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Nervous system immunohistochemistry of the parasitic cnidarian *Polypodium hydriforme* at its free-living stage

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ABSTRACT

Polypodium hydriforme, the only species in Polypodiozoa, which is currently considered a class of Cnidaria, and likely a sister group to Medusozoa (together with Myxozoa), is a cnidarian adapted to intracellular parasitism inside sturgeon oocytes. Free-living *P. hydriforme* lives on river bottoms; it walks on supporting tentacles and uses sensory tentacles to capture food and bring it to the mouth. The nervous system of free-living *P. hydriforme* was studied by confocal microscopy and immunohistochemistry using antibodies to FMRF-amide and α-tubulin combined with phalloidin-staining of F-actin fibres. A sensory FMRF-amide immunoreactive (IR) nerve net and an α-tubulin IR nerve net have been identified. The FMRF-amide IR nerve net underlies the epidermis along the tentacles and around the mouth; it consists of neurites emanating from epidermal sensory cells and basiepidermal ganglion cells, and it connects with cnidocytes. A deeper-lying α-tubulin IR nerve net occurs only in tentacles and looks like chains of different-sized beads crossing the mesoglea and entwining muscles. Anti-α-tubulin staining also reveals microtubules in muscle cells following the longitudinal muscle fibres or the thin circular F-actin fibres of the tentacles. Cnidocytes in the tentacles are embedded in a regular hexagonal non-neural network formed by the tubulin IR cytoskeleton of epidermal cells. Cnidocils of the cnidocytes around the mouth and in walking tentacles are identical, but those in sensory tentacles differ in length and width. The possible homology of the tubulin IR nerve net with motor nerve nets of cnidarians is discussed. The absence of a classic nerve ring around the mouth and the lack of specialised sense organs are considered to be plesiomorphic characters for Cnidaria.

1. Introduction

Cnidarians are diploblastic animals possessing stinging cells (cnidocytes). They are under intensive study in order to unravel their phylogeny (Kayal et al., 2013) as well as to find the sister group to Bilateria (Anderson et al., 2004; Technau and Steele, 2011). The cnidarian nervous system also attracts much attention, especially regarding its origin (Watanabe et al., 2009), its development at various stages of the life cycle (Piraino et al., 2011), sense organs (Nakanishi et al., 2008), nerve ring formation (Koizumi, 2007; Koizumi et al., 2015), neuromediators (Grimmelikhuijzen et al., 1991, 1996), morphology of neurons (Westfall, 2004; Satterlie, 2011), cnidocyte structure, cnidogenesis and evolution of cnidocytes (Östman, 1999, 2000; David et al., 2008; Fautin, 2009; Hwang et al., 2008; Özbek, 2011; Holstein, 2012).

Polypodium hydriforme used to be the only known intracellular parasite among cnidarians until Myxozoa, a phylum formerly placed in Protozoa, was recognized as cnidarian (Foox and Siddall, 2015; Okamura and Gruhl, 2015). All myxozoans (about 2000 species known to date) are extremely simplified parasites, including intracellular ones, of aquatic vertebrates and invertebrates.

P. hydriforme spends the major part of its life cycle (several years) in developing oocytes of sturgeons. All its embryonic stages are parasites inside fish oocytes, just the same as the planula which forms numerous buds and thus grows into a progressively elongating stolon. The planula and the stolon stages display inverted germ layers. Just before the spawning of the host fish, the *P. hydriforme* stolon everts, so that it acquires the normal position of the germ layers (Lipin, 1911) and all the yolk of the infected sturgeon oocyte is transferred into the forming gastral cavity of the everted stolon. Free life in the fresh water begins after release of the parasite (as everted stolon) from the infected eggs of the host fish and

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lasts probably for one summer season (Raikova, 1994). In water, the everted stolon immediately fragments into free-living specimens, with tentacles whose numbers are multiples of six. After depletion of the yolk in the gastral cavity, each individual forms a mouth at the place of the former attachment of the bud to the stolon, and begins to feed on small freshwater invertebrates. Free-living *P. hydriforme* walk on supporting tentacles and use sensory tentacles to capture food and bring it to the mouth. Despite the fact that the free-living specimens are devoid of an umbrella and have a polypoid appearance, after Shimkevitch (1890) we consider this generation medusoid. It follows a parasitic generation represented by the budding stolon, which likely corresponds to the actinula stage of the Narcomedusae, considered homologous with the polypoid stage. Also, free-living *P. hydriforme* is a propagation stage with gonads, and after release of the gametophores they die (Raikova, 1994, 2002).

Parasitic and free-living *P. hydriforme* have been previously studied by light and electron microscopy. These studies have defined characters that are presumably determined by the parasitic way of life and some unique ones not connected to parasitism (Raikova, 2008). Due to the inversion of germ layers during its parasitic stages, *P. hydriforme* became famous among coelenterates as an "enantiozoon". Besides, it possesses gonoducts in endodermal female gonads but has neither true eggs nor true sperm cells. Further, *P. hydriforme* completely lacks epitheliomuscular cells (Lipin, 1911); instead, independent muscle cells form a subepidermal layer. Thus, *P. hydriforme* displays a triploblastic state during most of its life cycle (Raikova et al., 2007).

Traditionally, *P. hydriforme* was assigned to the hydrozoan family Narcomedusae of the order Trachylina based on similarities in life cycle and embryonic stages with parasitic narcomedusae (Shimkevitch, 1890; Hyman, 1940). Later, taking into account morphological and cytological data, Raikova (1988, 1994) concluded that *P. hydriforme* did not fit into the class Hydrozoa and deserved being considered a separate class of Cnidaria. The validity of the class Polypodiozoa has been supported by Bouillon and Boero (2000) and Bouillon et al. (2004), but its phylogenetic relations with other Cnidaria still remain unclear.

Despite its typical cnidarian morphology and the presence of stinging cells, some phylogenomic studies caused doubts whether *P. hydriforme* was really a cnidarian. Most of the early phylogenetic analyses based solely on partial 18S rRNA sequences recovered *P. hydriforme* as sister group to triploblastic Bilateria (Zrzavý et al., 1998; Zrzavý, 2001; Collins, 2002) outside the phylum Cnidaria (Zrzavý and Hypša, 2003). However, Evans et al. (2008) later performed phylogenetic analyses of 18S and partial 28S rDNA sequences in a large dataset that included *P. hydriforme* and a comprehensive sampling of cnidarian taxa, and *P. hydriforme* was recognized as a real cnidarian, likely the sister taxon to Hydrozoa.

