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Spermatozoon and spermiogenesis in *Mesocoelium monas* (Platyhelminthes: Digenea): ultrastructure and epifluorescence microscopy of labelling of tubulin and nucleus

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Abstract. Spermiogenesis and the spermatozoon were studied in the digenean *Mesocoelium monas* Rudolphi, 1819 (from the toad *Bufo* sp. in Gabon). An ultrastructural study revealed that spermiogenesis follows the usual pattern found in digeneans, i.e. proximo-distal fusion of axonemes with a median cytoplasmic process followed by elongation. The spermatozoon has two fully incorporated axonemes with the 9 +"1" trepaxonematan pattern. Indirect immunofluorescence localization of tubulin and fluorescent labelling of the nucleus were used to obtain additional information on the structure of the spermatozoon. It was thus shown that one of the axonemes is slightly shorter than the other (190 versus 220 μm) and that the filiform nucleus (65 μm in length) is located at the distal extremity of the spermatozoon (220 μm in length). Various monoclonal and polyclonal antibodies, specific to alpha, beta, acetylated-alpha, or general tubulin, were used and produced similar labelling.

Spermatozoon ultrastructure is a valuable tool for phylogeny in the parasitic Platyhelminthes. It has provided information for the phylogeny of the Platyhelminthes as a whole (Ehlers 1985, Justine 1991a), the "Turbellaria" (Watson and Rohde 1995), the Monogenea (Justine 1991b) and the Eucestoda (Euzet et al. 1981, Bâ and Marchand 1995). However, in the Digenea, spermatozoon ultrastructure is relatively homogeneous (Justine 1995), and thus sperm ultrastructure has provided few characters of significance for phylogeny. Digenean spermatozoa are all filiform, with two incorporated axonemes, and thus conform to the plesiomorphic pattern found in the Cercomeridea (Justine 1991a, 1995); schistosomes constitute the single exception to this general pattern (Justine et al. 1993).

However, the number of species in which sperm ultrastructure has been described is lower in the Digenea than in the Monogenea (40 versus 60, see Table 1 in Justine 1995 and Podvyaznaya 1996) and it is not unlikely that the description of spermatozoa in new taxa of digeneans will lead to the discovery of different structures. Moreover, the use of several techniques in addition to transmission electron microscopy may provide new results useful for phylogeny. Immunocytochemistry of tubulin has given valuable information on the structure of spermatozoa of Crustacea (Tudge and Justine 1994), Nematoda (Mansir and Justine 1995) and Platyhelminthes (Iomini et al. 1995, Iomini and Justine 1997, Raikova et al. 1997).

Here we report observations on a digenean *Mesocoelium monas* Rudolphi, 1819, from an African toad.

MATERIALS AND METHODS

Materials

Toads, *Bufo* sp., collected at Franceville, Gabon were brought back alive to our laboratory (registration number of specimens 147SE). Specimens of *Mesocoelium monas* were collected from the intestine. Several specimens were fixed in 10% formalin and stained with carmine for determination, and have been deposited in the collection of the MNHN under registration number 66TN.

Transmission electron microscopy

Living specimens were fixed in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer, the worms were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in Spurr's resin. Ultrathin sections were contrasted with lead citrate and uranyl acetate, and observed with a Hitachi H600 electron microscope.

Immunocytochemical labelling, labelling of nucleus and epifluorescence microscopy

Germ cells were obtained by squashing worms in a drop of PBS (phosphate buffer saline, Sigma) on a pit slide previously washed with alcohol and acetone. The slides were allowed to

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Table 1. Antibodies used for immunocytochemical experiments on spermatozoa of *Mesocoelium monas*.

Antibody	Antigen used for antibody production	Reference	Concentration	Labelling of spermatozoa
Monoclonal anti alpha-tubulin	chicken embryo brain microtubules	Clone DM1A, Sigma (Blose et al. 1984)	1/200	+
Monoclonal anti beta-tubulin	rat brain tubulin	Clone TUB 2.1, Sigma (Gozes and Barnstable 1982)	1/200	+
Monoclonal anti-tubulin	cytoskeleton of *Trypanosoma brucei*; specific to alpha- and beta-tubulin	Clone TAT 1 (Woods et al. 1989)	1/200	+
Monoclonal anti-tubulin	bovine brain microtubules	Clone 16D3 (supernatant) (Gallo and Précigout 1988)	1/10	+
Monoclonal anti acetylated alpha-tubulin	acetylated alpha-tubulin from the outer arm of sea urchin (*Strongylocentrotus purpuratus*) sperm axonemes	Clone 6-11B-1 Sigma (Piperno and Fuller 1985)	1/200	+
Polyclonal anti-tubulin	chicken embryo brain microtubules	Sigma, T3526	1/40	+

