspects separately in the text.

General description of spermiogenesis and spermatozoa in Trepaxonemata. Following spermatogonial and spermatocyte divisions accompanied by incomplete cytokinesis, spermatids usually remain joined in clusters by cytoplasmic bridges or cytophores. The spermatid nucleus moves towards the apical plasma membrane, initially with scattered clumps of chromatin that then become more dispersed and filamentous. The apical region becomes a zone of differentiation (ZD) in which two centrioles develop into the basal bodies of free flagella growing out in opposite directions from one another. A banded structure known as an intercentriolar body (ICB) develops which, when seen between the basal bodies, usually consists of a dense central band with a series of lighter, thinner bands on either side (e.g. Fig. 5). In cross section, the plates are circular, i.e. the structure in three dimensions is a cylinder with discs of various densities. Rootlets, either well developed and sometimes multipartite, or small and indistinct, usually attach to the sides of the basal bodies and extend in the direction of the apex of the nucleus or along the sides of it. Microtubules line the plasma membrane in the ZD. An apical projection, often with electron dense material within it, develops distal to the basal bodies. The main spermatid body elongates, carrying the distal projection and flagella distally (except in *Kronborgia* and Neodermata - see below). The nucleus, with increasingly condensed chromatin, moves into the shaft, along with mitochondria or a single fused mitochondrion, and dense bodies of one or more kinds. Flagella may bend proximally to lie alongside or fuse with the spermatid shaft. In some taxa the flagella also rotate around the shaft so that they emerge adjacent to each other. Aflagellate sperm develop by elongation of the spermatid, lined by microtubules. The nucleus elongates, chromatin condenses and dense bodies and mitochondria become distributed within the shaft.

TABLE 1. — Characteristics of sperm and spermiogenesis of Trepaxonemata (minus Neodermata)

ICB: intercentriolar body; 1 = present; 0 = absent.

R: rootlets or striated structures attached to the proximal sides of basal bodies; 1 = present (D divided); 0 = absent.

DP: dense plates located on the distal sides of basal bodies (may not be homologous in triclads and proseriates; 1 = present; 0 = absent).

ST: axoneme insertion; 1 = axoneme insertion becomes sub-terminal as a result of formation of a distal process, \pm = slightly sub-terminal, 0 = no distal process, 1-0 = distal process present but disappears.

BBD: Basal body; 1 = basal body region moves distal during spermiogenesis; 0 = remains proximal.

AN: axoneme number; 2-1 = initially 2 but only one in mature sperm.

AS: axonemes state; F = free, A = adhering; I = incorporated in shaft, I-F = most of axoneme incorporated, short free region.

AP: axonemal placement; A = adjacent to one another; O = on opposite sides of the shaft to one another.

SF: split tips of flagella; 1 = present.

M: mitochondria; M = multiple; R = in rows; D = altered, membranous derivative; F = fused into 1, 2 or 3+ long mitochondria.

NN: nature of nucleus; L = lobed; 2 = 2 components coiled around one another; Rods = dense chromatin rods; E = membranous elaborations; Env = nucleus partially envelops a string of mitochondria.

DB: dense bodies present in main shaft; 1 = present; 0 = absent; 2 = 2 kinds noted.

PM: peripheral microtubules; 1 = present, 1Sp = full peripheral row plus inner spiral group (see text); + = some additional internal microtubules present in some parts; few = widely spaced; inc = incomplete outer row where axoneme(s) are adjacent to the plasma membrane and/or remain outside the ring of microtubules.

G: granules (approximately 25 nm diameter) in conspicuous longitudinal rows beneath the cortical microtubules of the shaft.

Wherever two or more states are separated by a stroke (/), different states were found in different species.

- = structure not present because sperm are aflagellate.

