







RESEARCH ARTICLE

The ultrastructure of the apical organ of the Müller's larva of the tiger flatworm *Prostheceraeus crozieri*

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Abstract

The tiger flatworm *Prostheceraeus crozieri* (Polycladida) develops via an eight-lobed, and three-eyed planktonic Müller's larva. This larva has an apical organ, ultrastructural details of which remain elusive due to a scarcity of studies. The evolution and possible homology of the polyclad larva with other spiralian larvae is still controversial. Here, we provide ultrastructural data and three-dimensional reconstructions of the apical organ of *P. crozieri*. The apical organ consists of an apical tuft complex and a dorso-apical tuft complex. The apical tuft complex features a central tuft of five long cilia, which emerge from four or five individual cells that are themselves encircled by two anchor cells. The necks of six multibranching gland cells are sandwiched between ciliated tuft cell bodies and anchor cells. The proximal parts of the ciliated cell bodies are in contact with the lateral brain neuropil via gap junctions. Located dorsally of the apical tuft complex, the dorso-apical tuft complex is characterized by several long cilia of sensory neurons, these emerge from an epidermal lumen and are closely associated with several gland cells that form a crescent apically around the dorsal anchor cell, and laterally touch the brain neuropil. Such ciliated sensory neurons emerging from a ciliated lumen are reminiscent of ampullary cells of mollusc and annelid larvae; a similar cell type can be found in the hoplonemertean decidula larva. We hypothesize that the ampullary-like cells and the tuft-forming sensory cells in the apical organs of these spiralian larvae could be homologous.

KEYWORDS

apical organ, ciliary tuft, frontal organ, larva, Polycladida, ultrastructure

1 | INTRODUCTION

Larval development via a trochophore or trochophore-like larva is known in many spiralian (which we define here as the monophyletic group of spirally cleaving phyla, sister to the Gnathozoa within the

Lophotrochozoa). These include Annelida, Mollusca, Nemertea, Platyhelminthes, and Entoprocta (Nielsen, 2012). The most common features of these larvae are ciliary bands and an apical organ. The apical organ in the comparatively well-studied mollusc larva has a presumed neuro-sensory function and typically consists of several

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cell types: ciliary tuft cells, ampullary cells, and parampullary cells (Croll & Dickinson, 2004). Similarly, in annelids ciliary tuft cells and ampullary-like cells have been described (Lacalli, 1981; Marlow et al., 2014). One of the central questions of spiralian evolutionary developmental biology is whether some of their larval features like the apical organ, are homologous (Marlow et al., 2014; Nielsen, 2004, 2005; Rawlinson, 2014); if so, this would suggest that the larvae themselves are homologous.

While most free-living flatworms are direct developers, some polyclads develop via one of several planktonic larval types known as Müller's, Goette's, and Kato's larvae. These larvae are characterized by different numbers of lobes, eyes, and general body shapes (Martín-Durán & Egger, 2012; Rawlinson, 2014). The most striking peculiarity of polyclad larvae are leg-like lobes that protrude from the body, ciliary bands at the margins of these lobes, a posterior tuft, and an apical organ (also referred to as a "frontal organ") (Lacalli, 1982, 1983; Lapraz et al., 2013; Martín-Durán & Egger, 2012; Rawlinson, 2014). In three transmission electron microscopical studies, the ultrastructure of the apical organ of three Müller's and a Goette's larvae was described as an apical tuft of long cilia with associated gland cells at the anterior pole (Lacalli, 1982, 1983; Ruppert, 1978). In the Müller's larva of the polyclad *Prostheceraeus crozieri*, a second tuft of long cilia was found just dorsal to the apical tuft in a scanning electron microscopical study (Lapraz et al., 2013). Here, we provide new ultrastructural data concerning the nature of this second dorso-apical ciliary tuft, a detailed description and three-dimensional (3D) reconstruction of the apical organ, and a comparison with other spiralian apical organs.

2 | MATERIALS AND METHODS

2.1 | Transmission electron microscopy

For transmission electron microscopy, 1-day-old larvae of *P. crozieri* were anaesthetized in 7.14% aqueous magnesium chloride for 10 min and then fixed in glutaraldehyde and osmium tetroxide in cacodylate buffer following the protocol of Eisenman and Alfert (1982), detailed in Salvenmoser et al. (2010) (for collection of adult animals, see Lapraz et al., 2013). Samples were dehydrated in a graded ethanol series, and embedded in EPON (Sigma-Aldrich) (see Gammoudi et al., 2016; Salvenmoser et al., 2010). Semithin sections (0.35 μm) as well as ultrathin sections (80 nm) were cut using a Leica ultracut UCT microtome. Semithin sections were stained using Richardson's methylene blue azure II disodium tetraborate decahydrate (Richardson et al., 1960). Ultrathin sections from two different individuals were contrasted using lead citrate and examined with a Zeiss Libra 120 energy filter transmission electron microscope using a Tröndle 2 \times 2k highspeed camera with ImageSP software (Tröndle). Image processing was performed with ImageSP and Adobe Photoshop 7.

2.2 | Serial block-face scanning electron microscopy (SBEM)

For SBEM, 1-day-old larvae of *P. crozieri* were fixed following a protocol published by Deerinck et al. (2010), embedded in Durcupan ACM resin (Sigma-Aldrich) hard formula, and processed as detailed in Bertemes et al. (2021). Sections from one individual were made and examined with a 3View system from Gatan (Gatan) (Zankel et al., 2009, 2014) using a backscattered electron detector from Gatan on an environmental scanning electron microscope (ESEM) Quanta 600 FEG (FEI) equipped with a Schottky emitter. The work was carried out in the low vacuum mode of the ESEM, to avoid charging on the specimen's surface. The SBEM image stack as a movie is available as Supporting Information: File 1. 3D reconstruction was created in Dragonfly software, Version 2021.1 for Windows (Object Research Systems [ORS] Inc., 2020; software available at <http://www.theobjects.com/dragonfly>).

3 | RESULTS

The apical organ of *P. crozieri*'s Müller's larva consists of two connected parts, which are termed "apical tuft complex" and "dorso-apical tuft complex" which we describe in turn.

3.1 | Ultrastructure of the apical tuft complex

The apical tuft complex is located right at the anterior pole (Figure 1). It comprises three key components: (1) a cluster of monociliated sensory cells (apical tuft sensory cells, or ATS cells), surrounded by (2) a circle of gland cell necks and the associated gland cells (apical tuft gland cells, or ATG cells), which are anteriorly (above the basal membrane) enclosed by (3) two anchor cells (Figures 2c–f and 3a,b).

The gland cell necks of the apical tuft complex form a circle surrounding the ATS cells and elongate posteriorly in a cup-like shape (Figures 2 and 3). In total, there are six gland cells in the apical tuft complex, each branching unequally into between one and four gland cell necks, 13 in total (Figure 3d). This cup-shaped construct is composed of two halves facing each other (Figure 3d, inset). Each half is built by a bundle of three ATG cells and their corresponding cell necks (Figure 3d). Within each bundle, the individual ATG cells are closely intertwined: the tips of the ATG cell necks alternate, that is, tips of the same ATG cell are (mostly) not adjacent (Figure 3d). The apical tuft complex of *P. crozieri* consists of two different types of gland cells, distinguishable by the size and electron density of their granules (Figure 4a–c). Granule type 1 (ATG1) stretches about 450–650 nm in the longest diameter and is not electron-dense (Figure 4b). Granule type 2 (ATG2) is smaller (about 150–350 nm in diameter) and is electron-dense (Figure 4b). In addition to the presence of granules, the gland cells can be identified by the occurrence of peripheral microtubules (Figure 5c).