dry for 1 h under a fan, then kept at 4°C and processed within 24 h. Cells were permeabilized in acetone (10 min, room temperature). Slides were then rinsed (PBS, 3 × 5 min). Non-specific antigens were blocked with 2% Bovine Serum Albumin (Sigma) in PBS (BSA-PBS) for 45–90 min at room temperature. A monoclonal or a polyclonal anti-tubulin antibody (see Table 1) was applied for 40 min at room temperature. After rinsing (PBS 3 × 5 min), a FITC-conjugated antibody (Goat anti-mouse, Nordic, 1/40 in PBS for monoclonal anti-tubulin antibodies, or Goat anti-rabbit, Nordic, 1/40 in PBS for polyclonal antibodies) was applied for 40 min at room temperature. The nuclear dye Propidium Iodide (10 µg/ml in PBS) was used for labelling the nucleus (10 min). After a final rinse (PBS 3 × 5 min), mounting was done in Citifluor (Citifluor Ltd., London, UK) and slides were sealed with nail enamel. Controls were done by omitting the first antibody and applying the second fluorescent anti-mouse or anti-rabbit antibody, or by using non-relevant monoclonal or polyclonal antibodies: they were negative and thus are not further mentioned or illustrated. Observations were made with a Nikon Optiphot epifluorescence microscope equipped with a mercury lamp and a single band Nikon filter for FITC channel (B-2A), TRITC channel (G-2A) or Hoechst channel (UV-1A), or a double-band (FITC/TRITC) Omega filter (XF 52).

Computer processing of immunocytochemistry micrographs

Figure 2b has been processed for enhancement of contrast in order to determine the relative length of the axonemes. The photographic print was scanned at 600 dpi and 256 grey levels with a Hewlett-Packard scanner, and processed on an Apple Power Macintosh 7100/66 computer with Adobe Photoshop. The image was inverted to produce a negative, with tubulin labelling appearing as black on a white background. This is more easily printed than images with large dark areas. Only the commands "brightness" and "contrast" were used, to avoid any distortion of the information contents of the image. The processed file was finally printed on a Hewlett-Packard Deskjet 660C ink jet printer.

RESULTS

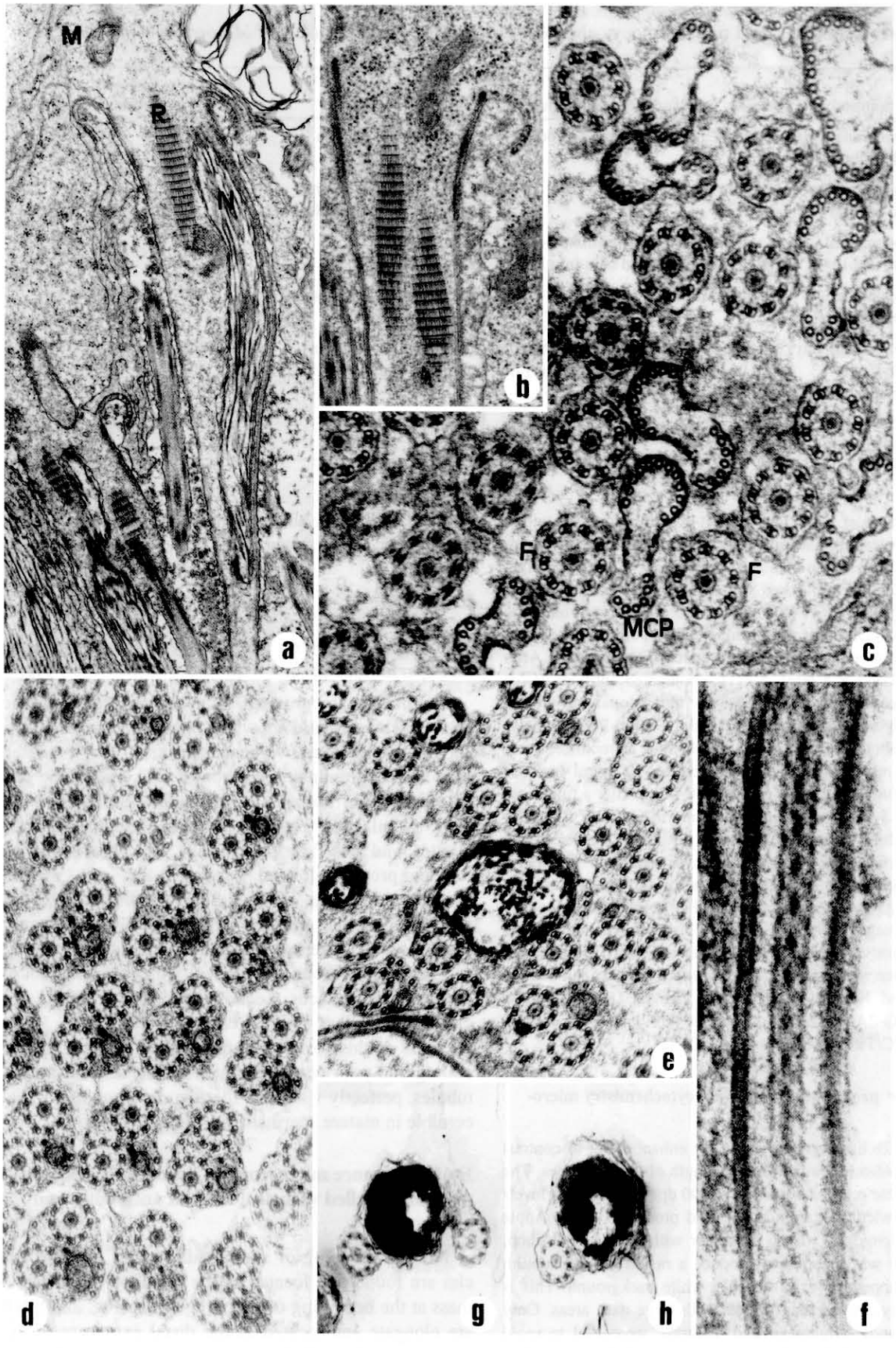
Transmission electron microscopy of spermiogenesis and spermatozoon — Fig. 1

