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VERSITA

Topography and ultrastructure of the tegument of *Aphallus tubarium* (Rodolphi, 1819) Poche, 1926 (Digenea: Cryptogonimidae), intestinal parasite of the common Dentex dentex (Linnaeus 1758) from Valinco Gulf

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Abstract

The tegument ultrastructure of the intestinal fluke *Aphallus tubarium* was studied for the first time with the use of scanning and transmission electron microscopy. New details on morphology were recorded. The ultrastructural study revealed that the tegument of *A. tubarium* had a syncytial organization with a distal cytoplasm lying over a basal matrix and cytons. The surface of the tegument is covered with pectinate spines arranged quincuncially. As anterior–posterior differences were observed, particular attention was given to spines. Spines decrease in size and density from the anterior part of body to posterior part. Two types of sensory structures were identified, uniciliated and dome-shaped. Type 1 sensory receptors were outgrowths bearing groups of papillae with shorter and rigid apical seta visible on the anterior part of body surface, encircling the worm. Type 2 sensory receptors was dome-shaped papillae devoid of cilia, found mainly around the oral sucker. Diagrams of spines and sensory receptors were made to help in understanding the nature of these structures. Surface morphology may prove to be useful in distinguishing *Aphallus* spp with other Cryptogonimidae.

Keywords

Aphallus tubarium, tegument, syncytium, spines, sensory structures

Introduction

Infections with helminths are common in wild fishes (Scholz, 1999). The present work describes the ultrastructure of the tegument of *Aphallus tubarium* (Rudolphi, 1819) Poche, 1926 (Digenea: Cryptogonimidae), a trematode that parasitizes the digestive tract of the common dentex *Dentex dentex* (Linnaeus 1758). As far as can be determined, no data on the ultrastructure of this digenean have been published.

The Cryptogonimidae Ward, 1917 is one of the many digenean families reported to infect species of Sparidae. It is a large family of trematode having a worldwide distribution, and comprising 66 genera with more than 200 species (Tkach and Bush, 2010). These parasites are mainly found in the digestive tract (stomach, pyloric caeca or intestine) of marine, brackish water, and freshwater fish (Miller and Cribb 2008; Foata *et al*., 2011). In the Mediterranean, only seven species have been reported. These belong to four genera: *Anoiktostoma Stossich*, 1899, *Metadena* Linton, 1910, *Aphallus* Poche, 1926 and *Paracryptogonimus* Yamaguti, 1934 (Bartoli and Gibson, 2001).

Surface morphology, especially the numbers, shapes, sizes, and distribution of various tegumental structures such as papillae, sensory receptors, or spines may be helpful in distinguishing closely related trematode species (Choudhury and Nelson, 2000; Moravec, 2002; Zdarska and Nebesarova 2003). Ultrastructural studies of many trematodes give also a clue to the understanding of their morphological structures, and have shown characters that help to determine

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the correct taxonomic position of those species (Han *et al*., 2003).

The aim of the present work was to perform a detailed description of *A. tubarium* in order to reveal some previously unreported tegument structures in this species using electron transmission microscopy (TEM) and scanning electron microscopy (SEM). This investigation complements previous descriptions of the adult *A. tubarium* (Bartoli and Bray, 1987; Korniychuck and Gaevskaya, 2004), and offers information on the tegumental topography. We gave a special consideration to the tegument features and sensory receptors, in order to establish a comparison with some other Cryptogonimidae species. It is important to investigate the tegument of each individual in order to elucidate questions on maintenance and survival of the parasite on their microhabitat.

Materials and Methods

Sampling and examinations of fish

Adult specimens of *A. tubarium* were collected alive from the common dentex *D. dentex* Linnaeus, 1758) caught in the Valinco gulf (Mediterranean Sea). Fish were caught for parasitological examinations. Biometrical measures, sex and maturity stage of each specimen of *D. dentex* were recorded. The fish were brought alive to the laboratory, anesthetized in ice, killed, and dissected the day of the capture. During necropsy, helminths from the intestine were collected alive with a Pasteur pipette and immediately fixed in absolute ethanol. Parasites were identified using a light microscope according to the description done in previous studies (Bartoli and Bray, 1987; Korniychuck and Gaevskaya, 2004). Some specimens were fixed in glutaraldehyde and prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations.