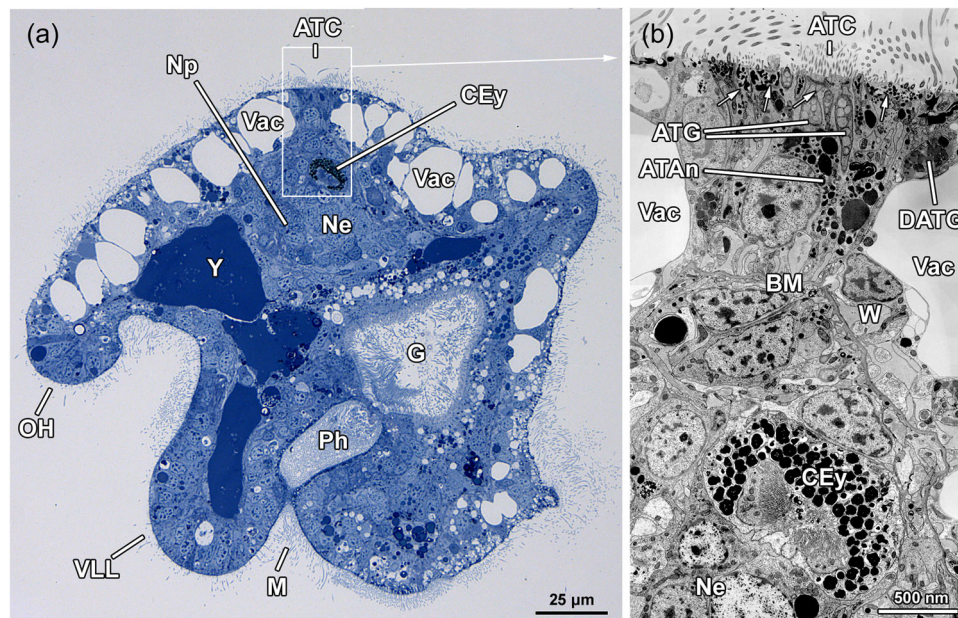


FIGURE 1 Sagittal sections of the Müller's larva of *Prostheceraeus crozieri* (specimen #1) at the level of the cerebral eye. (a) Semithin section (0.35 μm) of the whole larva. (b) Corresponding electron micrograph of the apical organ. Ultrathin section (80 nm). ATAn, apical tuft anchor cell; ATC, apical tuft complex; ATG, apical tuft gland cell; BM, basal membrane; CEy, cerebral eye; DATG, dorso-apical tuft gland cell; G, gut; M, mouth; Ne, neurons; Np, neuropil; OH, oral hood; Ph, pharynx; Vac, vacuole; VLL, ventro-lateral lobe; W, basal web; Y, yolk. Arrows point to a thin apical layer of small vesicles and granules.

The two anchor cells are part of the epidermal layer, but do not reach beneath the basal membrane (Figures 1b, 4a,b, 5a, and 6a). The anchor cells bear multiple cilia and are equipped with surface microvilli and a thin apical layer of small vesicles and granules (Figure 1b). In contrast to the neighboring epidermal cells, the anchor cells show no big vacuoles (Figures 1, 4a,b, 5a, and 6a). Apically, the gland cell necks are tightly enveloped, but not completely encompassed by the anchor cells; each gland cell neck is in contact with at least one anchor cell (Figures 2c and 3b). Each anchor cell harbors ATG cell necks of both ATG cell bundles, as the anchor cells are rotated by about 90° to the ATG cell bundles (Figure 3a,b,d). ATS and ATG cells pass from the epidermal layer through the basal membrane and extend posteriorly to the brain (Figures 2e,f, 4a,b, and 5a).

The ATS cell nuclei are located in the basal part of the cell, almost on top of the brain (Figures 5a, 7a, and 8a). They are monociliated and bear microvilli. Although there are five ATS cell necks, just four ATS cell nuclei are clearly observable in our stack (Figures 2i and 5a), but at the posterior-most part of the stack, there may be a fifth ATS cell nucleus (Figure 2j). The ATG cells reach further posteriorly (Figures 5a, 7a,b, and 8a). Separated in two bundles, consisting of three cells each, they run laterally to the brain (Figures 4e, 7a,b, and 8a). Both ATG cell bundles are pierced by a thin posterior cell process coming from one of the ATS cells (Figures 3c, 4e, and 7, Layer III). The posterior ATS cell process is connected to the brain via a synapse (Figure 4d,e). The space between the ATG cell bundles in the region between the basal membrane and the anterior tip of the brain (Figure 7, Layer II) is occupied by the cell bodies of the apical cells (Figures 7a and 8a). More posteriorly, the ATG cell bundles are

dorsally adjacent to four further gland cells (Figures 7b, Layer III), which are part of the dorso-apical tuft complex and are therefore termed the “dorso-apical tuft gland cells” or DATG cells.

3.2 | Ultrastructure of the dorso-apical tuft complex

The dorso-apical tuft complex is located about 15 μm dorsal to the apical tuft complex, measured from ciliary tuft to ciliary tuft (Figures 4a and 6a). It comprises two key components: (1) a cluster of ciliated ampullary sensory neurons (AmSN, making the tuft), which is in the middle of (2) a rough half-circle of gland cell necks and their associated gland cells (DATG cells) (Figures 2c, 4a,b, 6a–c, 7, Layer I, and 8b).

At least some of the cilia of the AmSN are recessed into the epidermal layer, forming a ciliated lumen (Figures 6a,b and 8b). Besides cilia, the AmSN also have microvilli, they are rich in mitochondria and have axons posteriorly extending above the basal membrane (Figures 6a–d and 8b).

The granules of the DATG cells measure about 450–750 nm and can be distinguished from the granules of the ATG cells based on their size and electron density—the DATG cell granules are similar in size to the ATG1 cell granules, but are more electron-dense; in contrast, the DATG cell granules are similar in electron density to the ATG2 cell granules, but larger (Figures 4a–c, 5a,e, and 6a). The DATG cell necks extend into the epidermal layer (Figures 4a,b, 5a, 6a, and 8b). These cell necks spread out dorsally around the apical tuft