As early as in 1938, Weill hypothesized a kinship between *P. hydriforme* and Myxozoa due to similarities in life history (e.g., parasitism) and morphology (e.g., the presence of atrichous isorhiza nematocysts, homologous to the polar capsules of some myxozoans) (Weill, 1938). The same hypothesis later received much attention in molecular phylogenetic studies (Siddall et al., 1995; Zrzavý and Hypša, 2003; Shpirer et al., 2014). The inclusion of *Polypodium* in a sequencing made by Siddall et al. (1995) entailed the demise of Myxozoa as a phylum of protists, removing Myxozoa and placing them as sister group to *Polypodium*. Despite the critical assessments of this work, recent phylogenomic analyses have recovered the Myxozoa and *P. hydriforme* within or as sister groups to the subphylum Medusozoa, a group comprising scyphomedusae ("true" jellyfish), stauromedusae (stalked jellyfish), cubomedusae (box jellyfish), and hydromedusae (see reviews by Foox and Sidall, 2015; Okamura and Gruhl, 2015).

Quite recent phylogenomic analyses by Zapata et al. (2015), unfortunately omitting *P. hydriforme*, support traditional relationships within Cnidaria, namely the monophyly of Medusozoa and the sister group relationship of Hydrozoa and a clade of Staurozoa, Cubozoa and Scyphozoa. Interestingly, within the Hydrozoa the earliest branch is Trachylina, including Narcomedusae, a group to which *P. hydriforme* had been traditionally assigned (Shimkevitch, 1890; Hyman, 1940). If *Polypodium* is indeed a separate clade from Hydrozoa (Raikova, 1994), then the common features of *P. hydriforme* and parasitic Narcomedusae could be explained as plesiomorphic or homoplasic due to parasitism.

Nevertheless, the results of various molecular analyses only strengthen our conviction that *P. hydriforme* is a somewhat "special" animal among the living cnidarians. But recently a similar fossil form has been described: *Haootia quadriformis* n. gen., n. sp., from Newfoundland, interpreted as a muscular cnidarian impression from the Late Ediacaran period (approx. 560 mya) (Liu et al., 2014). This species provides the earliest fossil evidence for both metazoan musculature and for Eumetazoa in the geological record and it had independent muscle cells running longitudinally up the length of the tentacles in an arrangement strikingly similar to that of *P. hydriforme* (Raikova et al., 2007).

P. hydriforme is not a common species in fresh waters (Jankowsky et al., 2008) and, in comparison with *Hydra,* is very poorly known by the scientific community. Therefore, any news on its organization is very important. Recently we have published some ultrastructural data on its nervous system focusing on the parasitic stages (Raikova, 2013). The aim of the present paper is to expand our observations on this topic, paying principal attention to the localization and distribution of nerve and receptor cells (ganglion, sensory cells, cnidocytes) at the free-living stage of the life cycle.

2. Materials and methods

Infected sterlet (*Acipenser ruthenus*) eggs were sampled in different fisheries on the Volga River, Astrakhan district, Russia. Free-living specimens of *P. hydriforme*, grown from infected oocytes in Petri dishes (Raikova, 1994), were used for the study. In culture, during the first week they ingested the yolk of the sterlet egg which had been drawn into their gastral cavities during evertion before spawning. After all the deposited yolk had been consumed, a mouth opened at the top of the body. Oligochaetes (*Tubifex tubifex*) were used as food for the free-living *P. hydriforme* which remained in the same Petri dishes as before; the food was given by forceps once per day.

Microphotographs of the fixed animals were made with a Leica microscope equipped with Nomarsky optics in order to obtain an overview of the animal.

For immunocytochemistry and phalloidin staining the samples were fixed with Stefanini solution (2% paraformaldehyde and 15% picric acid in 0.1 M sodium phosphate buffer, pH 7.6). The animals were stored in the fixative for several weeks, then incubated for 24–48 h in 0.1 M sodium phosphate buffer with 20% sucrose, washed three times for 5 min with PBS with 0.2% Triton X-100 (PBS-T) and incubated in 30 mm embryo dishes Non-specific antigens were blocked with 2% bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) in PBS (BSA-PBS) for 45–90 min at room temperature.

To localize anti-FMRF-amide IR neuropeptides, the animals were incubated with rabbit antibodies to FMRF-amide (ImmunoStar 20091; ImmunoStar Inc., Hudson, WI, USA) diluted 1:400 in PBS-T for 48–72 h at 10 °C on a shaker. Then, samples were washed three times for 5 min with PBS-T and incubated for 1–2 h with FITC-labelled swine anti-rabbit secondary antibody (DAKO F0205)

diluted 1:40 in PBS-T. After washing with PBS-T, the animals were stained with phalloidin-TRITC (Sigma–Aldrich P1951, diluted 1:100 in PBS) for 2 h at room temperature on a shaker. After rinsing in PBS 3 × 10 min, the animals were mounted in glycerol-PBS (2:1) and either examined directly or kept in a freezer at −20 °C before microscopic examination.

To localize α-tubulin, the animals were incubated for 24 h in mouse anti-α tubulin conjugated with FITC (Sigma–Aldrich F-2168) dilution 1:100 in PBS-T. Then, after rinsing in PBS-T, the samples were counter-stained with phalloidin-TRITC (Sigma–Aldrich P1951, diluted 1:100 in PBS) for 2 h at room temperature on a shaker, then mounted as described above.

As a control for specificity, some animals were incubated for a week in PBS-T solution only without any primary antibodies; then the secondary antibodies were applied in the usual way. No staining of the nervous system was obtained in controls.

The preparations were examined with a confocal scanning laser microscope (CSLM) Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany). The maximum projection option was used to make reconstructions from a whole series or from several adjacent optical sections in a series (thus resulting in a thicker optical section). The files obtained were processed with Leica LAS AF lite software, as well as Adobe PhotoShop v. 7.0.

3. Results

3.1. General morphology

Free-living specimens of *P. hydriforme* are biradially symmetrical; they usually have 12 or 24 tentacles situated in two symmetrical lateral groups on the sides of the sac-shaped body (Fig. 1A and B). The tentacles are of two kinds: slender sensory tentacles and thicker supporting (walking) tentacles. The sensory tentacles are situated at the base of the mouth cone, one pair above the other on the oral side, and two pairs of walking tentacles are situated on the aboral side. In living animals, the sensory tentacles are constantly moving to catch prey and bring it to the mouth. In fixed preparations, they are very often found screening the mouth (Fig. 1B). The supporting (walking) tentacles are thicker and are used for locomotion (Fig. 1A and B); they are distinguishable from sensory tentacles by dense aggregations of cnidocytes at the tips, looking whitish in living animals (Fig. 1A). Both kinds of tentacles possess a central axis (Fig. 1B). Free-living specimens do not have any umbrella. The mouth is located at the top of the mouth cone (Fig. 1B). *P. hydriforme* specimens multiply by fission beginning from the aboral pole; the mouth cone divides last (Fig. 1B).