Electron microscopy

Twenty specimens of *A. tubarium* were studied using scanning electron microscopy. Parasites were removed alive from their hosts, washed repeatedly with seawater to free them from mucus, fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, dehydrated through a graded acetone series (30%, 50%, 75%, 90% and 100%), critical point dried and sputter coated with gold/palladium. Samples were examined under a HITACHI S-3400-N scanning electron microscope operated at an accelerating voltage of 20 kV in the Service of Study and Research in Electronic Microscopy of the University of Corsica (Corte, France).

Transverse sections were made in both anterior and posterior parts of *A. tubarium* using transmission electron microscopy. Anterior sections were made in the oral sucker region. Posterior ones were made between the genital and excretory pores. Sections parts of *A. tubarium* were fixed for at least 1 hour in cold (4°C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.2, postfixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in ethanol and propylene oxide, embedded in Spurr (Spurr 1969), and then polymerized at 60°C for 24 hr. Ultrathin sections of tegument (60–90 nm) were obtained with the use of an RMC Boeckeler Power Tome PC ultramicrotome, placed on 300-mesh copper grids, and double stained with uranyl acetate and lead citrate according to Reynolds (1963). Sections were examined on a Hitachi H600 transmission electron microscope, operating at an accelerating voltage of 75 kV in the Service of Study and Research in Electronic Microscopy of the University of Corsica (Corte, France).

Data analysis

We chose to define 3 parts of the fluke body. The anterior part extends from the mouth to the genital pore. The median part is the portion between the genital pore and the two third of the inferior part of body. The posterior part extends from two third of the inferior part of body to the excretory pore. For characterization of spines, 5 specimens were examined. For each one, measurements (length, width, numbers (*N*) of pectinate spines) in micrometers of 30 spines were made on each part of body. Density values (*D*) of tegumental spines were obtained by counting them from randomly selected areas of 100 μm^2 each. The density is given as a range followed by the number of areas analysed.

We use one-way analysis of variance (ANOVA) to test differences in distribution between sets of values on various part of the body. If data failed to meet parametric testing requirements, comparisons of the mean values were performed using the Fisher exact test (Sprent and Ley, 1992). Significance for all the statistical analyses was established with 95% confidence intervals. Calculations were performed using the data analysis and statistical software Statgraphics.

Results

General morphology

The general morphology of *A. tubarium* was already described in previous studies (Bartoli and Bray, 1987; Korniychuck and Gaevskaya, 2004), but there is no detailed description of tegumental features. The body of *A. tubarium* was fusiform. The oral sucker was situated at the anterior extremity on the ventral surface (Fig. 1A). The ventral sucker, located on the anterior third of the body, was more developed than the oral one (Fig. 1A). The tegument of *A. tubarium* has a syncytial organization (Fig. 1B). It consisted of a distal cytoplasm delimited by an outer membrane and a basal matrix, lying over a nucleated region with circular and longitudinal muscle layers and cytons. The cell bodies containing cytons lie beneath a su-