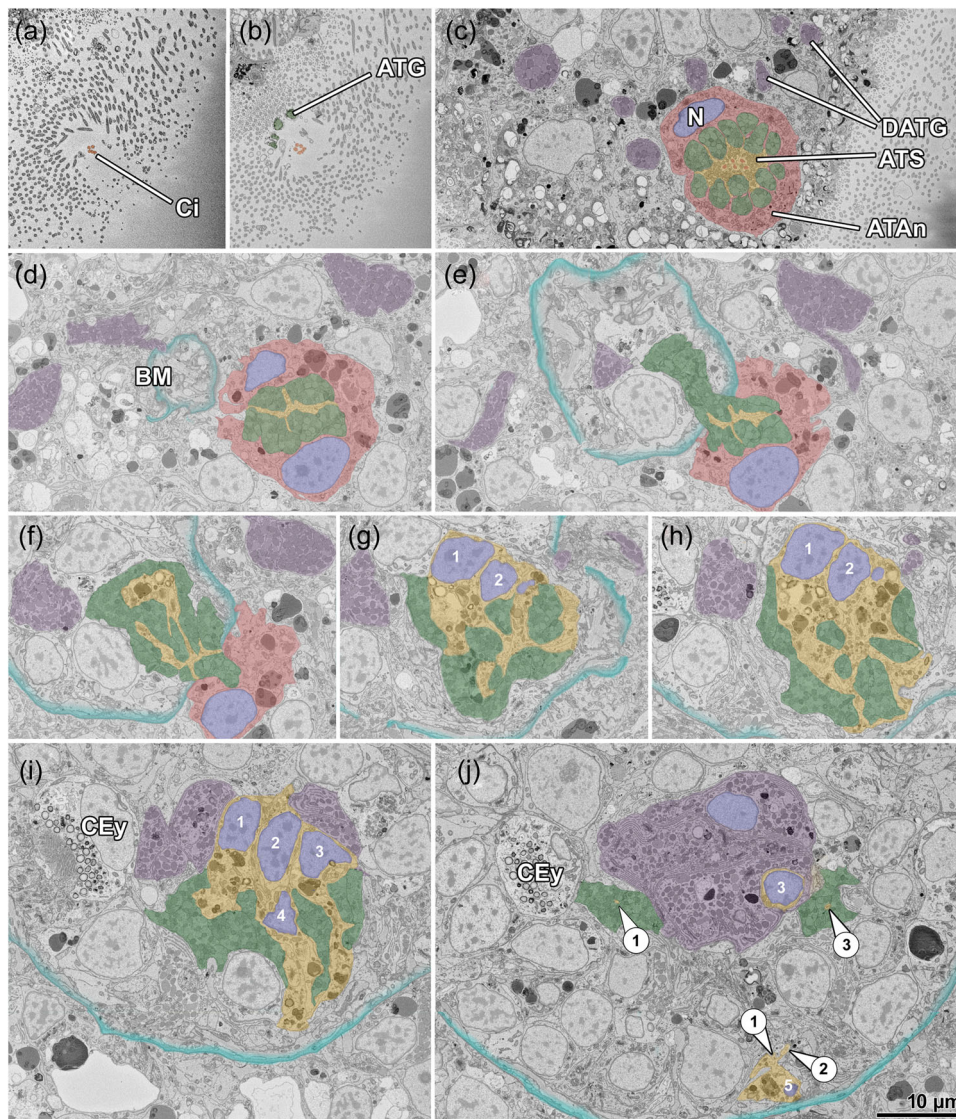


FIGURE 2 Serial block-face scanning electron micrographs (cross sections) of the larval apical organ (specimen #2). (a) Anterior-most part of cilia (Ci, orange) of the apical tuft sensory cells. (b) Ci and apical tuft gland cell (ATG, green) necks. (c) Apical tuft sensory cells (ATS, yellow), surrounded by circle of ATG necks, enclosed by two apical tuft anchor cells (ATAn, red; nucleus: N, blue), and a crescent of dorso-apical tuft gland (DATG, purple) cell necks. (d) More posterior section of the anterior part of the apical organ (BM, basal membrane, turquoise). (e, f) ATG necks and ATS pass through BM. (g–j) Posterior part of the apical tuft complex located posterior to the BM. CEy, cerebral eye. Numbers refer to individual ATS cells (j) or nuclei (g–j).

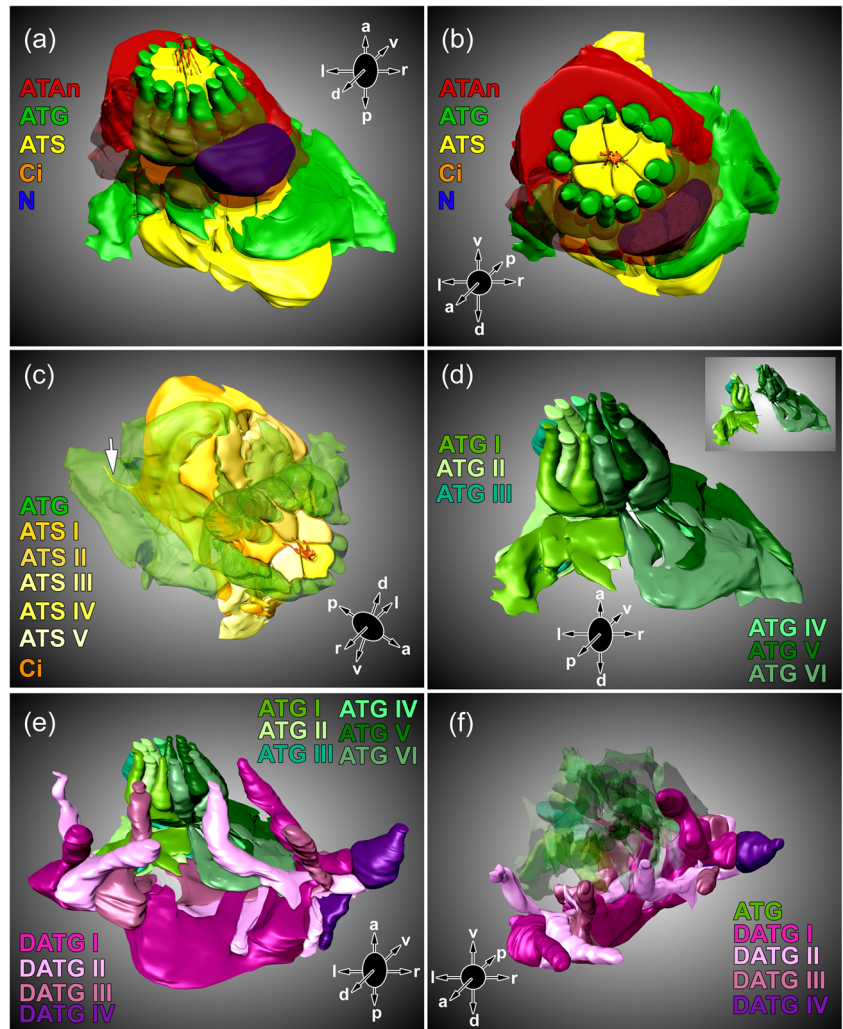
complex in a loose half-circle (Figures 2c, 3e–f, and 7, Layer I). In total, four DATG cells contribute to the dorso-apical tuft complex with a variable number of necks: one DATG cell is trifurcated, two are bifurcated, and one is unbranched—in total there are eight DATG cell necks (Figure 3e,f). Three DATG cells branch first posterior to the basal membrane and one of these branches a second time in the epidermal layer (Figure 3e). The DATG cell necks form two bundles, each containing at least one gland cell neck from each gland cell (except for the unbranched DATG cell, which contributes to only one bundle). The DATG cell bodies are located dorsally and slightly lateral to the brain (Figures 5a, and 7b, Layer III). Along the cell membrane of the DATG cells, there is a row of peripheral microtubules (Figure 5d).

4 | DISCUSSION

4.1 | The apical tuft complex

In general, there is a broad agreement between our data and the observations of Ruppert (1978) and Lacalli (1982, 1983). The apical tuft complex of the Müller's larva of *P. crozieri* includes the two main elements described by Ruppert (1978) and Lacalli (1982, 1983): an apical cluster of monociliated sensory cells and a surrounding circle of gland cell necks. Unlike these authors, however, we think that the apical tuft complex consists of an additional key component: two anchor cells surrounding the ATS cells and encompassing the ATG cell necks. Based on their ultrastructural features, the anchor cells of the

FIGURE 3 3D reconstruction of the larval apical tuft complex and the glandular part of the dorso-apical tuft complex (specimen #2). (a). Lateral view of the apical tuft complex. (b). Anterior view of the apical tuft complex. (a, b). 3D, three-dimensional; ATAn, apical tuft anchor cell; ATG, apical tuft gland cell; ATS, apical tuft sensory cell; Ci, cilia of the ATS; N, nucleus of ATAn. (c) ATS and ATG, green. Different ATS are colored in different shades of yellow (ATS I–V). Arrow points to thin posterior cell process of one of the ATS. (d). Cup-shaped arrangement of ATG. Different ATG are colored in different shades of green (ATG I–VI). Insert shows lateral symmetry of the ATG bundles forming the two halves of the cup (ATG I–III left, ATG IV–VI right). (e). Lateral view of ATG I–VI and the dorso-apical tuft gland cells (DATG I–IV, different shades of purple). (f). Anterior view of the ATG (here translucent green) and DATG I–IV. Black oval with arrows showing the orientation, anterior (a), posterior (p), ventral (v), dorsal (d), right (r), left (l).



apical tuft complex seem to be modified epidermis cells, that is, epidermal cells that lack big vacuoles. A difference between the ATG cells in Goette's larva and in Müller's larva of *P. crozieri* is the presence of peripheral microtubules in the latter (Ruppert, 1978). We note that the different granule types in ATG cells could not be distinguished in our SBEM images (Figure 2), in contrast to our TEM images (Figures 4 and 6a).