Taxon	Characteristics of spermiogenesis					Characteristics of spermatozoon									
	ICB	R	DP	ST	BBD	AN	AS	AP	SF	M	NN	DB	PM	G	
Polycladida															
Cotylea					1	2	F	O		F					
Acotylea	1	1		1	1	2	A	O		MR		1	1		
Lecithoepitheliata															
Prothynchidae	1	1		1-0	1	2	F/A	O		M		2	1		
Prolecithophora															
(except <i>Urastoma</i>)	-	-	-	-	-	0				D	L	0/1	1+		
<i>Urastoma</i>						2	1	A?		F-2		0	1		
Proseriata															
Coelogynoporidae	1	1-D	1	1	1	2	F			M		1	1		
Nematoplanidae	1	1		1		2	F					1	1		
Monocelididae	1	1-D	1	1	1	2	F			MR/F		1	1		
Otoplanidae	1	1	1	1		2	F			MR/F		1	1		
Tricladida															
Terricola	1	1	1	1	1	2	F	A	1	F-1	2	0	1		
Maricola	1	1	1	1	1	2	F	A		F-1	2	0	1+		
Paludicola	1	1	1	1	1	2	F	A	1	F-1	2	0	1+		
Typhloplanida															
Typhloplanidae	1	1/?		±	1	2	MF	A		M		1	1-inc/1		
Promesostomidae						2	F								
Solenopharyngidae						2	F			MR	E	2?	1	1	
Byrsophlebiae	1	1		1	1	2	F	A		MR	Env	2	1	1	
Trigonostomidae						0									
Kalyptrorhynchia															
Eukalyptrorhynchia				±	1	2	1	O		F		2	1		
Schizorhynchia	1	1		±	1	2-1	1			F	Rods	0	1		
Dalyciida															
Dalyciidae	1	1-D		1	1	2	F	A		MR	Env	2	1		
Provorticidae						0									
Graffillidae	3/-1	3/-1		1/-±	1/-1	2/0/2	F/-1	3/-A		3/F-2/R		1/0/2	1/1/1-inc	1	
Luridae	-	-	-	-	-	0				M		1	1 few		
Umagillidae	1/-	3/-			1/-	2/0	F/-	O	1/-	M		1	1		
Pterastericolidae	1 (weak)	0		0	1	2	1-F	O		M		1	1-inc		
Temnocephalida															
Temnocephalidae	1	1		1	1	2	F	A		M		1	1Sp	1	
Actinodactylellidae	1	1		1	1	2	F	A		M		1	1Sp		
Scutariellidae	1	1		1	1	2	F	A		F-2		0	1	1	
Didymorchidae	1	1		1	1	2	F	A	1	M		1	1Sp	1	
Fecampiida															
Fecampiidae	0	0	0	0	0	2	1	O		F-3+		0	1		

Polycladida

There are many LM studies of Polycladida [e.g. 21, 22], but only one EM study of spermiogenesis (*Notoplana japonica*) [38] and few EMgraphs of sperm [e.g. 27]. Flagella remain free in Cotylea but superficially united with the shaft in Acotylea, sometimes part of an undulating membrane but never wholly incorporated beneath the plasma membrane. Flagella arise from and remain on opposite sides of the shaft very close to the distal tip of the spermatozoon [22]. There are multiple dense bodies [e.g. 27] and multiple mitochondria, some apparently fused into larger aggregates [38]. Longitudinal microtubules lie in a complete ring beneath the plasma membrane, apparently for the whole length of the spermatozoon.

Spermiogenesis in *Notoplana japonica* [38] is basically as described above for the Trepaxonemata. KUBO-IRIE & ISHIKAWA [38] described additional proximal centrioles attached at right angles to the main ones during spermiogenesis, but the micrographs are not clear. There has been no other report of four centrioles in the ZD of a platyhelminth spermatid.

Lecithoepitheliata

There is one EM study of sperm and spermiogenesis of *Prorhynchus* sp. [79] (Table 1). Sperm have an irregular contour, and flagella lie in grooves of wide lateral extensions of the shaft (Fig. 13). The testes of some Baikal endemic *Geocentrophora* species (*G. interstitialis*, *G. wagini* and *G. wasiliewi*) were examined [7] and sperm found to have two free flagella with 9+1 axonemes, longitudinal microtubules and 1-5 longitudinal folds. No members of Gnosesimidae have been investigated.

Prolecithophora

Mature sperm have been examined in several species [e. g. 11, 14, 16, 22, 43, 60] and spermatid development in *Multipeniata* [60]. Sperm are aflagellate, have a row of cortical microtubules, lack dense bodies (except for *Multipeniata*) and have a complex intraspermial membranous system (mitochondrial derivative?) closely associated with the lobed nucleus in a spiralling arrangement in some species. Sperm of *Urastoma cyprinae* do not resemble those of the other prolecithophorans examined [46]. There are two initially free axonemes that become completely incorporated in the mature sperm body, peripheral microtubules that are crowded into the centre of the shaft at one end, at least two mitochondria of regular appearance, no dense bodies and no membranous system. It is possible that *Urastoma* may not belong to the Prolecithophora, as suggested by NOURY-SRAÏRI *et al* [46].

Proseriata

There are several reports and a few micrographs of some aspects of sperm [22, 25, 65, 69] and three reports of some aspects of spermiogenesis from three of the seven families [65-67, 69]. Sperm have two free, sub-terminally inserted axonemes, peripheral microtubules, an elongate nucleus, numerous dense bodies, and mitochondria arranged in a tightly packed longitudinal row or rows (Fig. 4). During spermiogenesis, a well developed ICB lies between the basal bodies, and rootlets (sometimes multipartite) extend from the basal bodies towards and alongside the nucleus. In three species of Parotoplaninae (Otoplanidae), a distinctive striated distal appendage to the ICB forms during cell elongation. SOPOTT-EHLERS [67] suggested the structure to be an autapomorphy of Parotoplaninae. Changes in the ICB were also documented in a nematoplanid [66] and a coelogynopodid [65, 69], involving considerable stretching and partial splitting in the first, and transformation of some elements and partial splitting in the second.