3.2. FMRF-amide immunoreactivity

Immunostaining with antibodies to FMRF-amide reveals nerve nets in both the sensory and supporting tentacles (Fig. 2) as well as in the mouth cone (Fig. 3). FMRF-amide IR sensory cells (about 25–30 μm long) are present in abundance in the tentacles of both kinds and around the mouth (Fig. 2A–C); only the aboral part of the body seems to be devoid of them and of other nerve elements. The sensory cells send basal neurites into a nerve net with bi-, tri- and multipolar neurons (about 4–8 μm in size) located basiepidermally, at the border with the mesoglea (Fig. 2C and D). The FMRF-amide IR neurites often appear as strings of beads of different sizes (up to 4 μm), due to numerous varicosities along the neurites (Fig. 2A and D). Sometimes the beaded appearance of the nerve net is less pronounced (Fig. 2C). The beaded neurites run parallel to the tentacle surface (Fig. 2A and C) and along the mouth cone (Fig. 3A and B), directly under the epidermis. In the tentacles, neurons of the nerve net appear to make contact with the muscles (Fig. 2D). At the tips of the walking tentacles, the neurons are interspersed between cnidocytes (Fig. 2E).

The tips of the sensory tentacles display a cluster of FMRF-amide IR cells forming a hemisphere around a vacuole or an empty space situated at the very tip (Fig. 4A). Distal ends of muscles running along the tentacles reach the nerve cluster (Fig. 4A).

The sensory cells are rod-shaped, spindle-shaped or flask-shaped with one end (usually the basal one) thicker than the other (Figs. 2A–C, E, and 4A). The cytoplasm contains numerous granules (Fig. 2B) about 100 nm in diameter, as measured on the corresponding TEM preparations (Raikova, 2013).

The nervous system of the mouth cone is represented by a FMRF-amide IR nerve net with large (12–15 μm) bi-, tri- and multipolar neurons of both sensory and ganglion types and beaded neurites connecting them (Fig. 3A–D). The rim of the mouth displays a more concentrated nerve net than the rest of the mouth cone (Fig. 3A–C), but there are no neurite bundles characteristic of classic nerve rings (see Section 4). There is an ectodermal bolster (a kind of a lip) around the mouth, which also lines the mouth rim on the inside (Fig. 3D). It is covered with cnidocytes (Fig. 3C, E, and F) and displays numerous sensory cells (Fig. 3D).

3.3. α-tubulin immunoreactivity

Immunostaining with α-tubulin antibodies was observed in all kinds of cells, namely in epidermal cells (Figs. 4C, D and 5C), in cnidocytes (Figs. 4C–E and 5A–C), in nerve and in muscle cells (Figs. 4D, F and 5D–F).

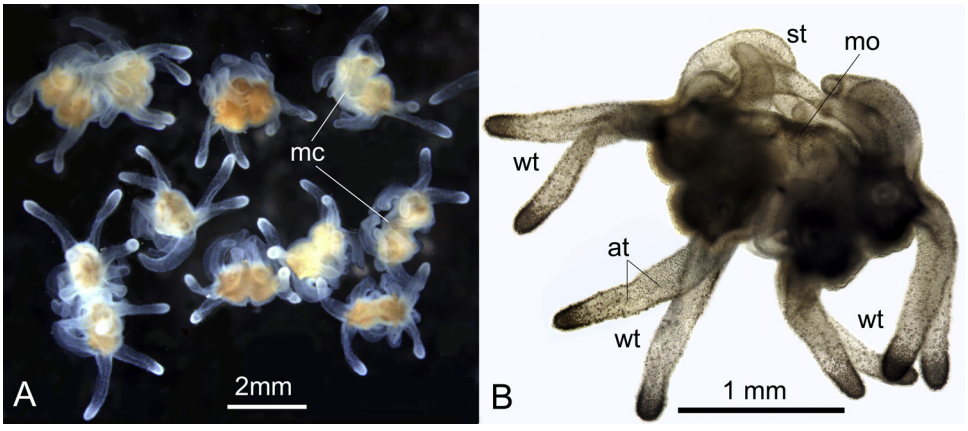

Fig. 1. (A) Free living 24-tentacled *P. hydriforme* in fission with yellow yolk in gastral cavities and mouth cones (mc) screened by tentacles. (B) *P. hydriforme* in fission, with mouth cone the last to divide. Note the longer walking tentacles (wt) with visible central axis (at) and the shorter sensory tentacles (st) screening the mouth opening (mo).