4.1.1 | The number of anchor cells

The anchor cells of the apical tuft complex can, in fact, be recognized in published work. In Ruppert (1978), a slightly oblique transversal TEM section through the very anterior tip of a Goette's larva contains a part of a putative anchor cell (Ruppert, 1978; his figure 4a). A more posterior transverse section shows at least three anchor cells surrounding the circle of gland cell necks (Ruppert, 1978; his figure 4b). The number of anchor cells may apparently vary between species or at least between different larval types: at least three in some Goette's larvae (Ruppert, 1978), and two in some Müller's larvae (this study).

4.1.2 | The number, branching, and arrangement of the ATG cells

Lacalli (1983) describes and illustrates the shape of the entire mass of gland cell necks as a "flat-bottomed flask." In his drawing (Lacalli, 1983; his figure 2), the ATG cell necks point straight anterior. He describes 12 ATG cell necks resulting from the trifurcation of four thick gland cell cords emerging from at least two ATG cells. In the larva of *P. crozieri* described here, the gland cell necks do not point straight anterior, but rather surround the ATS cells in a cup shape. We hypothesize that the interleaving of gland cell necks serves a stabilizing function, even more so, in that the two interleaving gland cell neck bundles occupy one-half of each anchor cell. This stresses the importance of the anchor cells as a key component of the apical tuft complex. Regarding the cell bodies of the ATG cells, Ruppert (1978) simply notes that they are located above, below, and behind the brain, while the ATG cell necks run posterior, dorsal, and ventral to the neuropil. It is not clear, however, whether DATG cells are also part of his observations. In *P. crozieri*'s Müller's larva, the ATG cell nuclei are below the brain, but the cell bodies and necks run laterally to both sides (see Figures 7a,b and 8a). Only the DATG cells are

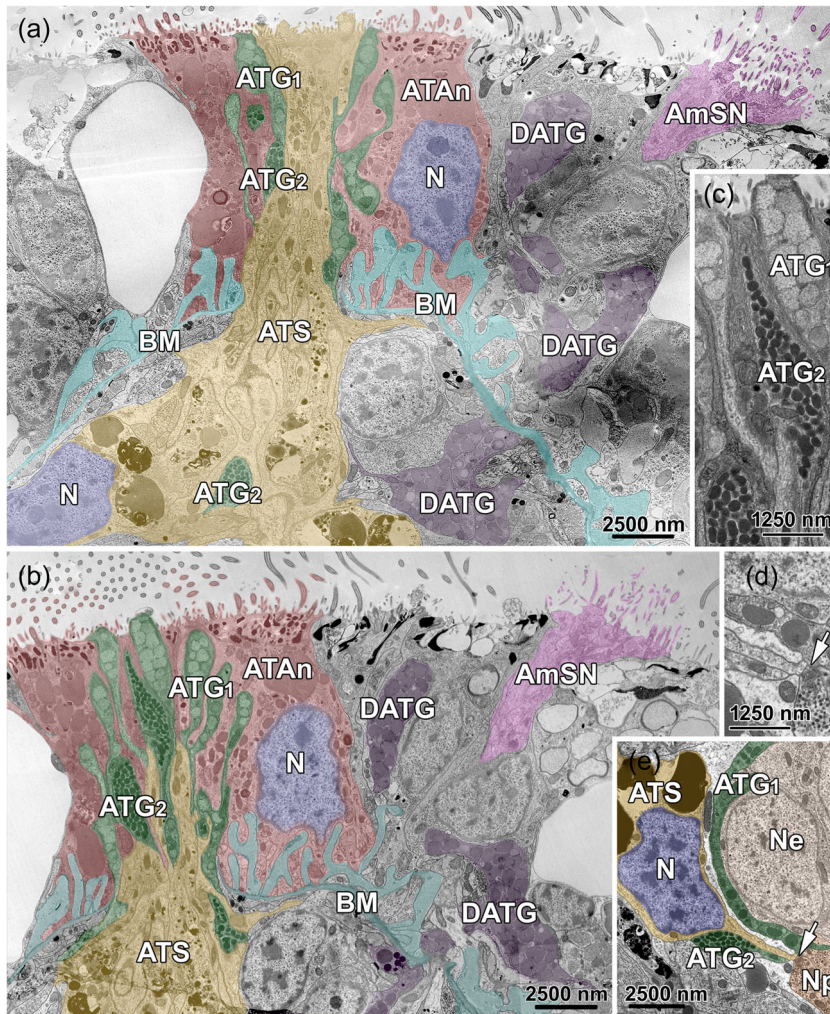


FIGURE 4 Sagittal ultrathin section through the apical organ (specimen #1). (a) Median sagittal section of the apical organ. (b) More lateral sagittal section of the apical organ. (c) Detail of apical tuft gland cells granule type 1 (ATG1) and granule type 2 (ATG2) (green). (d) Detail of the synapse of the apical tuft sensory cell. Arrow points to synapse. (e) Detail of the apical tuft sensory cell (ATS, yellow). Arrow points to synapse. AmSN, ampullary sensory neurons (pink); ATAn, apical tuft anchor cell (red); BM, basal membrane (turquoise); DATG, dorso-apical tuft gland cell (purple); N, nucleus (blue) of the ATAn; Ne, neurons (light peach); Np, neuropil (peach).

mainly distributed dorsal to the brain (Figures 5a and 7b), and it may be that Ruppert's dorsally located ATG cells correspond to our DATG cells since Ruppert did not distinguish between ATG and DATG cells.

4.1.3 | The number, branching, and arrangement of the ATS cells

A similar issue shows up with the number of ATS cells. In contrast to the Müller's larva of *P. crozieri*, where the ATS cells consist of five apical cell processes and five cilia (Figures 2a-c and 3a-c), the ATS cells in the Müller's larvae of Ruppert (1978) comprises at least six cilia and hence at least six monociliated apical cell processes (Ruppert, 1978; his figure 3a,b). In the Müller's larva of *P. crozieri*, there are just four apical cell bodies and nuclei clearly observable beneath the basal membrane (Figures 2g-i and 7a), while a potential fifth cell body and the start of the nucleus appear only in the last section of the SBEM image stack (Figure 2j). Lacalli (1983) describes the same phenomenon in Müller's larva of *Pseudoceros canadensis*. Even though he observed five ATS cell appendices and five corresponding cilia, he was only able to detect four cell bodies on top of the brain. He traced this discrepancy back to a bifurcation of

one of the ATS cell bodies, making one of the ATS cells biciliated, similar to the multiciliated ATS cells of a Goette's larva (Ruppert, 1978). In *P. crozieri*, we think that the missing fifth ATS cell body is not located on top of the brain as are the other four, but rather more ventral. The number of ATS cells is not consistent between the studied species: six or more ATS cells in Ruppert's undetermined Müller's larva and five in *P. canadensis* and in *P. crozieri*.

4.2 | The dorso-apical tuft complex

In all other ultrastructural studies concerned with polyclad larvae, only a single apically located organ, either termed "frontal organ" (Ruppert, 1978) or "apical organ" (Lacalli, 1982, 1983), was described. In a light and scanning electron microscopical study, Lapraz et al. (2013) observed for the first time a second tuft of about 15 long apical cilia, shifted slightly dorsal to the first tuft of long apical cilia. We were able to find this second apical ciliary tuft in our semi and ultrathin sections (Figures 6a and 8c,d).