Tricladida

Species from all three sub-taxa (Paludicola, Maricola and Terricola) have been examined by EM [17-19, 28, 30, 31, 37, 40, 59, 62, 63] and some common characteristics identified [59]. All have two free, sub-terminally inserted flagella which arise together on one side of the shaft, a distal projection beyond the flagellar insertion, and nucleus and a single fused mitochondrion (Fig. 8) extending throughout most of the remaining length of the sperm (in contrast to many other turbellarians, in which the nucleus is restricted to a shorter proportion of the sperm body). The nucleus has two distinct components, one (the protein component [62]) less dense than the other, coiled around each other in screw-like fashion (Figs 6, 8). In at least some species, nucleus and elongate mitochondrion also coil around each other. Flagella of mature sperm contain many small granules and the tips split into smaller units (Fig. 6) (Paludicola, Terricola). Mature sperm have a short inner row of microtubules in addition to the full peripheral row (Paludicola, Maricola) and lack the numerous dense bodies characteristic of many turbellarians (Paludicola, Maricola, Terricola). During spermiogenesis, the nucleus has a dense apical layer, there are short cross-striated rootlets from the basal bodies to the nucleus, and dense, half-moon shaped plates around the basal bodies opposite the rootlets (Fig. 5) (Paludicola, Maricola, Terricola). In at least two paludicolans and one maricolan the ICB splits into two halves attached to the basal bodies during rotation [18, 59].

Rhabdozoela

Typhloplanida. There are LM reports of sperm with and without free flagella [e. g. 22], but only detailed EM reports of three species, *Bothrosostoma personatum* [13] and *Phaenocora anomalocoela* [80], both Typhloplanidae, and *Anthopharynx sacculipenis*, Solenopharyngidae [68]. *B. personatum* sperm have two free, subterminally inserted flagella emerging together on the same side of the shaft, dense bodies, mitochondria and a complete ring of cortical microtubules. During spermiogenesis, dense heels form at the ends of the basal bodies which then rotate around the shaft, resulting in compression of one semicircle of microtubules into a tight double row. *P. anomalocoela* sperm (Fig. 12) have two fully incorporated axonemes of unequal length, numerous mitochondria and dense bodies. Basal bodies are close together and axonemes also remain adjacent for their entire length, situated between the plasma membrane and the row of microtubules. The nucleus extends almost the entire length of the sperm. During spermiogenesis, dense heels form at the ends of basal bodies, and massive rootlets are attached to the sides of basal bodies as they rotate around the shaft, compressing some of the peripheral microtubules into a central double row. The manner in which the compressed row rejoins the outer microtubules does not result in a spiral formation (cf. Temnocephalida below). *A. sacculipenis* sperm are filiform, totally enclosed by cortical microtubules, possess two free flagella, one or two rows of tightly packed mitochondria, and have regular rows of granules beneath the plasma membrane. There are numerous dense bodies along the shaft and terraced elaborations of the nuclear membrane. Similarly, two free flagella, regular rows of granules, dense bodies and a tightly packed row of mitochondria are present in the sperm of *Maehrentalia* sp. (Byrsophlebidae) [75]. *Typhloplana virida* has two free, 9+“1” flagella [42].

Kalyptorhynchia. By LM and EM, no sperm have been found with free flagella [22, 25, 39].

— *Eukalyptorhynchia*. EMgraphs of sperm of *Gyratrix* sp. (Polycystidae) and *Odontorhynchus* sp. (Fig. 14) (Gnathorhynchidae) [52] and *Polycystis naegeli* (Polycystidae) [39] show dense bodies, probably of two kinds, an elongate mitochondrion, peripheral microtubules and two fully incorporated axonemes. In *P. naegeli* the nucleus contains a number of dense bodies (long rods or short rounded structures?). Basal bodies are staggered and slightly