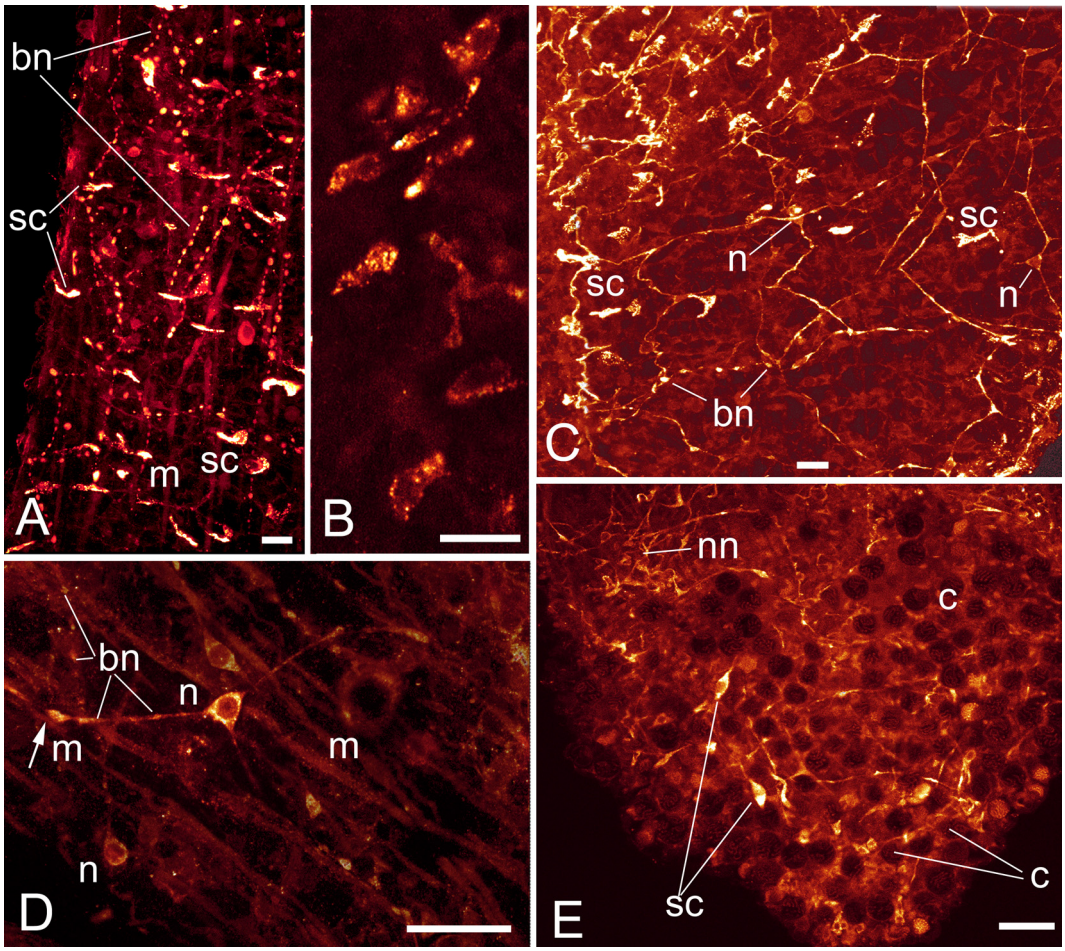

Fig. 2. FMRF-amide immunoreactivity in (A–D) sensory and (E) walking tentacles of *P. hydriforme*. (A) Sensory cells (sc) connected by beaded neurites (bn) running above the muscles (m). (B) Sensory cells with granular cytoplasm. (C) Nerve net with tripolar neurons (n) connecting numerous sensory cells (sc). (D) Tripolar neuron (n) contacting (arrow) muscle (m). Note beaded neurites (bn) of the bi- and tripolar neurons. (E) Nerve net (nn) connecting cnidocytes (c) and sensory cells (sc). Scale bars = 20 μm.

In the mouth cone, no distinct α-tubulin IR nerve net was observed (Fig. 4B). There is a dense ring of holotrichous isorhiza cnidocytes around the mouth opening, with cnidocils strongly α-tubulin IR (Fig. 4B, inset).

In the tentacles, we observed prominent chains of α-tubulin IR neurons, which do not form a regular net such as the FMRF-amide IR nerve elements, but mostly look like separate strands of beads of different sizes, from 2 to 4 μm and up to 6.3 μm (Figs. 4D, F and 5D and E). The strands run both in longitudinal and cross direction at the level of the muscles, i.e. in mesoglea, and seem to contact the muscles on the way (Figs. 5D,E).

Anti-α-tubulin immunostaining reveals intracellular microtubular structures both in contracted and relaxed muscles (Figs. 4D; 5D, E) (in accordance with TEM observations by Raikova (1984) and Raikova and Napara (1999), see also Section 4). Unlike the nerve strands they are never beaded. Besides, tubulin IR microtubules can be observed among thin circular F-actin fibres crossing the tentacle (Fig. 5D and E).

In the epidermis of the tentacles, tubulin immunoreactivity was observed along the lateral borders of penta- or hexagonal epidermal cells, likely due to microtubules lining the cell membrane on the inside, as observed earlier in electron micrographs of epithelial cells of *P. hydriforme* (Raikova, 1980: Fig. 15; Raikova, 1984). Thus a hexagonal tubulin IR cytoskeletal network is formed, with strongly stained cnidocytes at the net knots (Figs. 4C, D and 5C). The epidermal cell borders are also stained by phalloidin (Fig. 4C and D).

All stinging capsules (nematocysts) in *P. hydriforme* are spherical with cnidocils situated right above the operculum of the capsule (Figs. 3E, F and 4F). Cnidocytes in walking tentacles (atrichous isorhiza; Ibragimov and Raikova, 2004) have cnidocils of similar length and width. Stinging thread here fills all the space of the capsule (Fig. 4C and D). Cnidocytes in sensory tentacles and around the mouth (holotrichous isorhiza; Ibragimov and Raikova, 2004) are different: the stinging thread begins under the capsular lid, makes two incomplete turns and coils at the bottom of the capsule (Figs. 3E, F; 5B, C). The cnidocils in sensory tentacles show both pointed and obtuse tips, some are shorter and thicker, others are bent (Figs. 4E and 5A), while around the mouth all cnidocils seem to be similar (Figs. 3F and 4B).

All the cnidocytes in double-stained preparations display both α-tubulin immunoreactivity and phalloidin staining of F-actin fibres. Cnidocils of cnidocytes are α-tubulin IR (Figs. 4E, F and 5A). Around the base of each cnidocil, a phalloidin-stained ring is observed (Figs. 4E and 5A); our previous TEM observations permit to identify this ring as a collar of stereocilia which contain F-actin microfilaments (Raikova, 1990). Below the phalloidin-stained ring, there is a wider α-tubulin IR ring with several radiating IR spokes (Figs. 4C, 5A and B). This ring can be interpreted as a strongly α-tubulin IR cnidocil apparatus: a ring of radiating supporting rods and numerous microtubules interspersed between them (Raikova, 1990).

At a deeper level, the stinging cells are triangular in cross-section with thick tubulin IR microtubule lining along the cell borders