The distinction of these two anterior cilia tufts is difficult without electron microscopical approaches as they are located quite close (ca. 15 μ m) to each other (Figures 4a,b and 6a; see also Lapraz et al., 2013;

FIGURE 5 Sagittal ultrathin section through the lateral region of the apical organ and the brain (specimen #3). (a) Overview. (b) Detail of the brain neuropil (Np). (c) Detail of the margin of an apical tuft gland cell (ATG) type 1. Arrows point to peripheral microtubules. (d) Detail of the margin of a dorso-apical tuft gland cell (DATG). Arrows point to peripheral microtubules. (e) Detail view of an ATG type 1 neighboring a DATG. AmSN, ampullary sensory neurons (pink); ATAn, apical tuft anchor cell (red); ATS, apical tuft sensory cell (yellow); BM, basal membrane (turquoise); N, nucleus of the ATAn (blue); Ne, neurons (light peach); Np, neuropil (peach).

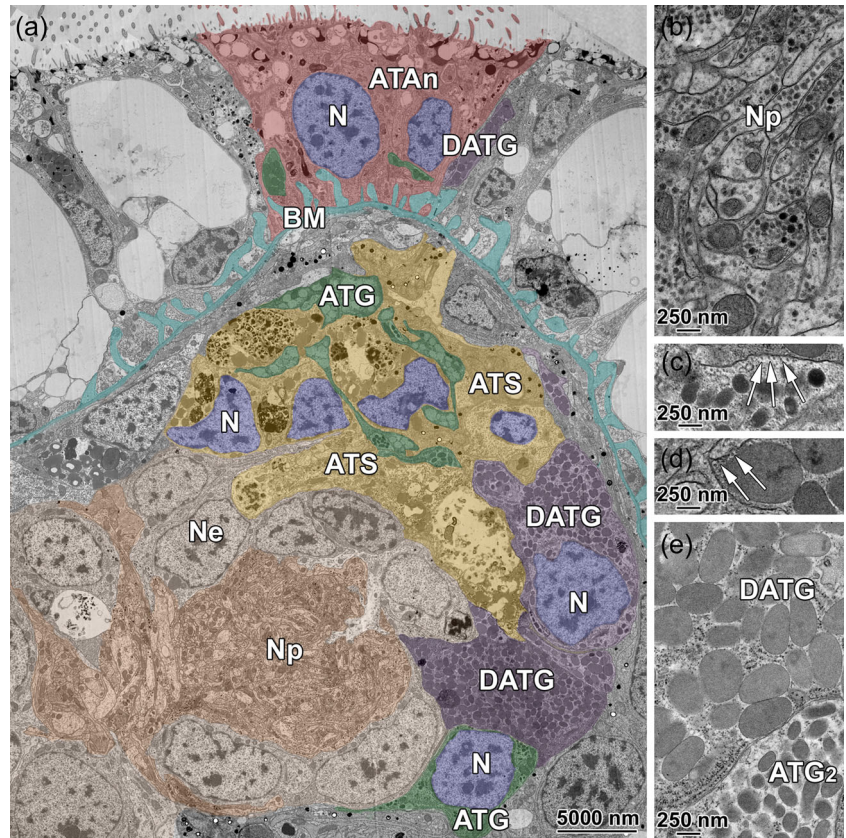
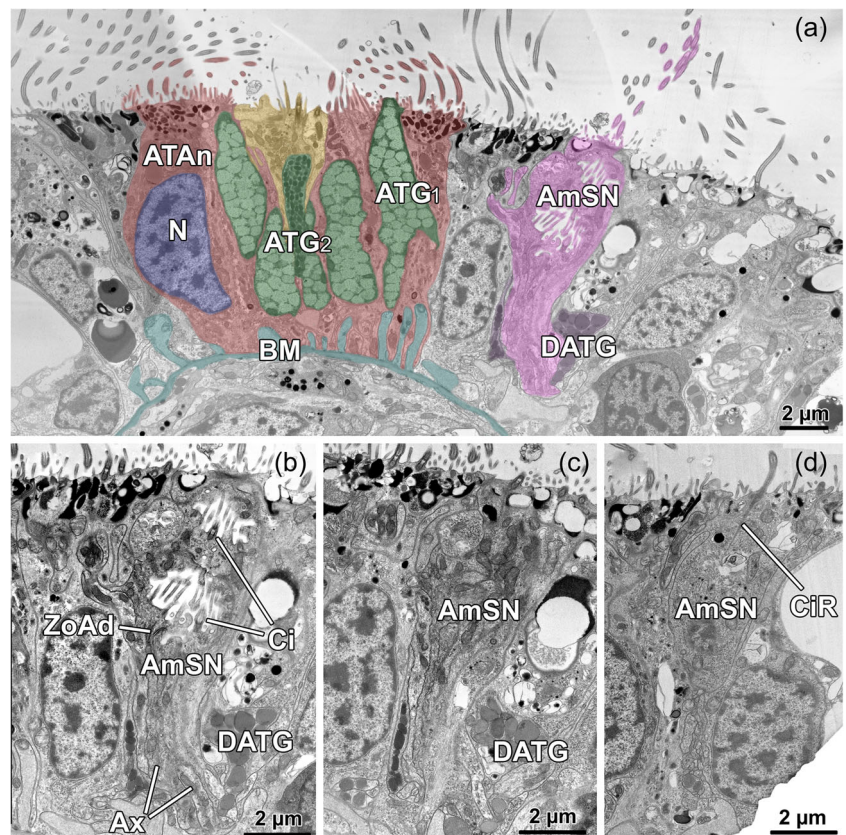


FIGURE 6 Sagittal ultrathin section through the apical organ (specimen #3). (a) Color-coded overview. (b–d) Details of the AmSN in consecutive sections. AmSN, ampullary sensory neurons; ATAn, apical tuft anchor cells (red); ATS, apical tuft sensory cells (yellow); ATG1, apical tuft gland cell granule type 1 (green); ATG2, apical tuft gland cell granule type 2 (green); Ax, axons of the AmSN; BM, basal membrane (turquoise); Ci, cilia of the AmSN; CiR, ciliary rootlet of the AmSN; DATG, dorso-apical tuft gland cell (purple); N, nucleus of the ATAn (blue); ZoAd, zonula adhaerens.