Spermatids are grouped in a common cytoplasmic mass. Each spermatid shows an elongate zone of differentiation, containing two centrioles, each with a striated root, and longitudinal microtubules (Fig. 1a,b). The distal extremity of the zone of differentiation bears a median cytoplasmic process, with longitudinal microtubules, and two flagella (Fig. 1c). The three elements fuse and produce elongating spermatids (Fig. 1d,e). The mitochondrion (Fig. 1d) and the nucleus (Fig. 1e) elongate and migrate into the spermatid. The axonemes show the 9 +"1" trepaxonematan structure (Fig. 1c,f).

The spermatozoon shows two axonemes, the mitochondria and nucleus. Sections at the level of the nucleus show either one or two axonemes (Fig. 1g,h). Sections at this level are often coiled. Owing to this coiling and poor conditions of fixation, cortical microtubules, perfectly visible in spermatids, were hardly discernible in mature spermatozoa, though present.

Epifluorescence microscopy of spermatids and spermatozoa labelled with anti-tubulin antibodies and nuclear dyes — Figs. 2–4

Nuclear labelling of spermatids reveals that the nuclei are round and located in the common cytoplasmic mass at the beginning of spermiogenesis (Fig. 2a,b), but are elongate and located in the distal extremity of the spermatid at the end of spermiogenesis (Fig. 2c, d).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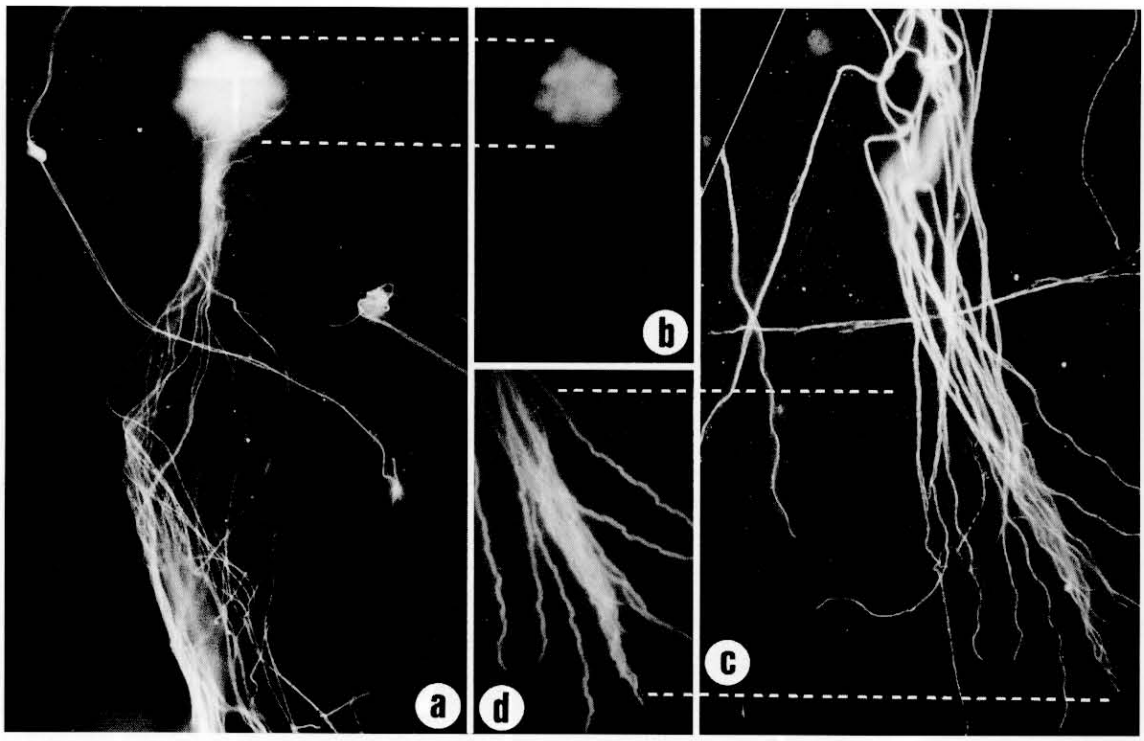

Fig. 2. Immunocytochemistry of tubulin and nuclear labelling of spermatids of *Mesocoelium monas*. **a,b** – early spermatids. Tubulin labelling (**a**) with monoclonal antibody DM1A against beta-tubulin. The nuclei (**b**) are located in the common cytoplasmic mass; **c,d** – late spermatids. Tubulin labelling (**c**) with monoclonal antibody 6-11B-1 against alpha-acetylated tubulin. The nuclei (**d**) have migrated in the distal extremity of the spermatids. The limits of nuclei are indicated by white dots on all figures; tubulin labelling observed with FITC filter, nuclear labelling observed with TRITC filter (**a–d** × 600).

Anti–tubulin labelling reveals that the spermatozoon is a filiform cell, 220 µm in length (Fig. 3a). The two axonemes generally appear as a single labelled line (Fig. 3a), but in some cases (Fig. 3b,d,e), a part of the length of the spermatozoon shows two parallel lines which correspond to the two axonemes.