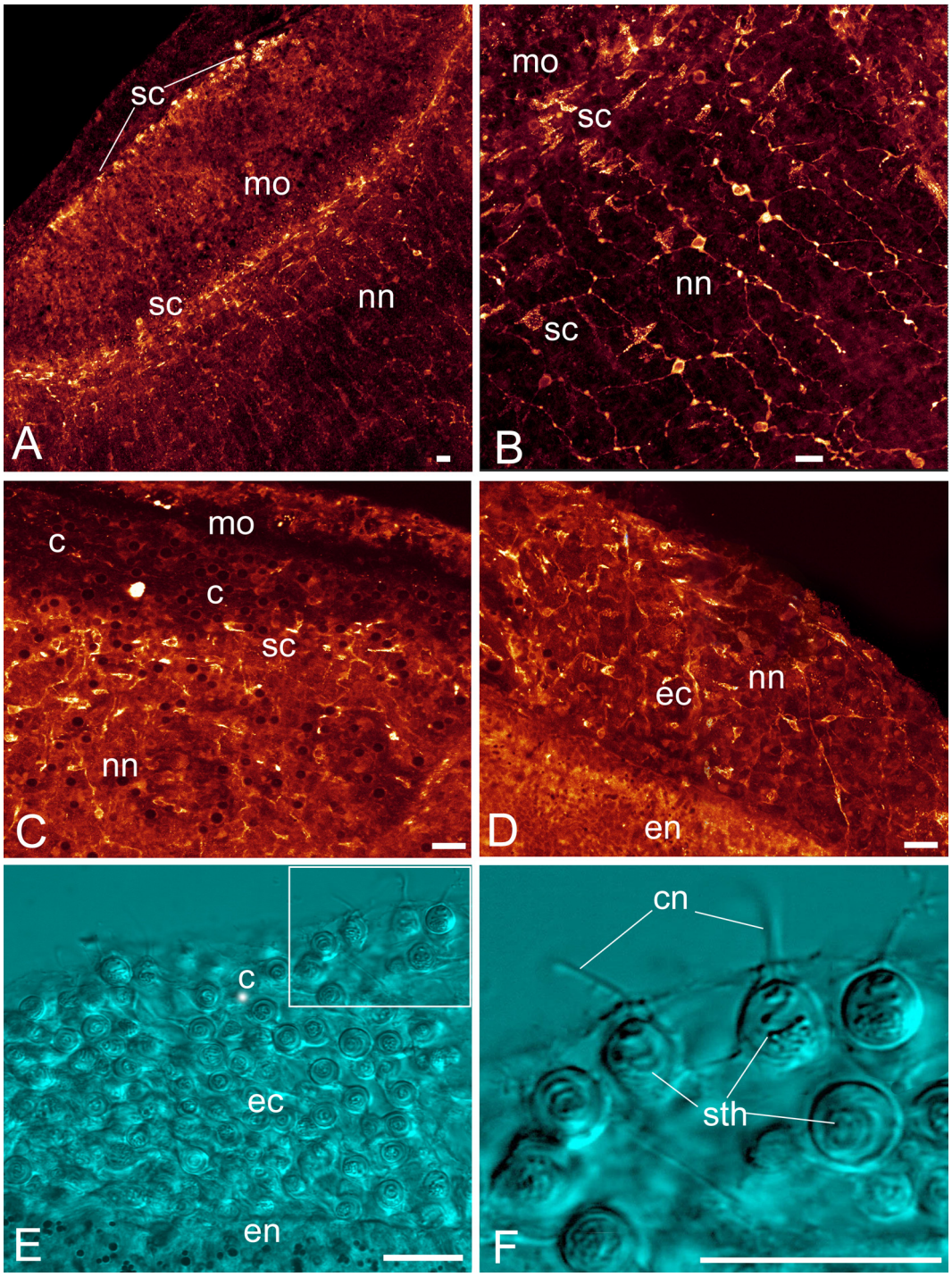

Fig. 3. FMRF-amide IR nerve net (nn), sensory cells (sc) and cnidocytes (c) in the mouth cone. (A) Mouth opening (mo) with bordering line of sensory cells. (B) detail of (A), sensory cells and nerve net. (C) Cnidocytes, sensory and nerve net cells around the mouth opening. (D) Boundary between ectodermal (ec) bolster and endoderm (en) within the mouth. Note the presence of the nerve net in the ectoderm and its absence in the endoderm. (E) Differential contrast microphotograph of cnidocytes covering the ectodermal bolster around the mouth. Endoderm contains dark yolk granules. (F) Detail of (E), cnidocytes with coiled stinging threads (sth) and cnidocils (cn) situated above the operculum of the capsule. Scale bars = 20 μm.

(Figs. 4C, D; 5C, F). The cnidocytes are united in a common hexagonal tubulin IR network formed by the microtubular borders of epidermal cells as described above (Fig. 4C).

4. Discussion

The nervous system of *P. hydriforme* was first described by Lipin (1911) as a network of nerve cells. This author studied macerated animals stained with methylene blue at the stage of the parasitic stolon, when the cell layers were inverted, i.e. the endoderm was outside and faced the yolk of the sturgeon's egg, whereas the ectoderm was inside. Lipin believed that the nervous system of inverted *P. hydriforme* was of endodermal origin (as in "enantiozoon"), while other non-inverted cnidarians possessed an ectodermal nervous system. Lipin also described bi- and tripolar neurons and demonstrated their junc-