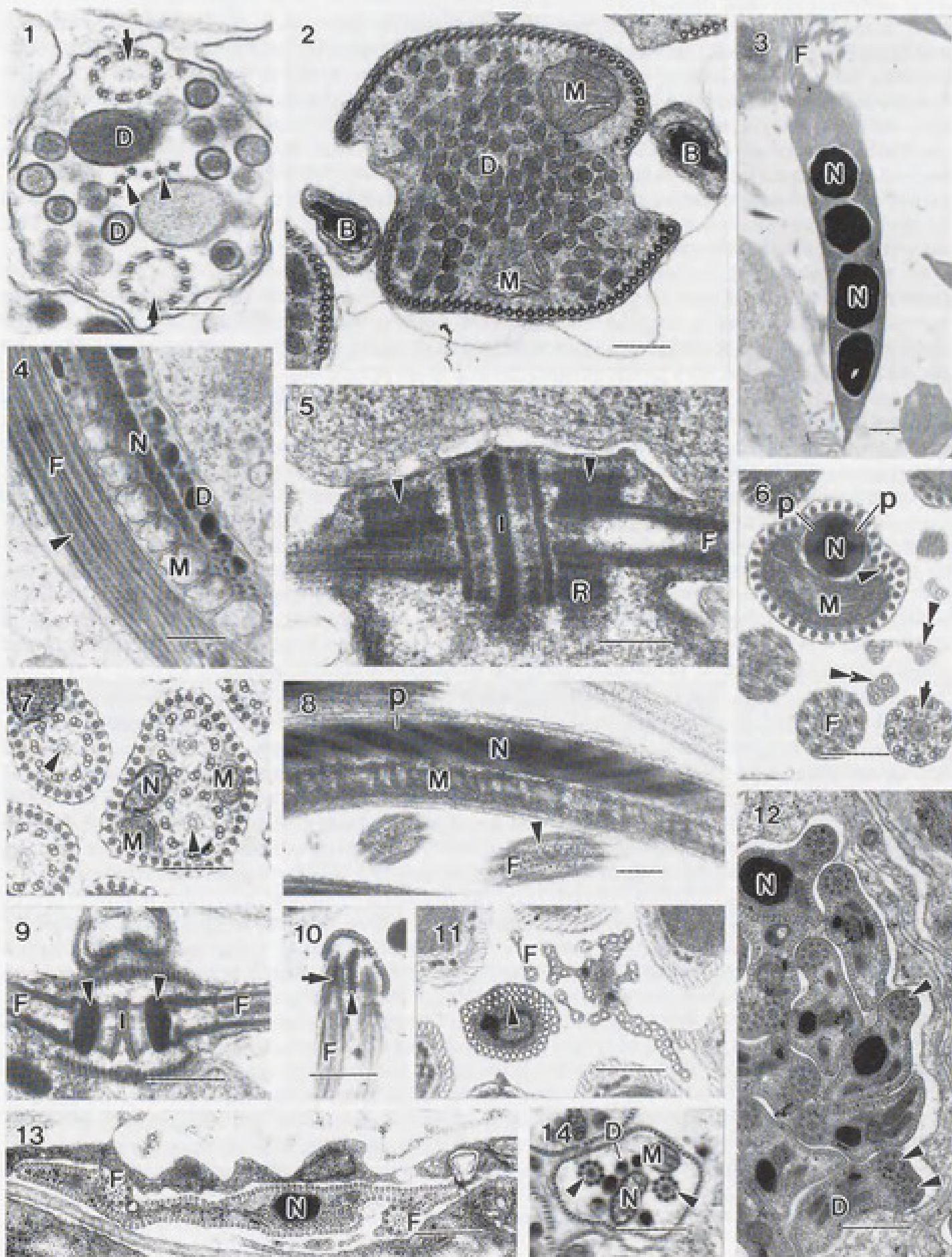
subterminal. L'HARDY [39] also studied spermiogenesis in *Polycystis naegeli* - initially free flagella are incorporated in a distal to proximal manner.

— *Schizorhynchia*. *Baltoplana magna* sperm have a single incorporated axoneme [23, 25]. WATSON & L'HARDY [76] found two basal bodies in the ZD, with an ICB between them. Only one develops into a normal flagellum - the other remains as a short bud. Both are carried distally by the elongating shaft and one is incorporated into the shaft in a distal to proximal direction while the spermatid is still pyriform. The other degenerates and mature sperm have only a single axoneme, from one end to almost the other end. The shaft is surrounded by cortical microtubules, there is an elongate mitochondrion, no dense bodies and the nucleus has an unusual arrangement of long dense chromatin rods. Preliminary observations by SCHOCKAERT (personal communication) suggest that several schizorhynch families may contain taxa with monoaxonemal sperm.

Dalyelliida. This diverse group contains free living, symbiotic, and parasitic species, but there are few reports of detailed EM investigations.

— *Dalyelliidae*. Two micrographs of sections of *Microdalyellia* sperm [27] and a full study of spermiogenesis and sperm of *Gieysztor* sp. [82] show two free axonemes, multiple mitochondria, dense bodies (2 kinds in *Gieysztor*), and in *Gieysztor* the nucleus almost completely envelops a string of mitochondria along the shaft. During spermiogenesis in *Gieysztor*, an ICB lies between the basal bodies, dense heels develop at the bases of basal bodies, and flagella rotate around the shaft to lie adjacent to one another. Rotation causes compression of one semicircular row of peripheral microtubules, so that they lie in a double row in the middle of the spermatid, in the region of the basal bodies. The manner in which the compressed row rejoins the outer row does not result in a spiral formation (cf. Temnocephalida).