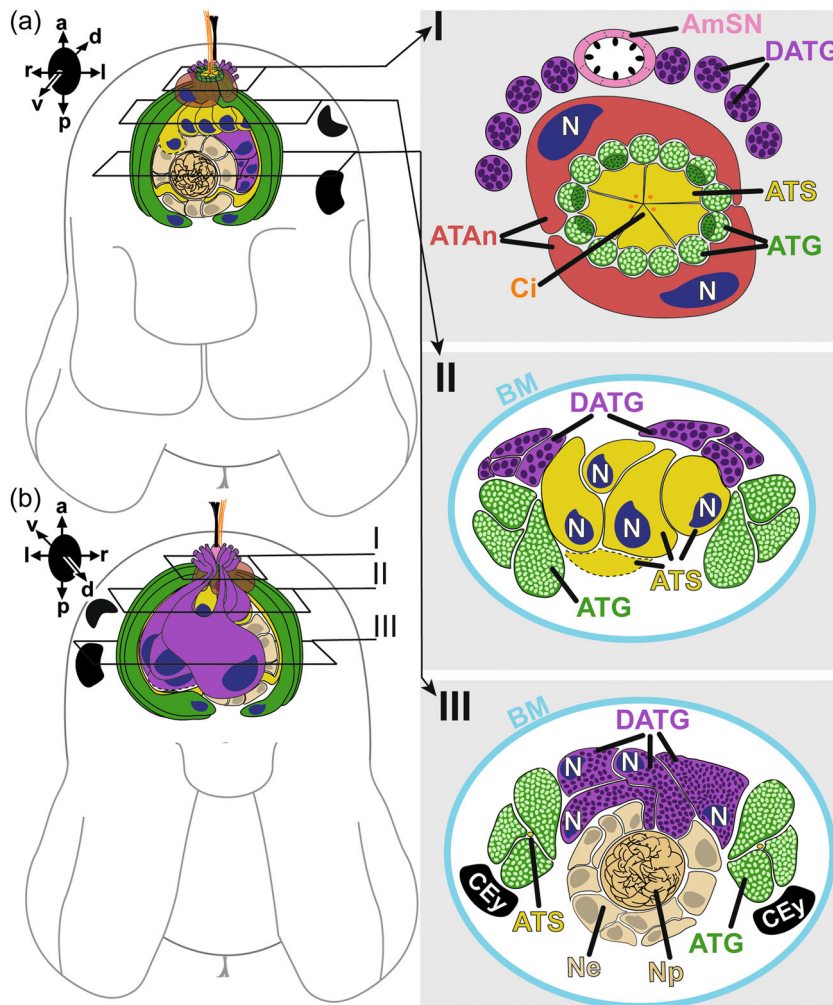


FIGURE 7 Reconstruction and cellular arrangement of the apical organ of the Müller's larva of *Prostheceraeus crozieri*. (a) Ventral view of the apical organ. I–III. Cross section of the indicated layers. (b) Dorsal view of apical organ. AmSN, ampullary sensory neurons (pink); ATAn, apical tuft anchor cells (red); ATS, apical tuft sensory cells (yellow); ATG, apical tuft gland cells (green); BM, basal membrane (turquoise); Ci, cilia of the ATS (orange); CEY, cerebral eyes (black); DATG, dorso-apical tuft gland cells (purple); N, nuclei of the ATAn (blue); Ne, neurons (light peach); Np, neuropil (peach). Estimated cells shown with dashed lines. Black oval with arrows showing the orientation, anterior (a), posterior (p), ventral (v), dorsal (d), right (r), left (l).

their figure 8b,e) and have possibly been overlooked in studies using only light microscopy. Another possibility for the absence of a second apically located ciliary tuft in the literature is that one of the tufts may be reduced at an early time point in the larval development. The larvae used in Ruppert (1978) are of planktonic origin with unknown age, but the larvae of Lacalli (1983) were fixed within 1 day of hatching like the specimens used in the present study. Curiously, in the specimen used for SBEM, the AmSN cells, and their ciliary tuft could not be detected either, only the associated DATG cells.

4.3 | Neuronal differences in the apical and the dorso-apical tufts in polyclad larvae

The function of the apical/frontal organ in polyclad larvae is a much-discussed topic (Kato, 1940; Lacalli, 1982, 1983; Rawlinson, 2010, 2014; Ruppert, 1978; Younossi-Hartenstein & Hartenstein, 2000). Kato (1940) suggested that the frontal organ is used to break the eggshell at hatching in polyclad larvae. In contrast, Ruppert (1978) hypothesized a sensory and glandular function based on ultrastructural data. Lacalli (1982) observed that the bases of the ciliated apical cells in *P. canadensis* is located on top of the brain, but not directly connected to the brain

and he hence determined that these ciliated apical cells were not sensory neurons (Lacalli, 1982), or that it was not clear if they were sensory neurons or sensory cells (Lacalli, 1983). In *P. crozieri*, we found neuronal axons in the basal region of the AmSN (Figure 6b), thus suggesting that the ampullary cells are neurons. We found no axons in ATS cells, but a synapse between the posteriormost tip of an ATS cell and the neuropil of the brain (Figure 4d,e) implies that ATS cells are not neurons, but sensory cells.

Together, apical and dorso-apical tuft complexes constitute the apical organ. Both organs of *P. crozieri* likely have a sensory function, with the main difference that the apical tuft complex features neurons, while the dorso-apical tuft complex does not, probably reflecting their different (but so far unknown) functions.

4.4 | Comparison of the apical and frontal organs in different spiralian larvae

4.4.1 | Entoprocta

The structural similarities of the apical tuft complex in polyclad larvae and the frontal organ in some entoproct larvae are an

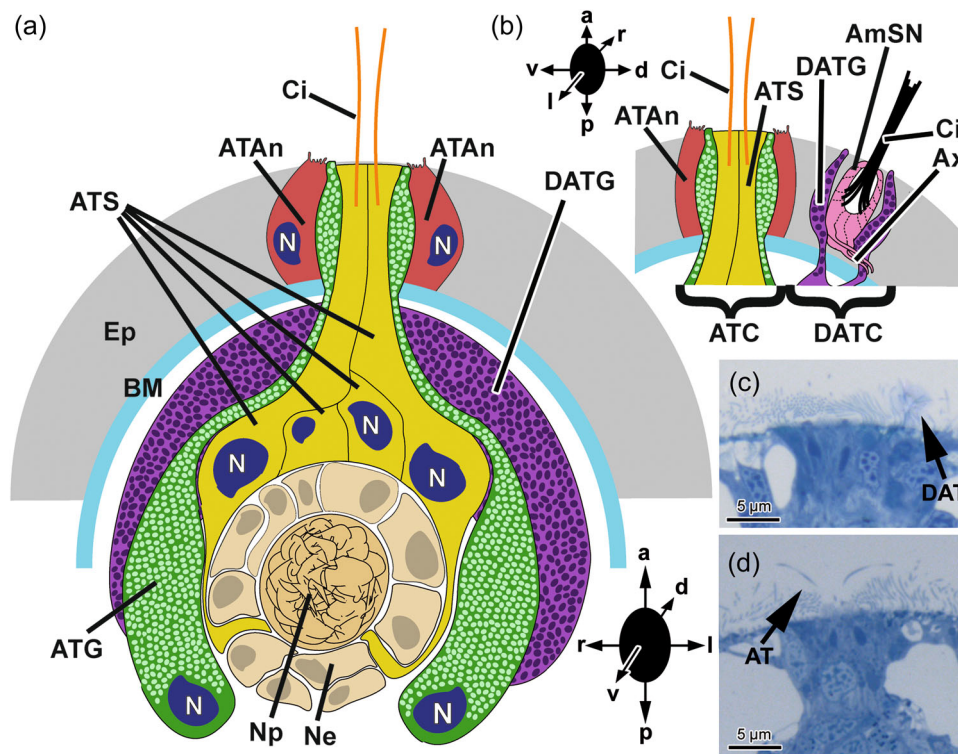


FIGURE 8 Reconstruction of the apical organ of the Müller's larva of *Prostheceraeus crozieri*. (a) Horizontal view; (b) sagittal view; (c) sagittal semithin section showing the DAT; (d) sagittal semithin section showing the AT. AmSN, ampullary sensory neurons (pink); AT, apical tuft; ATAn, apical tuft anchor cells; ATC, apical tuft complex; ATG, apical tuft gland cells (green); ATS, apical tuft sensory cells (yellow); BM, basal membrane (turquoise); Ci, cilia of the ATS (orange) and the AmSN (black); DAT, dorso-apical tuft; DATC, dorso-apical tuft complex; DATG, dorso-apical tuft gland cells (purple); N, nuclei of the ATAn (blue); Ne, neurons (light peach); Np, neuropil (peach). Black oval with arrows showing the orientation, anterior (a), posterior (p), ventral (v), dorsal (d), right (r), left (l).

apical (polyclads) or frontal (entoprocts) ciliary tuft surrounded by gland cells, closely associated with a brain (or larval ganglion) and a pair of cerebral eyes. The entoproct larva of *Loxosomella* has an apical organ as well as a frontal organ (Figure 9e) (Merkel et al., 2015; Nielsen, 1971; Woollacott & Eakin, 1973). The frontal organ in *Loxosomella* larvae marks the anterior pole, while the apical organ is located dorsally (see Merkel et al., 2015; their figure 2, Wanninger et al., 2007). The apical organ of the entoproct larva of *Loxosomella murmanica* shows a high number of serotonin-expressing cells (14–16), but no serotonin expression has been described or shown in the *Loxosomella* larval frontal organ (see Wanninger et al., 2007). In another *Loxosomella* larva, a frontal organ ganglion was even described (Woollacott & Eakin, 1973). Comparing the data of the entoproct larva of *L. murmanica* with our current knowledge of the polyclad Müller's larva of *P. crozieri*, the following similarities can be found: (1) an anteriorly located, ciliated, glandular organ with a secretory character (*P. crozieri*: apical tuft complex, *L. murmanica*: frontal organ) and (2) a second ciliated organ, located dorsal of the frontal organ, with (sensory) neurons (*P. crozieri*: dorso-apical tuft complex, *L. murmanica*: apical organ).