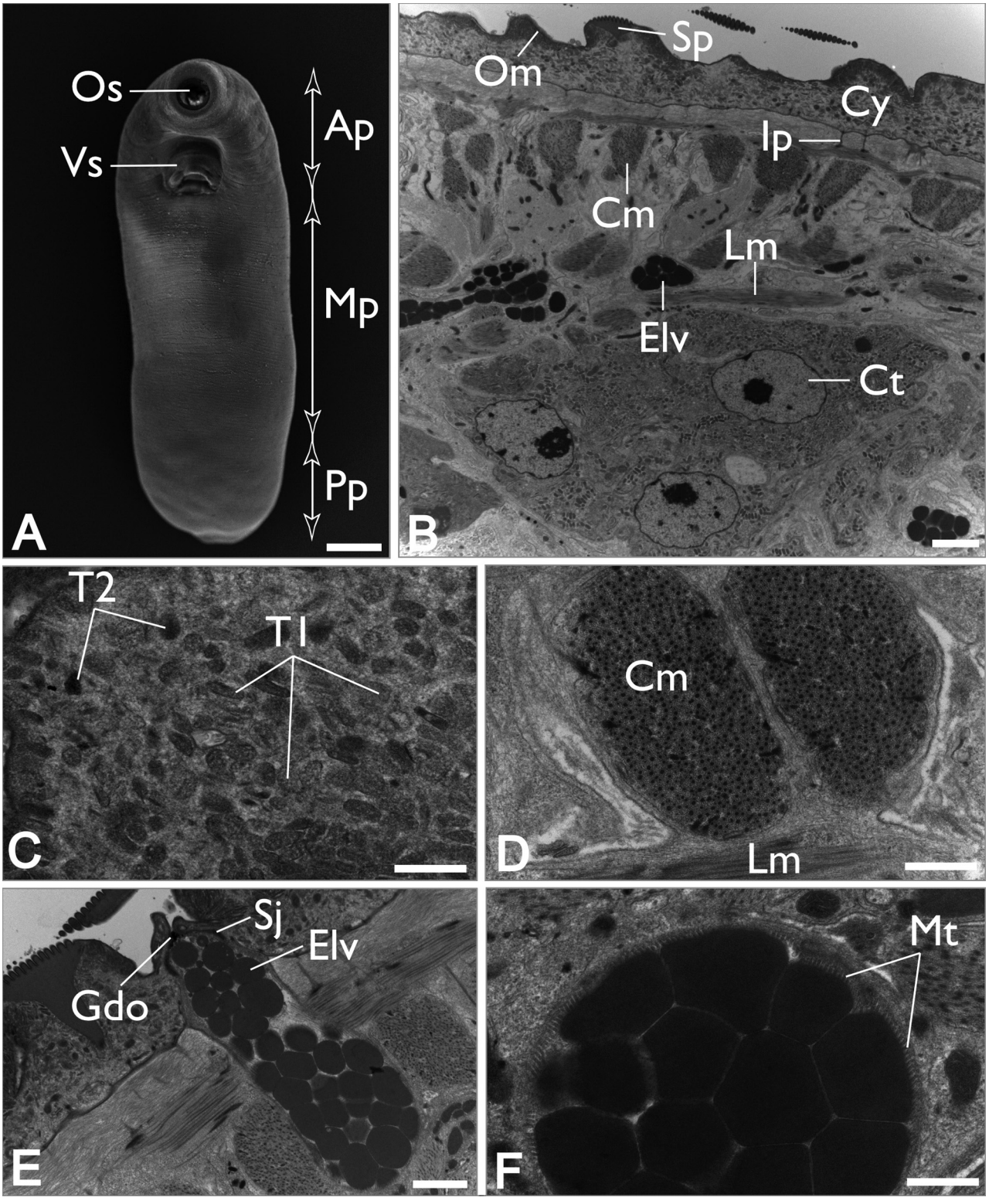

Fig. 1. Tegument of *Aphallus tubarium*. **A –** General view of *A. tubarium* showing anterior part (*Ap*), median part (*Mp*), posterior part (*Pp*) of body, oral (*Os*) and ventral sucker (*Vs*); **B –** Tegument showing distal cytoplasm (*Cy*) and pectinate spines (*Sp*) delimited by an outer membrane (*Om*). Internuncial processes (*Ip*) link the distal cytoplasm to the nucleated region; circular (*Cm*) and longitudinal (*Lm*) muscle layers, electron-lucent secretory vesicles (*Elv*), cytons (*Ct*); **C –** Type 1 secretory vesicles (*T1*), Type 2 secretory vesicles (*T2*); **D –** Details of circular (*Cm*) and longitudinal (*Lm*) muscle layers; **E –** Eccrine secretion of electron-lucent secretory vesicle (*Elv*); septate junction (*Sj*) and gland ducts opening (*Gdo*); **F –** The ducts are supported by longitudinal microtubules (*Mt*). Scale bars: A = 500 μm; B = 1 μm; C = 500 nm; D = 500 nm; E = 1 μm; F = 500 nm.