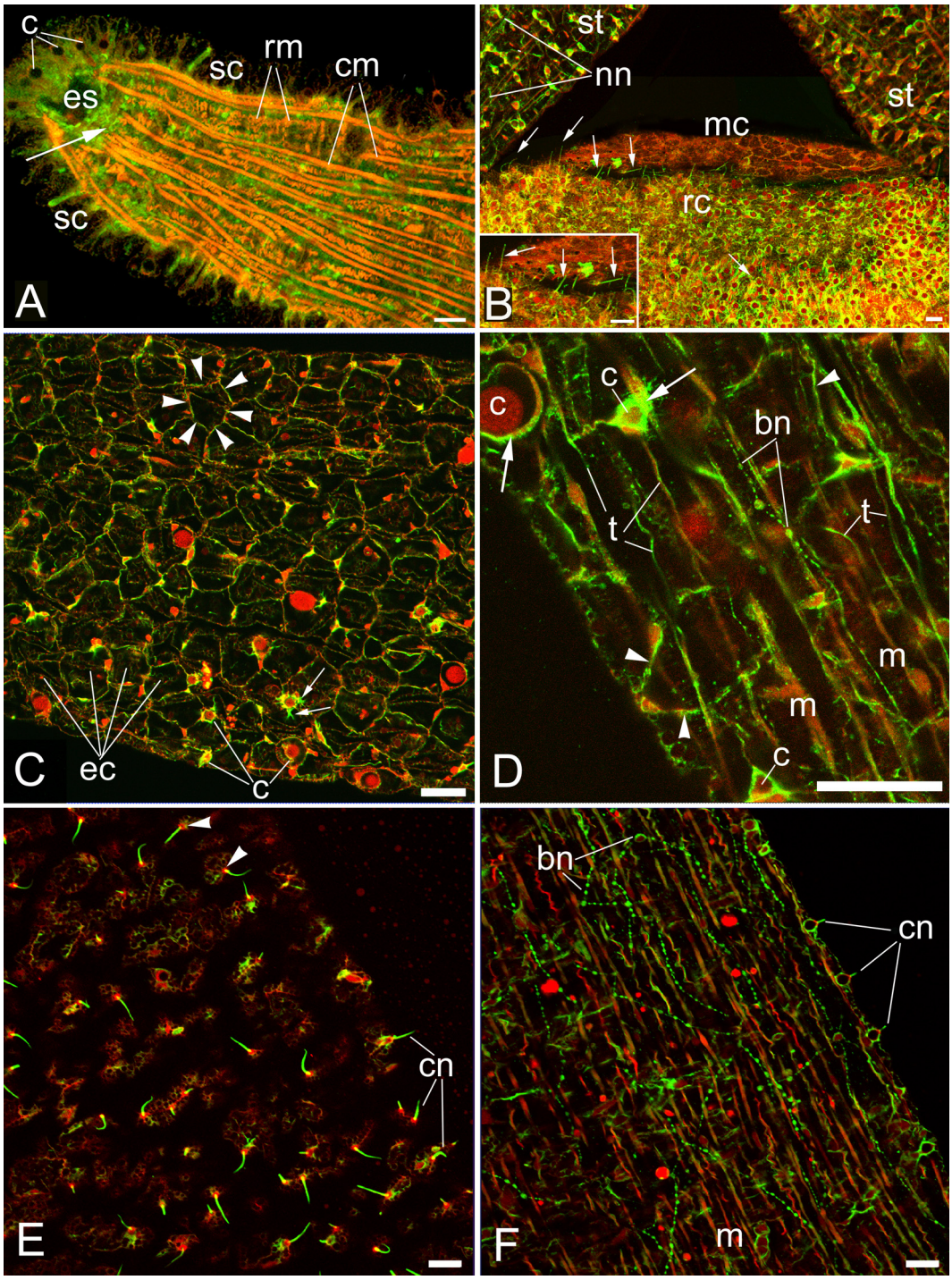

Fig. 4. Mouth and tentacles. (A) Double staining (anti-FMRF-amide in green and phalloidin in red) of the tip of a sensory tentacle. Note empty space (es) at the very tip, the cluster of nerve cells around it (arrow), cnidocytes (c), sensory cells (sc), contracted (cm) and spiralling relaxed muscles (rm). (B–F) Double staining (anti-α-tubulin in green and phalloidin in red). (B) Mouth cone (mc) with ring of cnidocytes (rc) around the mouth opening. Arrows point to cnidocils. Note the presence of a nerve net (nn) in the sensory tentacles (st) and its absence in the mouth cone. (B, inset) Higher magnification of tubulin-IR cnidocils at the mouth rim. (C) Walking tentacle with borders (arrowheads) of penta- or hexagonal epidermal cells (ec) showing both tubulin IR and phalloidin staining. Note cnidocytes (c) with tubulin IR radiating supporting rods (arrows). (D) Walking tentacle showing muscles (m) with tubulin structures (t) and beaded neurites (bn). Note tubulin IR cnidocyte (c) cytoplasm (arrows) and borders of epidermal cells (arrowheads). (E) Surface of sensory tentacle showing green tubulin cnidocils (cn) with red phalloidin-stained rings of stereocilia around their bases (arrowheads). (F) Deeper section of the same tentacle showing beaded neurites (bn) of tubulin IR nerve net innervating muscles (m). Note cnidocils (cn) located just above the capsule. Scale bars = 20 mm.

tions with muscle cells. However, he could not discover any sensory cells (Lipin, 1911). Sensory and neurosecretory cells were recognized later under an electron microscope and the ectodermal origin of *P. hydriforme*'s nerve cells was ascertained (Raikova, 2013).

Confocal microscopy extended our knowledge on the nervous system of *P. hydriforme*, confirming Lipin's main observation that it was a subepidermal network and contributing the characterisation of nerve cell types. Apart from the "neurons" described by Lipin (1911), which are currently classified as ganglion cells, sensory cells