4.4.2 | Mollusca

Based on the complexity of the expressed serotonergic pattern in the apical organ, there may be a link between mollusc and entoproct apical organs and even a common ancestral condition (Wanninger et al., 2007). In molluscs, serotonergic immunoreactivity has been shown in parampullary cells (Croll & Dickinson, 2004; Kempf et al., 1997; Marois & Carew, 1997), and the serotonergic cells in the apical organ of a polyplacophoran larva have been homologised with the serotonergic cells in an entoproct larval apical organ (Wanninger et al., 2007). We suggest that the serotonergic apical organ cells of the polyplacophoran and the entoproct larva are also parampullary cells.

With regard to *P. crozieri*'s Müller's larva, it is interesting that the apical organ of several molluscs shows sensory neurons with a deep multiciliated lumen, usually called ampullary cells (Bonar, 1978; Croll & Dickinson, 2004; Page, 2002; Page & Parries, 2000), similar to the cells building the dorso-apical tuft of the Müller's larva of *P. crozieri*. Kempf et al. (1997) noted a similarity between mollusc ampullary cells and ampullary-like cells in some annelid larvae. Most mollusc larvae also feature at least one nonampullary ciliary tuft, the cells of which are probably not neuronal and are not serotonin-positive (Kempf et al., 1997;

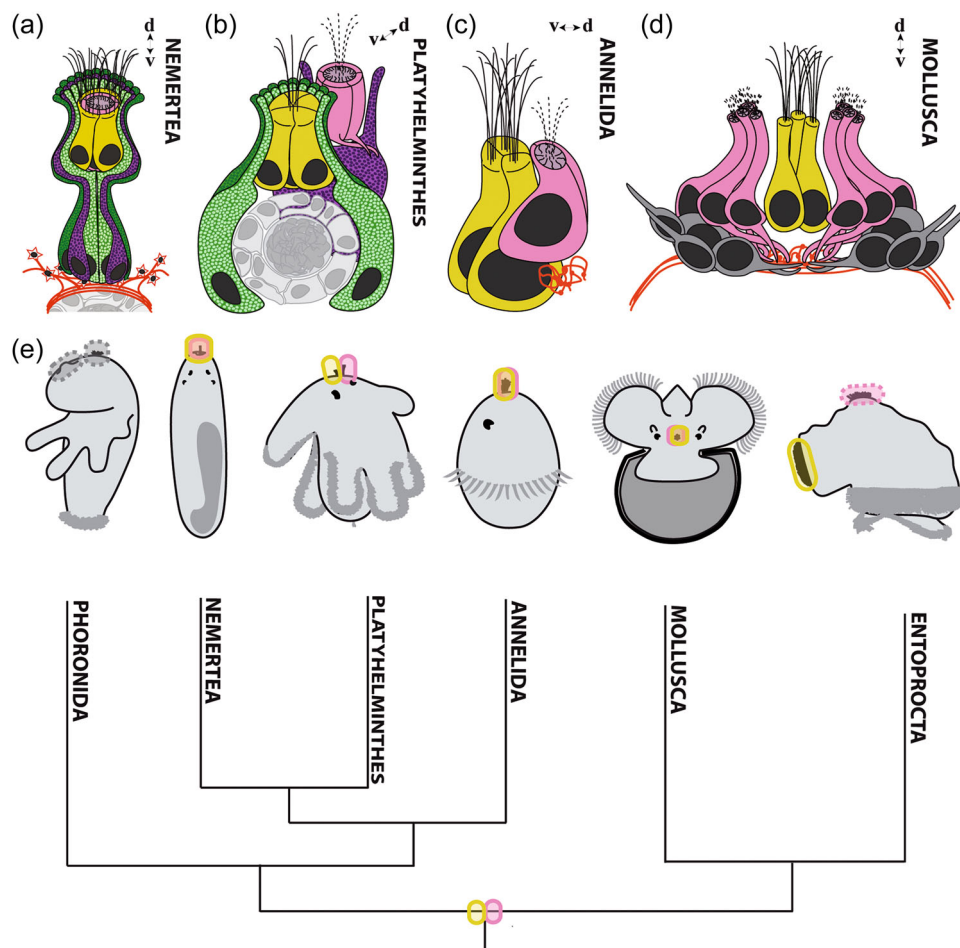


FIGURE 9 Apical organs of marine invertebrate larvae of several spiralian phyla. (a) Reconstruction of the apical organ of the hoplonemertean decidula larva (after Magarlamov et al., 2020). (b) Reconstruction of the apical organ of the polyclad Müller's larva. (c) Reconstruction of the apical organ of an annelid trochophore (after Lacalli, 1981). (d) Reconstruction of the apical organ of molluscan larvae (after Croll & Dickinson, 2004). (a–d) Ciliated tuft arising from sensory cells (yellow) and ciliated ampullary cells (pink). Cilia from ampullary cells only sometimes emerging above the epidermal surface in (c, d) (cilia drawn with dashed lines). Nerves associated with the apical organ drawn in red, glands in green and purple. (e) Spiralian larvae in a tree after Marlétaz et al. (2019) with apical organs highlighted. Yellow circle marks presence of ciliated sensory cells, pink circle marks presence of ampullary cells. The homology of apical and frontal organs in phoronid larvae remains unclear. In Entoprocta, the apical organ (marked with dashed pink circle) is not yet studied and the presence of ampullary cells is only hypothesized.

Ruthensteiner & Schaefer, 2002; Uthe, 1995). These ciliary tuft cells are usually an integral part of the apical organ and have been suggested to be homologous in most spiralian larvae (Ruthensteiner & Schaefer, 2002). Such non-neuronal cells were also found in polyclad larvae (Lacalli, 1982, 1983; Ruppert, 1978; this study: ATS cells).

4.4.3 | Annelida

Similar to Müller's and mollusc larvae, the apical organs of the polychaete trochophores of *Phyllodoce* and *Platynereis* feature multiciliated tuft cells emerging from a deep multiciliated lumen (Lacalli, 1981; Marlow et al., 2014) (Figure 9c). These tuft cells are similar and have been homologised to the ampullary cells (sensory neurons) of molluscs (Marlow et al., 2014), but their neuronal

character in annelids is still unclear (Lacalli, 1981; Marlow et al., 2014). The second part of the apical organ is formed by multiciliated tuft-forming cells ventral to the ciliated lumen in *Phyllodoce* (Lacalli, 1981), while in *Platynereis* there is a crescent of multiciliated cells dorsal to the ampullary cells (Marlow et al., 2014). The ampullary cells in polychaete trochophores are similar to the AmSN found in the polyclad Müller's larva (both feature a ciliated lumen), while the annelid (nonampullary) tuft-forming cells are similar to the polyclad ATS cells (Figure 9b,c).

4.4.4 | Phoronida, Brachiopoda, Ectoprocta

In phoronid larvae (actinotrochs), the presence of an apical organ and a frontal organ has been reported (Lacalli, 1990; Temereva, 2017).