The nucleus is filiform, twisted and 65 µm in length (Fig. 3a,c,f). It is located at the distal extremity of the spermatozoon, with its broader extremity at the level of the tip of the spermatozoon; one of the two axonemes, which are twisted around the nucleus, extends up to the distal extremity of the nucleus, but the other is interrupted approximately at mid–length of the nucleus, 30 µm before the end (Fig 3b). This is more apparent on a computer-processed image (Fig. 4). One of the axonemes is thus 220 µm in length, and the other 190 µm in length.

The different antibodies used for labelling the spermatozoon gave similar results, which are summarized in Table 1.

DISCUSSION

Structure of spermiogenesis and spermatozoa

The general ultrastructure of spermiogenesis in *Mesocoelium monas* is similar to that described in most digeneans, consisting of a proximo-distal fusion of flagellar axonemes with a median cytoplasmic process followed by an elongation of the spermatids (Justine 1991a, 1995). No special feature was found in the present species. The present observations thus confirm the relative homogeneity of the sperm structure previously observed in the Digenea.

In *Mesocoelium*, one of the two axonemes is longer than the other, thus producing transverse sections containing a single section of axoneme. In filiform spermatozoa, the interpretation of such sections is difficult when transmission microscopy alone is used. Here, the use of immunocytochemistry and nuclear labelling demonstrates the spatial relationships of the axonemes with the nucleus. Sections of digenean spermatozoa

← **Fig. 1.** Transmission electron microscopy of spermiogenesis and spermatozoon of *Mesocoelium monas*. **a,b** – zone of differentiation with migrating nucleus and mitochondrion, longitudinal section (a × 20 000, b × 24 000); **c** – median cytoplasmic processes and flagella, transverse section (× 60 000); **d** – elongating spermatids, transverse section (× 40 000); **e** – elongating spermatids, transverse sections at the level of the nucleus (× 40 000); **f** – axoneme showing the 9 +"1" trepaxonematan pattern, longitudinal section in spermatid (× 100 000); **g,h** – spermatozoa, transverse section at the level of the nucleus, showing two (g) and one (h) section of axoneme (× 40 000). **Abbreviations**: F – flagellum; M – mitochondrion; MCP – median cytoplasmic process; N – nucleus; R – striated root.