FIGS 1-14. — Spermatozoa of "Turbellarian" Platyhelminthes. 1: Transverse section of sperm of *Amphiscolops* sp. (Acoela). Note incorporated axonemes (arrows) without central element, two sizes of dense bodies (D), central array of microtubules (arrowheads). Bar = 200 nm. 2: Transverse section of sperm of *Macrostomum tubum* (Macrostomida). Note numerous dense bodies (D), mitochondria (M), bristles (B) and contralateral rows of peripheral microtubules. Bar = 200 nm. 3: Longitudinal section of sperm of *Macrostomum tubum* (Macrostomida). Note terminal fringe of filaments (F) and separate nuclei or nuclear fragments (N). Bar = 500 nm. 4: Longitudinal section of sperm of *Monocelis* sp. (Proseriata). Note nucleus (N), dense bodies (D), row of mitochondria (M) and flagella (F) with double-helical central element. Bar = 200 nm. 5: Zone of differentiation of *Romankenkius libidinosus* (Tricladida). Note intercentriolar body (I), rootlet (R), flagellum (F) and dense plates (arrowheads). Bar = 200 nm. 6: Transverse section of sperm of *R. libidinosus* (Tricladida). Note nucleus (N) with dark and lighter (p) components, some additional internal microtubules (arrowhead), free flagellum (F), split tips of flagellum (double arrowheads) and dense granules between spokes of flagellum (arrow). Bar = 200 nm. 7: Transverse section through sperm of *Kronborgia isopodicola* (Fecampiida). Note nucleus (N), mitochondria (M), and incorporated axonemes with hollow central elements (arrowheads). Bar = 200 nm. 8: Longitudinal section of sperm of *R. libidinosus* (Tricladida). Note single long mitochondrion (M), nucleus with spiralling lighter component (p), and flagellum (F) with dense granules (arrowhead). Bar = 200 nm. 9: Zone of differentiation of *Decadidymus gulosus* (Temnocephalida). Note intercentriolar body (I) beginning to split, and dense heels (arrowheads) at the ends of the basal bodies of flagella (F). Bar = 500 nm. 10: Spermatid of *Decadidymus gulosus* (Temnocephalida) after rotation of flagella. Note inner compressed double row of microtubules (arrowhead) and spur on the basal body (arrow) of a flagellum (F). Bar = 500 nm. 11: Cross sections through sperm of *Craspedella spenceri* (Temnocephalida). Note spiral microtubule arrangement (arrowhead) and filaments (F) resulting from splitting of the proximal end of the sperm. Bar = 200 nm. 12: Cross sections through sperm of *Phaenocora anomalocoela* (Typhloplanida). Note nucleus (N), dense bodies (D) and one or two axonemes (arrowheads) incorporated just beneath the plasma membrane. Bar = 500 nm. 13: Transverse section of sperm of *Prorhynchus* sp. (Lecithoepitheliata). Note nucleus (N) and flagella (F) lying in grooves of the shaft. Bar = 500 nm. 14: Transverse section through sperm of *Odontorhynchus* sp. (Kalyptrorhynchia). Note nucleus (N), dense body (D), single mitochondrion (M) and incorporated axonemes (arrowheads). Bar = 500 nm.



— *Provorticidae*. *Provortex balticus* has elongate sperm, without flagella [22].

— *Graffillidae*. The sperm of the parasitic species *Paravortex cardii*, *P. karlingi*, *P. tapetis* and *Graffilla buccinicola* are aflagellate [12, 22, 45] while free-living *Pseudograffilla arenicola* has two free, sub-terminally inserted flagella [22]. In the three *Paravortex* species, regular longitudinal rows of small dense granules extend throughout the sperm, beneath the peripheral microtubules. They resemble regular rows found in temnocephalans and do not react positively to the Thiéry test for glycogen [45]. Similar rows of granules (also negative to the Thiéry test) are present in the sperm of *Bresslauilla relictia* [75] which have two superficially attached 9+“1” axonemes, two kinds of dense bodies (one occurring outside of the cortical microtubules) and a single row of mitochondria. NOURY SRAÏRI *et al.* also found small numbers of membrane-bound dense bodies in *P. cardii* and *P. tapetis* [45].

— *Luridae* [70]. In *Luriculus australiensis* [58] microtubules in a helical configuration surround the spermatid, and progressive elongation results in formation of an aflagellate, filiform spermatozoon, containing regular rows of dense bodies, mitochondria and a ring of a small number of longitudinal peripheral microtubules, interrupted where organelles are close to the surface.

— *Umagillidae*. From LM, *Syndesmis echinorum* sperm are reported to be aflagellate [22]. *Cleistogamia longicirrus* and *Seritia stichopi* sperm (EM) [56] have two free flagella, numerous dense bodies, multiple mitochondria and peripheral microtubules. *Syndisyrinx punicea* has similar sperm (and split flagellar tips) [54], an ICB is present during spermiogenesis, and although dense floccular material surrounds the basal bodies, no rootlets were seen [41].

— *Pterastericolidae*. Sperm of 5 species of *Pterastericola* and spermiogenesis of one of these (*P. astropectinis*) have been examined by EM [33, 83]. A faint ICB is present but apparently no rootlets. Basal bodies and free flagella are carried distally at the end of the elongating spermatid, before fusing with the shaft for most or all of their length. There are a few dense bodies, a small number of proximally positioned, elongate mitochondria and a row of peripheral microtubules which is discontinuous where one or sometimes both axonemes lie close to the surface. Regular centrioles composed of triplets were seen in mature sperm.

Temnocephalida. Sperm and spermiogenesis have been studied in one species from each of Actinodactylellidae, Scutariellidae and Didymorchidae and at least 12 from Temnocephalidae.