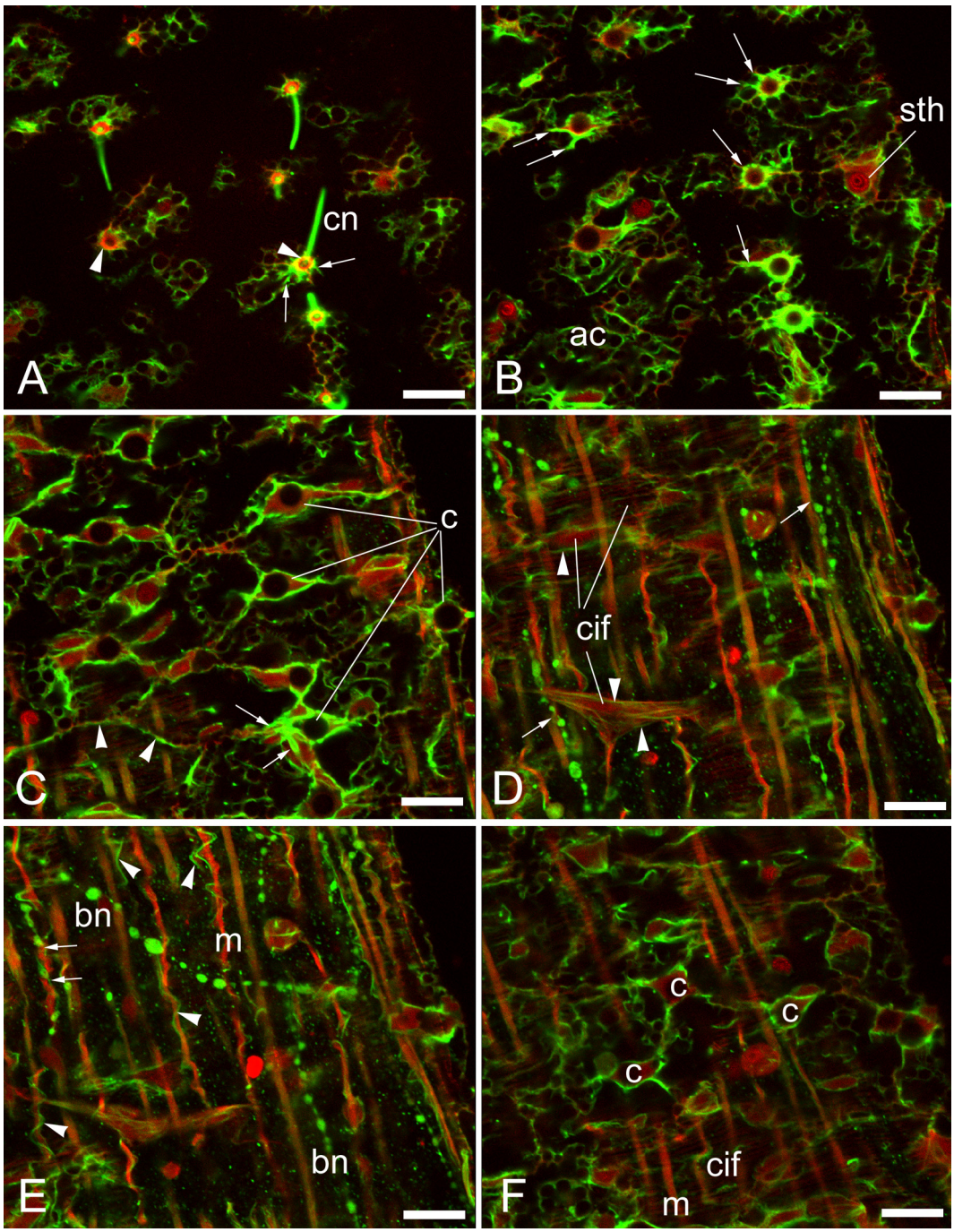

Fig. 5. (A–F) Double staining (anti-α-tubulin in green and phalloidin in red) of a sensory tentacle. Series of optical sections. (A) Surface; green cnidocils (cn) with red ring of stereocilia (arrowheads) and green supporting rods (arrows). (B) Supporting rods (arrows) radiating from capsules; stinging threads (sth) stained by phalloidin. Alveolar cytoplasm of epidermal cells (ac) is slightly tubulin IR. (C) Supporting rods (arrows) anchoring cnidocytes (c) in the network formed by tubulin IR limits of epidermal cells (arrowheads). (D) Beaded neurites of tubulin IR nerve net running alongside muscles (arrows); tubulin IR microtubules (arrowheads) are visible among thin circular phalloidin-stained fibres (cif). (E) Deeper level, demonstrating beaded neurites of tubulin IR nerve net (bn) contacting muscles (arrows). Note microtubules (arrowheads) spiralling around muscles (m). (F) Level closer to the other surface of the tentacle, showing muscles (m), circular fibres (cif), and the basal parts of cnidocytes (c) united in the tubulin IR net. Scale bars = 20 mm.

have been identified. We have demonstrated that *P. hydriforme*'s nerve cells contain FMRF-amide-like neuropeptides, which is characteristic for all Cnidaria (Grimmelikhuijzen et al., 1991). However, the methods we used were insufficient to discriminate neuropeptides in ganglion and sensory cells as it was done for *Hydra* (Koizumi et al., 2004) and for the nervous system of *Clava multicornis*'s planula (Piraino et al., 2011; Pennati et al., 2013a,b).

The main feature of sensory cells is the presence of neurosecretory granules containing FMRF-amide-like neuropeptides. These structures likely correspond to the dense-cored vesicles 80–120 nm in diameter identified by electron microscopy in an earlier study (Raikova, 2013). The difference between sensory and neurosecretory cells in *P. hydriforme*, when examined under a confocal microscope, is not well apparent. It is even possible that they are the same kind of cells referred to by different names only. Almost each sensory cell shows granular content, only some spindle-shaped ones seem to be free of granules. The presence of a cilium, common for sensory cells in free-living cnidarians (Westfall, 2004),

presumably distinguishes sensory cells from neurosecretory cells. However, in our material, the cilium could only be detected by electron microscopy (Raikova, 2013). The absence of significant differences between sensory and neurosecretory cells was also noted in *Hydra* (Westfall, 1973; Westfall and Kinnamon, 1978), where it was explained by the cells being multifunctional and primitive compared to specialized neurons in higher animals.

The essential question is whether *P. hydriforme* possesses a nerve ring around the mouth, which is typical of hydrozoans and considered an important phylogenetic character for cnidarians. According to the definition by Koizumi et al. (2015), a nerve ring is a ring of neurons whose neurites make up a bundle running circumferentially around the mouth. It is common for the head of the *Hydra* polyp where it is composed of about 30 neurite bundles and considered a central nervous system-like nerve structure (Koizumi et al., 2004, 2015; Koizumi, 2007). Hydromedusae have concentrations of hundreds of axons running in parallel forming nerve rings in the margin of the bell (Mackie, 2004).

As for *P. hydriforme*, despite the fact that there is a line of sensory cells around the mouth, there is no ring of neurite bundles running circumferentially around the mouth, i.e., the main characteristic of a classic nerve ring is absent. Thus, we consider the nerve ring to be absent in *P. hydriforme*. It should be noted, however, that the absence of the nerve ring in the newly hatched free-living *P. hydriforme* may be temporary. In fact, the animals studied mostly still lived on yolk accumulated in the gastral cavity and were not yet feeding properly.

Considering the phylogenetic implications of the absence of the nerve ring in *P. hydriforme*, it may be either a plesiomorphic character or the result of secondary loss due to parasitism. Koizumi et al. (2015) considered the presence of the nerve ring to be an ancestral feature in Hydrozoa, so in the unlikely case that *P. hydriforme* is a hydrozoan, a secondary loss would be probable. Alternatively, if *P. hydriforme* is a sister group to Hydrozoa (Evans et al., 2008) or a sister group to Medusozoa (see reviews by Foox and Sidall, 2015; Okamura and Gruhl, 2015), then the absence of the nerve ring is most likely plesiomorphic, because the ring is absent in a large part of Medusozoa, in such groups as Stauromedusae, Coronatae, Semaeostomea, Rhizostomeae (Marques and Collins, 2004), and thus is likely to be absent in basal Medusozoa.

The absence of specialised sense organs more complicated than sensory tentacles (e.g., rhopalia) is probably another plesiomorphic character of *P. hydriforme*; however, a secondary loss due to parasitism cannot be excluded. Sensory tentacles demonstrate a noticeable concentration of FMRF-amide IR cells around an empty space at the tip of the tentacle, the exact function of which is unknown. *P. hydriforme* specimens in aquaria consistently use sensory tentacles to touch the bottom with their tips (Ibragimov, 1999). Likely, these special nerve aggregations help to assess the substrate surface. It is appropriate to mention here that the quality of the substrate is very important for the animal during prey capture and when looking for an appropriate host to infect by gametophores (Raikova, 2002). *P. hydriforme*'s cnidocysts (holotrichous isorhizas) are capable of penetrating only a soft integument, e.g., that of *Tubifex* (Raikova, 2002; Ibragimov and Raikova, 2004), and are unable to catch chitinous crustaceans. Another possible function of the nerve aggregation at the tip of the sensory tentacle is the control of tentacle muscles. It is not yet clear whether the empty space inside the aggregation is a permanent structure or whether it appears only temporally, e.g., when the tentacle touches the substrate.

FMRF-amide nerve nets observed in tentacles and in the mouth cone appear to have primarily a sensory function, innervating numerous sensory cells and cnidocytes. Such peptidergic nets are a common feature among cnidarians (Grimmelikhuijzen et al., 1991, 1996; Satterlie, 2011; Satterlie and Eichinger, 2014).