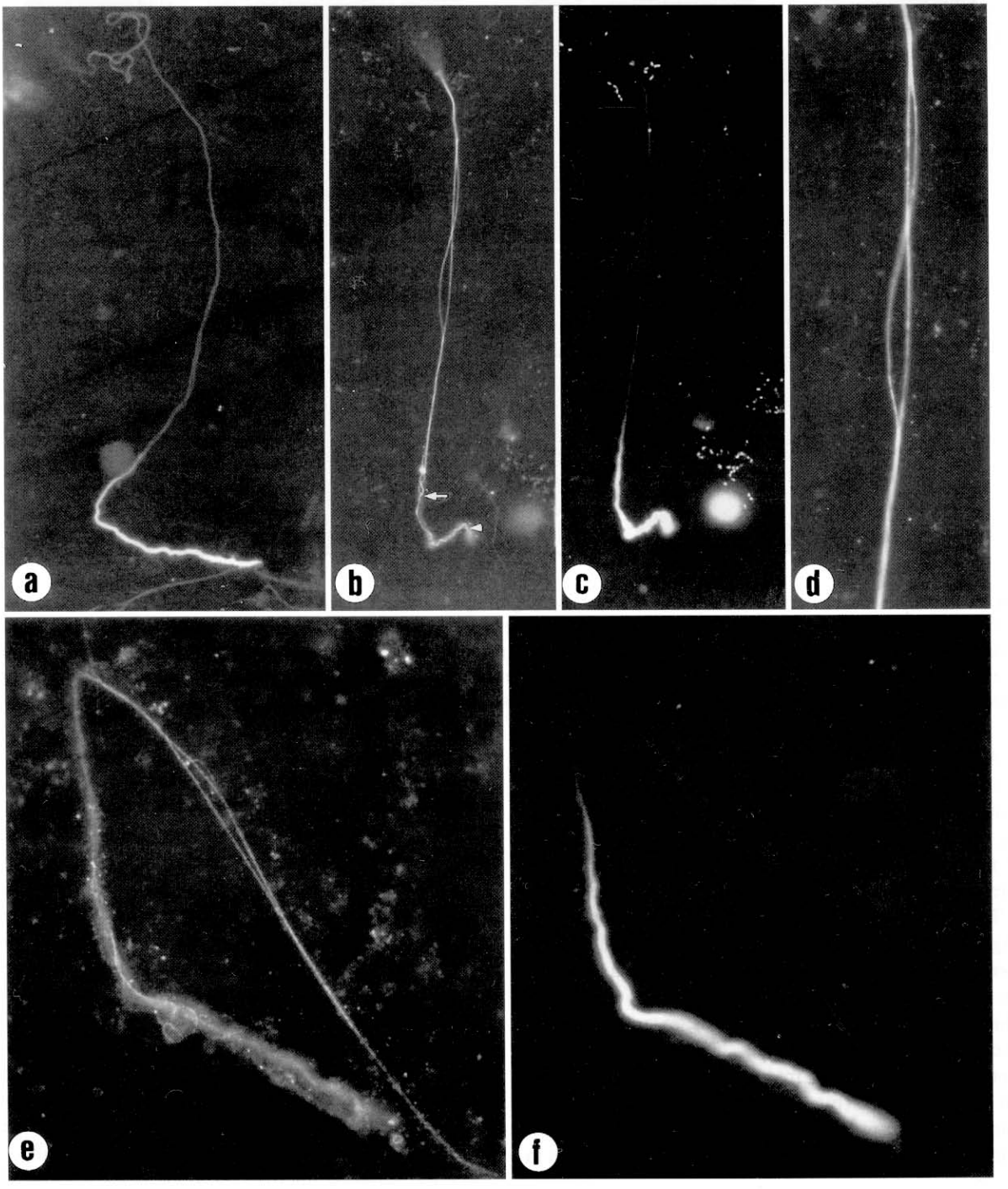

Fig. 3. Immunocytochemistry of tubulin and nuclear labelling of spermatozoa of *Mesocoelium monas*. **a** – spermatozoon, observation with double band filter, showing both tubulin and nucleus labelling. Monoclonal antibody DM1A against beta–tubulin; **b,c,d** – spermatozoon, observation of tubulin labelling with FITC filter (**b,d**) and of nuclear labelling with TRITC filter (**c**). One extremity of this cell (upper) is out of the plane of focus. Arrow – extremity of the shorter axoneme; arrowhead – extremity of the longer axoneme; **d** – higher magnification of a region showing two parallel lines, corresponding to the two axonemes. Monoclonal antibody 6-11B-1 against alpha–acetylated tubulin; **e,f** – nuclear extremity of a spermatozoon, showing twisting and pointed shape of nucleus; **e** – tubulin labelling, FITC filter; **f** – nuclear labelling, TRITC filter. Monoclonal antibody 16D3 against tubulin (**a,b,c**, × 600; **d,e,f**, × 1 200).

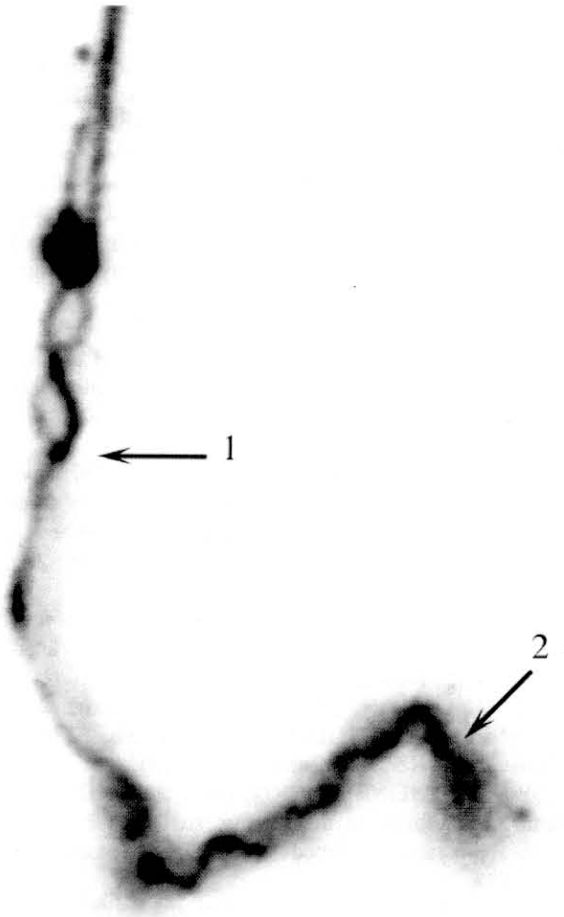

Fig. 4. Immunocytochemistry of tubulin in the spermatozoon of *Mesocoelium monas*. Computer-enhanced image produced from Fig. 2b. The axonemes appear as black lines. **1** – distal extremity of shorter axoneme; **2** – distal extremity of longer axoneme.

showing the nucleus and a single axoneme have been found in a number of digeneans such as *Brachylaimus* (Žďárská et al. 1991), *Clonorchis* (Jeong and Rim 1984), *Cryptocotyle* (Rees 1979), *Fibricola* (Sohn and Lee 1993), *Maritrema* (Hendow and James 1988) and *Echinostoma* (Iomini and Justine 1997), and it is likely that this