— *Temnocephalidae*. There are more or less comprehensive reports of sperm and spermiogenesis in seven species of *Temnocephala*, three of *Craspedella*, and *Decadidymus gulosus* and *Diceratocephala boschmai* [36, 82, 84-87]: they are compared by WATSON & ROHDE [82]. They have in common the formation of dense heels on the basal bodies (Fig. 9) and compression of a semicircle of microtubules by rotation of the basal bodies around the shaft as described above for *Gieysztoria*. However, the two edges of the compressed row rejoin the uncompressed row in an unequal manner, resulting in a region of the spermatid and mature sperm in which the peripheral microtubules are arranged spirally (Fig. 11). In addition, proximal ends of mature sperm have either a terminal flange of microtubules within the plasma membrane, or are split into a fringe of fine filaments containing microtubules (Fig. 11). It is likely that flange and split shaft represent modifications of the one feature, since both structures occur in the one genus (*Temnocephala*) [84] and there is a tendency for the flange in *T. dendyi* to break up into filaments (personal observation). Membrane-bound dense bodies and numerous smaller granules, in regular rows beneath the microtubules, are present in the sperm of most of the species examined. A dense body is usually located very close to the end microtubule of the inner spiral row.

— *Actinodactylellidae*. Spermiogenesis in *Actinodactylella blanchardi* [82] involves formation of an ICB, rootlets and a similar dense heel on the basal bodies, flagellar rotation and compression of one semicircular row of microtubules and a spiral region of microtubules where the compressed region rejoins the other microtubules. The proximal end of the mature sperm is split into a fringe of filaments, containing microtubule(s).

— *Scutariellidae*. Sperm of *Troglocaridicola* sp. have a similar proximal fringe of filaments and dense heel formation at the ends of basal bodies during spermiogenesis [29] and compression of a semicircular row of microtubules by rotation of the basal bodies. However, expansion of this row apparently does not result in a spiral arrangement. Mature sperm have regularly arranged longitudinal rows of small granules beneath the peripheral microtubules, similar to those found in *Paravortex* (Graffillidae) and *Temnocephalidae*, but no membrane-bound dense bodies. The distal, anterior region comprises an electron dense, not membrane-bound corkscrew originating from the apical process of the spermatid. This structure appears to be unique among the free-living platyhelminths but resembles terminal structures found in many cestodes [3] and the monogenean *Calicotyle* [71, 81].

— *Didymorchidae* [32]. Only *Didymorchis* sp. (from *Cherax destructor*) has been examined [82]. Sperm and spermiogenesis are as in *Actinodactylella*; the nucleus in the distal region also has a thin trailer which extends to the plasma membrane (also present in *Temnocephala minor* [84]).

Fecampiida. *Kronborgia isopodicola* is the only species in which sperm or spermiogenesis has been examined [77, 78, 88]. Sperm have two fully incorporated axonemes of unequal length, longitudinal, peripheral microtubules, no dense bodies and a small number of elongate mitochondria (Fig. 7). Spermiogenesis [78] involves formation of two free axonemes, incorporated into the spermatid in a proximal to distal manner. There is no ICB nor are there flagellar rootlets, but many microtubules attached to the basal bodies probably effect rotation of the basal bodies to lie parallel from their initial orientation at right angles to each other. Both ends of the sperm taper markedly, the proximal (anterior) end containing a dense rod which becomes horseshoe shaped in cross section and in which the ends of the longitudinal microtubules are embedded. The distal (posterior) end beyond the nucleus consists of a long narrow filament containing a few, and eventually no microtubules.

Phylogenetic implications

The validity of comparisons of sperm and spermiogenesis of different taxa depends on quality of fixation and "completeness" of the investigations. Concerning fixation, clarity of most cellular components differs in different studies even if specimens are processed similarly. For example, dense bodies sometimes appear very dense and/or membrane-bound and sometimes very indistinct. Reasons could be real differences in composition, fixation artefacts or changes with development. Smaller, not membrane-bound granules are sometimes noted in the cytoplasm of the sperm body (Table 1) arranged in very regular rows, best seen in tangential longitudinal section. Their absence in other groups could be of phylogenetic significance or a fixation artefact.

Concerning "completeness" of investigation, many studies were carried out to answer particular questions, such as whether sperm are biaxonemal and have the 9+1 arrangement. Such brief investigations allowed some important generalisations leading to the establishment of the taxon *Trepaxonemata*, the distinction between *Cotylea* and *Acotylea* sperm, and recognition of the complete incorporation of axonemes in kalyptorhynchids (indicating monophyly). Some studies are less complete than others because few cells of a particular stage were available or because serial sectioning was not used. Thus it is unclear whether certain structures such as rootlets, ICB, spiral of microtubules etc. were overlooked or are indeed absent. When comparing the morphology of the nucleus, for example, degree of maturation of the spermatid is crucial and sperm should be examined from the seminal vesicle or sperm ducts as well as from the testis.