Both these organs in actinotrochs show limited similarity to the frontal or apical organs of molluscs, entoprocts, and platyhelminths (Temereva & Tsitrin, 2014). The frontal organ of actinotrochs, also called the pyriform organ, is unciliated, of sensory character and in several species, is not outwardly visible (Temereva, 2017; Temereva & Tsitrin, 2014). In ectoproct larvae, a similar frontal/pyriform organ also exists in addition to an apical organ (formed of monociliated cells). Contrary to the frontal organ of phoronid larvae, the ectoproct larval frontal organ is ciliated, equipped with gland cells, and has a secretory character (Gruhl, 2009; Woollacott & Zimmer, 1971). A potential homology between the phoronid and the ectoproct frontal organ is inconclusive (Temereva & Tsitrin, 2014). Brachiopod larvae do not have a frontal organ, but only an apical organ (Hay-Schmidt, 1992; Santagata, 2011). The apical organ of brachiopods features monociliated apical sensory neurons in a broad field (Santagata, 2011). The apical organ of phoronids and ectoprocts has a central ciliary tuft emerging from a pit; in phoronids, apical sensory cells, not neurons, are described (Temereva & Tsitrin, 2014), while in ectoprocts, two types of apical sensory neurons have been shown (Gruhl, 2009).

4.4.5 | Nemertea

The apical organ in Müller's larvae of *P. crozieri* shares a very similar ultrastructural organization with the apical organ in the hoplonemertean decidula larvae of *Quasitetrastemma stimpsoni* (Magarlamov et al., 2020). Both main components are present: ampullary cells and ATS cells. Additionally, these cells are closely associated with glands in both animal groups. In the decidula, the apical organ consists of a ciliary tuft composed of several multiciliated cells surrounded by a cup-shaped structure built by gland cells and braced by the surrounding epidermal cells. The multiciliated tuft cells (called apical plate cells) are arranged in two layers forming an outer and inner concentric ring (Magarlamov et al., 2020). The inner layer forms a ciliated lumen of six to eight cells and is surrounded by an outer layer consisting of four multiciliated cells (Magarlamov et al., 2020). The inner layer forming a ciliated lumen is reminiscent of the AmSN that also form a ciliated lumen in *P. crozieri*, while the outer multiciliated layer in *Q. stimpsoni* and the ATS cells in *P. crozieri* are tuft-building sensory cells, in which the cilia do not emerge from a lumen (Figure 9). In contrast to Müller's larvae, the gland cells of the larva of *Q. stimpsoni* are not bifurcated. Interestingly, there are three different gland cell types in the apical organ in early rudiment hoplonemertean larvae (Magarlamov et al., 2020) and in Müller's larvae (Figure 4a,b), with similar granule types in both larvae: mucoïd cells in nemerteans correspond to ATG1 cells in Müller's larvae (large granules with low electron density), bacillary cells to DATG cells (large granules with high electron density), and granular cells to ATG2 cells (small granules with high electron density), respectively (Figure 9a,b). The apical plate cells forming the ciliated tuft in the early nemertean larvae are not neurons but are described as transforming into sensory neurons during larval development, while the ciliated lumen is lost (Magarlamov et al., 2020).

According to recent molecular phylogenies, Platyhelminthes and Nemertea are possible sister groups (Marlétaz et al., 2019; Philippe et al., 2019); this clade has been called Parenchymia (Nielsen, 1995). An interesting parallel in polyclad larvae and larvae of hoplonemerteans is that in both groups the multiciliated cells with a lumen, and the ciliated tuft cells of the apical organ are intimately associated with gland cells (Magarlamov et al., 2020). This is in contrast to other spiralian larvae, making this finding a possible synapomorphy of the hoplonemertean and the polyclad larvae. The overall architecture of the hoplonemertean and the polyclad apical organ is also very similar, with the exception that the tuft-forming, ciliated lumen is in the middle of the apical organ in the hoplonemertean larva, while in the Müller's larva it is slightly dorsally located.

4.5 | A revision of the apical organ in spirilians

Based on our data and on an extensive review of the literature, we propose a new definition of the spiralian apical organ, which we consider homologous. In many spiralian larvae, the apical organ consists of two main components: first, multiciliated sensory neurons forming a ciliated lumen (often referred to as ampullary cells). From this ciliated lumen, often (polyclads: this work; annelids: Marlow et al., 2014; molluscs: nemertean decidula larva: Magarlamov et al., 2020; molluscs: Bonar, 1978; Marois & Carew, 1997; Page, 2002; Page & Parries, 2000; Uthe, 1995), but not always (annelids: Lacalli, 1981; molluscs: Croll & Dickinson, 2004; Kempf et al., 1997; Ruthensteiner & Schaefer, 2002), a multiciliated tuft emerges above the epidermal surface. Second, a ciliated tuft arising from sensory cells, which are either monociliated (polyclads—Müller's larvae: Lacalli, 1982, 1983; Ruppert, 1978; this work; nemertean pilidium larva: Lacalli & West, 1985), or multiciliated (annelids, Lacalli, 1981; molluscs, Croll & Dickinson, 2004; nemertean decidula larva, Magarlamov et al., 2020; nemertean pilidium larva, Cantell et al., 1982). These two main components differ in their relative positions. They either form adjacent structures (entoprocts: Nielsen, 1971; Wanninger et al., 2007; molluscs: Page, 2002; annelids: Lacalli, 1981; Marlow et al., 2014; polyclads: this work), or a series of organs (molluscs: Croll & Dickinson, 2004; Kempf et al., 1997; Page & Parries, 2000), or a concentric complex (nemerteans: Magarlamov et al., 2020). Since these two main components are not universal, but are very common in four spiralian phyla (Platyhelminthes, Annelida, Mollusca, Nemertea, Figure 9a–d), we propose them to be an ancestral character for spirilians (Figure 9e). According to the proposed sister group relationship of Mollusca and Entoprocta, we hypothesize the presence of ampullary-like cells in entoprocts, the apical organ of which has not been studied ultrastructurally to date. In phoronids and other lophophorates, the apical organ may have been substantially modified. One of the components of this putatively homologous apical organ may be absent in some groups such as a ciliary tuft in some molluscs (see Ruthensteiner & Schaefer, 2002) or AmSN in some annelids

(Lacalli, 1981) and nemertean pilidium larvae (Lacalli & West, 1985; Magarlamov et al., 2020). For polyclads, AmSN have been described for the first time in the present study. The AmSN may either have been overlooked in these polyclad larvae from other species, or they may not be present at all, or they are not present in the examined developmental stages (Lacalli, 1982, 1983; Ruppert, 1978).

According to the current phylogeny of flatworms, polyclad larvae as a plesiomorphic character for the Platyhelminthes would imply the loss of a larva in the Catenulida, the Macrostomorpha, the Prorhynchida, and the Euneoophora (Egger et al., 2015). These losses are necessary to explain the homology of the larval forms in polyclads and the trochophore larva of the Trochozoa (Egger et al., 2015; Martín-Durán & Egger, 2012).

We hypothesize that the two main components of apical organs in many spiralians—the AmSN and the tuft-forming sensory cells—may comprise a homologous structure (Figure 9e). The alternative is that these components have arisen independently several times, which would suggest that they are required for functions that can best be fulfilled by structures with these forms. No functional studies have been made on the apical organs of polyclad larvae, which may further elucidate these questions.

AUTHOR CONTRIBUTIONS

Isabel L. Dittmann: Conceptualization, funding acquisition, methodology, investigation, imaging, visualization, validation, writing (original draft, review and editing). **Alexandra L. Grosbusch:** Investigation, imaging, writing (review and editing). **Magdalena Nagler:** Investigation, imaging, writing (review and editing). **Willi Salvenmoser:** Technical support, methodology, investigation, imaging, validation, writing (review and editing). **Armin Zankel:** Technical support, methodology, resources, writing (review and editing). **Maximilian J. Telford:** Resources, funding acquisition, writing (review and editing). **Bernhard Egger:** Conceptualization, supervision, resources, funding acquisition, methodology, investigation, imaging, validation, writing (original draft, review and editing).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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