character is present in many species. A comparative study may prove that the relative length of axonemes is a character useful for phylogeny in the Digenea, as the number of axonemes has already been used as a phyletic character in the Monogenea (Justine 1991b) and Cestoda (Euzet et al. 1981, Bâ and Marchand 1995).

The use of immunocytochemistry of tubulin and nuclear labelling for the understanding of spermatozoon structure

Interpreting the antero-posterior sequence of transverse ultrastructural sections of filiform spermatozoa is sometimes difficult. Immunocytochemistry of tubulin combined with nuclear labelling gives additional information on the relative length of the axonemes and position of the nucleus. In particular, this technique demonstrates that sections containing a single axoneme and the nucleus are the most distal.

Our study on *Mesocoelium* shows that various antibodies can be used for this technique. Table 1 shows that antibodies prepared against antigens of extremely varied phyletic origin are effective. This was not unexpected because tubulin is a protein which is highly conserved in evolution (Adoutte et al. 1985).

The demonstration of acetylated tubulin in *Mesocoelium* confirms the finding of this post–translational modification of tubulin in *Echinostoma caproni* (Iomini et al. 1995). It was shown in spermatozoa of *Echinostoma*, using post–embedding transmission electron microscope immunocytochemistry, that acetylated tubulin is found in the microtubules of the axonemes but not in the cortical microtubules (Iomini et al. 1995).

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