Considering all this, we must conclude that only a small number of species has been examined in appropriate depth for detailed phylogenetic considerations. Suitable data can be used in two ways for such considerations - to examine the monophyly of established taxa, and to look for evidence of relationships between such taxa. As a first step, autapomorphic feature states need to be identified (refer to Table 1).

1. *Flagella: presence, kind, number and location.* The primitive metazoan sperm is considered to be monoflagellate [20], while most platyhelminths possess two flagella or incorporated axonemes. The biaxonemal condition could be plesiomorphic for the whole phylum (lost in Catenulida and Nemertodermatida, highly modified in Macrostomida) or independently acquired in Acoela and Trepaxonemata (or in Acoela and Rhabditophora if the bristles of macrostomids are modified flagella). Absence of flagella, reduction to a single flagellum, and the state of incorporated axoneme(s) are undoubtedly apomorphic states in some taxa, and the unique central element is a synapomorphy for Trepaxonemata (unless the central element in some acoels also has the same structure, but see earlier note regarding possible misidentification). All Acoela, Kalyptorhynchia and Neodermata have axonemes incorporated in the sperm body, all acotylean polyclads have axonemes superficially attached to the sperm body, and all Prolecithophora (except *Urastoma*) lack axonemes. Paired incorporated axonemes in Acoela are not associated with the presence of an ICB as they are in Kalyptorhynchia and Neodermata, and are therefore likely to be autapomorphic. Incorporated axonemes are also found sporadically in other taxa (eg. Pterastericolidae, *Phaenocora* in Typhloplanidae, *Bresslauilla relict*a in Graffillidae), where they represent a derived condition relative to other species in the taxon. The same applies to aflagellate sperm. Thus, all Catenulida and Prolecithophora (except *Urastoma*) lack flagella, but so also do some species of rhadocoels, e.g. the only species of Luridae and Provorticidae examined, four of the six species of Graffillidae examined, and one species of Trigonostomidae. With so few taxa examined in detail, we cannot imply relationships between higher taxa on the basis of presence, absence, incorporation or not of flagella, especially since loss of structures is common in evolution and these traits may be more closely linked to reproductive biology than to ancestry. Table 1 shows that flagella arise on opposite sides of the shaft in most taxa but adjacent to each other in Tricladida, Dalyelliidae, Temnocephalida and Typhloplanidae. In the last three the condition is likely to be synapomorphic, since it is associated with a unique mode of rotation of the flagella during spermiogenesis; it may be of independent origin in Tricladida because different structures are present in the ZD.

In at least four species, all symbiotic, *Syndisyrinx punicea*, *Kronborgia isopodicola*, *Pterastericola asamushii* and the monogenean *Anoplodiscus cirruspiralis*, the central element of axonemes appears hollow in cross section instead of solid as in other trepaxonematan taxa, although it does have the double helical arrangement in longitudinal section. The phylogenetic significance of this difference is unknown.

2. *Mitochondrion/mitochondria.* Within the phylum a wide spectrum of mitochondrial formations is present ranging from numerous small mitochondria, through regular, tightly packed rows of individual mitochondria, to varying degrees of fusion and formation of mitochondrial rods and derivatives. The fused forms derive from many small mitochondria during spermiogenesis. Acoela all retain scattered individual mitochondria, triclad and kalyptorhynchs appear to have a single rod-like mitochondrion, prolecithophorans (except *Urastoma*) have a complex membranous derivative, at least two families of Proseriata have regular rows of tightly packed mitochondria, whereas within the Dalyelliida and Temnocephalida a range of states occurs. Presence of small and of fused mitochondria can be demonstrated, but it is very difficult to prove that only a single mitochondrial rod exists because of the considerable length of many sperm. It is likely that fused mitochondria have arisen many times independently. However, if it can be shown that *all* species in the taxon exhibit the derived state (such as in Prolecithophora (except *Urastoma* which may not be a prolecithophoran), Neodermata, Tricladida) then it is likely to be an autapomorphy for that taxon, especially if there are other sperm or spermiogenetic synapomorphies for the group.

Among other characters of sperm and spermiogenesis, a number of apomorphic states can be identified and used for phylogenetic deliberations. These are as follows:

a. The 9+1 axoneme in Trepaxonemata (Polycladida, Lecithoepitheliata, Proseriata, Tricladida, Typhloplanida, Kalyptorhynchia, Dalyelliida, Temnocephalida and Neodermata).