We have observed for the first time extensive beaded chains of α-tubulin IR nerve cells in the tentacles of *P. hydriforme*; they cross the mesoglea and sometimes closely associate with muscles. The beads likely represent nerve cell bodies which are interconnected by neurites, but the possibility that they are varicosities cannot be excluded without having confirmed the presence of nuclei. The tubulin IR strands are located deeper than the FMRF-amide net and are more widely spaced. According to Satterlie (2011), tubulin immunoreactivity is a principal character of motor nerve nets (MNNs): *"Tubulin stains the motor nerve net whereas FMRF-amide stains the diffuse nerve net"*. The α-tubulin IR nerve net observed in *P. hydriforme* could have been considered homologous to MNNs in other Cnidaria, but the anti-tubulin labelling is not exactly specific to MNN. Indeed, the anti-tubulin antibody has been used to label non-MNN nerve nets in planulae (Gröger and Schmid, 2001; Nakanishi et al., 2008; Piraino et al., 2011) and polyps (Nakanishi et al., 2008). The MNN is defined functionally and physiologically as a nerve net that conducts pacemaker-derived, regular action potentials, which generate periodic contractions of swimming muscles in scyphozoan and cubozoan medusae. In the absence of comparable functional and physiological data, we should refrain from using the term MNN to refer to the anti-tubulin IR nerve nets in the tentacles of *Polypodium*. The presence of a motor nerve net in constantly moving tentacles would be quite natural, albeit *P. hydriforme* seems not to be an active swimmer as are the owners of MNNs among scypho- and cubomedusae (Eichinger and Satterlie, 2014; Satterlie and Eichinger, 2014).

Preparations double-stained with anti-α-tubulin and phalloidin give us an additional insight into muscle structure. We have found earlier that muscle cells possessed microtubules forming a kind of skeleton for myofilaments (Raikova, 1984; Raikova and Napara, 1999). Confocal images demonstrate these structures very clearly, both in contracted and in relaxed (spiralled) muscles. Besides, α-tubulin IR microtubules were now found to follow the previously described thin F-actin filaments in muscle outgrowths going in cross direction in tentacles (Raikova et al., 2007). These features make *P. hydriforme*'s muscles even more unusual.

Cnidarian cnidocytes are currently considered components of the nervous system, i.e., highly derived neurosensory cells (Hausmann and Holstein, 1985; Miljkovic-Licina et al., 2004; Denker et al., 2008; Marlow et al., 2009; Watanabe et al., 2009). In *P. hydriforme*, cnidocytes are numerous in the tentacles (Ibragimov, 1999; Ibragimov and Raikova, 2004); around the mouth they form a stinging ring similar to the one found in some hydromedusae (Marques and Collins, 2004).

The cnidocytes of *P. hydriforme* are different from those in other cnidarians due to their spherical shape and to the position of the cnidocil directly above the operculum of the capsule. They are more similar to mechanoreceptors than to classic stinging cells (Raikova, 1990). Besides, two rows of spines characteristic for their capsular thread (Ibragimov, 1999; Ibragimov and Raikova, 2004) distinguish them from the cnidocytes of all other Cnidaria. The walking tentacles demonstrate a powerful armament of atrichous isorhiza cnidocytes covering their tips and scattered on the tentacles' surface. Sensory tentacles possess four categories of holotrichous isorhizas (Ibragimov, 1999). Different forms and lengths of the cnidocils, such as a dagger or a stump, straight or curved, suggest functional differences. Likely each kind of cnidocil is intended either for defense or for catching specific food depending on the size and density of its integument; it would be worthwhile to elucidate the functional differences of the four cnidocil categories in the future. Many cnidocils are curved, likely because they are devoid of a central element (Raikova, 1990) which is responsible for the rigidity of cnidocils (Hwang et al., 2008).

As in *Hydra*, the cnidocytes in *P. hydriforme* are innervated by sensory cells (Hobmayer et al., 1990; Holstein, 2012). The capsules

are mounted in a tubulin skeleton as the cytoplasm of the cnidocytes is filled by microtubules (Raikova, 1990). The cnidocytes are connected with each other by α-tubulin IR cytoskeleton of epidermal cells forming a kind of subcellular non-neural net.

Stinging cells in *P. hydriforme* (atrichous and holotrichous isorhizas) belong to the most primitive nematocyst type (Werner, 1965; Bozhenova, 1988; Östman, 1999, 2000; David et al., 2008; Fautin, 2009) and because of their unusual characteristics they are interesting as a step in the evolution of cnidocytes, especially as a possible link between Myxozoa and other Cnidaria. Myxozoan spores possess polar capsules, intracellular organelles strikingly concordant with cnidarian nematocysts, including their ultrastructural features (e.g., capsule wall and inverted tubule), formation (Golgi-secretory pathway, tubule invagination), and molecular architecture proteins involved in capsule walls (Okamura and Gruhl, 2015). Homology of the two structures appears highly likely (Weill, 1938; Uspenskaya, 1984; Okamura and Gruhl, 2015). The first analyses of minicollagens and nematogalectins (Shpirer et al., 2014) demonstrated that the cnidocytes of *P. hydriforme* share two of three minicollagens with the polar capsules of Myxozoa. Therefore, we share the opinion that the polar capsules of Myxozoa are homologous to the nematocysts of Cnidaria. The tubulin IR cnidocil of *P. hydriforme*'s cnidocysts, situated right above the capsule lid, could be homologous to the tubulin IR myxozoan polar cap observed at the spore pole, above the outlets of the polar capsule discharge channels (Uspenskaya and Raikova, 2004).

The epidermal cells of *P. hydriforme* contain alveolar cytoplasm. They are lacking a muscle part and a cilium at all stages of *P. hydriforme*'s life cycle (Raikova, 1994) and therefore represent a special kind of epithelium among Cnidaria. Their principal character is the presence of apical acid mucopolysaccharide granules contained in alveolae which are not stained by the methods used here. The epidermal cells display α-tubulin IR borders corresponding to microtubules lining the cell membrane on the inside observed in ultrastructural studies (Raikova, 1980: fig. 15; Raikova, 1984). Thus, a third, non-neural but cytoskeletal tubulin IR network is formed in addition to the two neural ones, tubulin IR and FMRF-amide IR.

In conclusion, we would like to highlight the significance of the available data on *P. hydriforme* for understanding cnidarian evolution and discussing the phylogenetic placement of Myxozoa. We are sure that without taking into account this peculiar organism any discussion on these topics will be incomplete.

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