Prolecithophora are aflagellate (except *Urastoma*), therefore indeterminate on this basis. Macrostomids are aflagellate or have a pair of stiff bristles, perhaps modified flagella, but there is no 9+“1” structure to suggest inclusion in this taxon, although secondary modification cannot be excluded. Some acoel species have a solid central element but its homology with 9+“1” has not been established;

b. The ICB is present in most species of Trepaxonemata possessing axonemes (notably absent in *Kronborgia* and monopisthocotylean monogeneans [35]). It does, however, appear to vary in composition and in the changes which it undergoes during spermiogenesis in different taxa, and these variations may be autapomorphic for smaller taxa;

c. Helical arrangement of two distinctive components of the nuclei of triclads;

d. Lobed nucleus of prolecithophorans;

e. Spiral arrangement of microtubules in the region of sperm adjacent to the flagellar insertion in a taxon consisting of Temnocephalida minus Scutariellidae;

f. A proximal terminal flange or fringe of microvilli-like processes on mature sperm of Temnocephalida. A terminal fringe is also present in some *Macrostomum* sp. and two acoels, but clear differences in other sperm characters suggest independent origin in these groups;

g. A suite of characteristics seen during spermiogenesis in Temnocephalida, Dalyelliidae, Byrsophlebiae and Typhloplanidae (dense heel formation at the end of basal bodies, rotation of flagella around the shaft resulting in compression of a semicircle of microtubules, formation of a spur on the basal bodies in mature sperm). In *Decadidymus gulosus* (Temnocephalidae) the ICB splits completely and one half accompanies each basal body during rotation - it is likely that the same occurs in the other taxa. This combination of characteristics suggests a common ancestor for these groups. It is not present in Pterastericolidae and Umagillidae where it may have been secondarily lost or the absence could indicate that these families are misplaced. Some dalyelliid families have species with aflagellate sperm, hence no ICB etc., some have not been studied, and no other typhloplanid families have been examined. It is therefore premature to draw phylogenetic conclusions, but it does appear that detailed studies of events in the ZD of many more species could help to clarify relationships within Rhabdocoela (minus Neodermata). The ICB also splits into two in some triclad species, but different accessory structures are involved;

h. The bristles, multiple nuclei and unusual spermiogenesis of *Macrostomum* have not been seen in any other platyhelminth taxa and also differ significantly from the primitive sperm and spermiogenesis models of many Metazoa: they must be seen as apomorphies.

The foregoing discussion indicates that sperm and spermiogenetic characters support the monophyly of each of the Acoela, Polycladida Acotylea, Prolecithophora (except *Urastoma*), Tricladida, Temnocephalida minus Scutariellidae, Kalyptorhynchia, Neodermata. They also provide significant evidence to suggest that some taxa may be misplaced, e.g. *Urastoma* may not be a prolecithophoran and *Kronborgia* is not a rhabdocoel.

Regarding relationships between higher taxa, an area of great interest concerns the origin of the Neodermata, without doubt a monophylum as shown by a range of characteristics including the following: sperm with completely incorporated axonemes (or single axoneme or none by secondary reduction), a single elongate mitochondrion (or none by reduction), no dense bodies, and incorporation of axonemes in the proximal to distal direction [4, 35]. Suggested sister group relationships to Neodermata have been proposed on the basis of the common occurrence of one or more of these character states in some turbellarian groups (in conjunction with other characters not related to sperm and spermiogenesis), e.g. with Fecampiidae [77] and with a clade comprising Pterastericolidae, Fecampiidae and Acholadidae [34]. However, the first has not been supported by DNA studies and at least the inclusion of Pterastericolidae in the second is not supported by the later finding of dense bodies and distal to proximal fusion in spermiogenesis of Pterastericolidae [83]. There is also no evidence from sperm or spermiogenesis in support of a close relationship of Temnocephalida and Neodermata as suggested by BROOKS [9], or of “Dalyellioida” and

Neodermata suggested by EHLERS [15]. The sister group of the Neodermata remains undetermined.

The question of the relationship between Catenulida, Acoelomorpha and Rhabditophora has been addressed by several authors [e.g. 2, 15, 64 and references in these] but recent studies of sperm and spermiogenesis only confirm the divisions, without shedding light on their interrelationships. Spermatogenesis and spermatozoa in the Catenulida are unlike those observed in any other platyhelminth group. If the monoflagellate condition of Nemertodermatida is plesiomorphic (i.e. if the last common ancestor of the Acoelomorpha was monoflagellate with a 9+2 flagellum), then presence of two flagella in Acoela and the remainder of the platyhelminths is likely to be of independent origin. However, if a second axoneme has been lost in Nemertodermatida, the biflagellate condition need only have arisen once in the stem species of Acoelomorpha and Rhabditophora. Some sperm with two axonemes have been found in *Nemertoderma* sp. A [73] but spermiogenesis has not been studied.

The taxa commonly known as rhabdocoels (Typhloplanida, Kalyptorhynchia, Dalyelliida and Temnocephalida) appear from DNA and protonephridial ultrastructure to constitute a monophylum, but relationships between the orders and placement of some families within orders are uncertain. JONDELIUS & THOLLESSON [34] used parsimony analysis of 21 LM and EM characteristics to derive a working hypothesis for the Rhabdocoela, but the homology of many of the characters utilised is questionable. Sperm and spermiogenesis studies may prove useful because certain taxa within the group have very distinctive structures and processes.

In conclusion, sperm and spermiogenesis data have the potential to contribute significantly to phylogenetic analysis within the turbellarians, but many more subordinate taxa must be examined for a parsimony analysis.

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