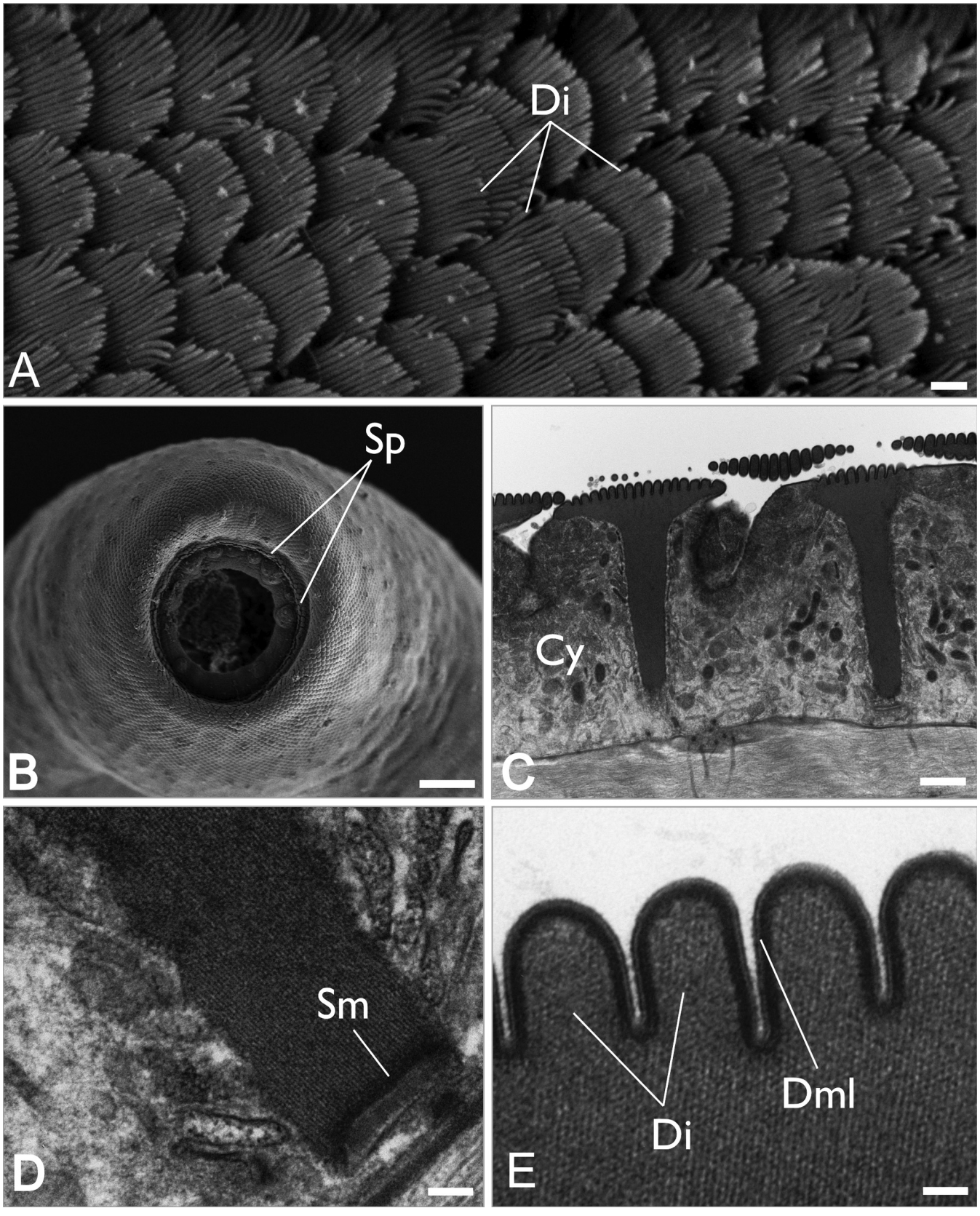

Fig. 2. Pectinate spines. **A –** View of pectinate spines with digitations (*Di*); **B –** location of spines (*Sp*) near the oral sucker; **C –** insertion of spines in distal cytoplasm (*Cy*); **D –** Spine matrix (*Sm*); **E –** details of spines. *Dml* dense material layer, *Di* digitations. Scale bars: A = 1 µm; B = 20 µm; C = 500 µm; D = 100 nm; E = 40 µm.

perficial layer of muscle, connected to the distal cytoplasm by way of internuncial processes (Fig. 1B). The ground substance of the surface syncytium consists of granular material and contains numerous mitochondria, ribosomes and vesicular inclusions more or less dense (Fig. 1B,C). Type 1 secretory vesicles are round or ovoid membranous bodies, variable in diameter,

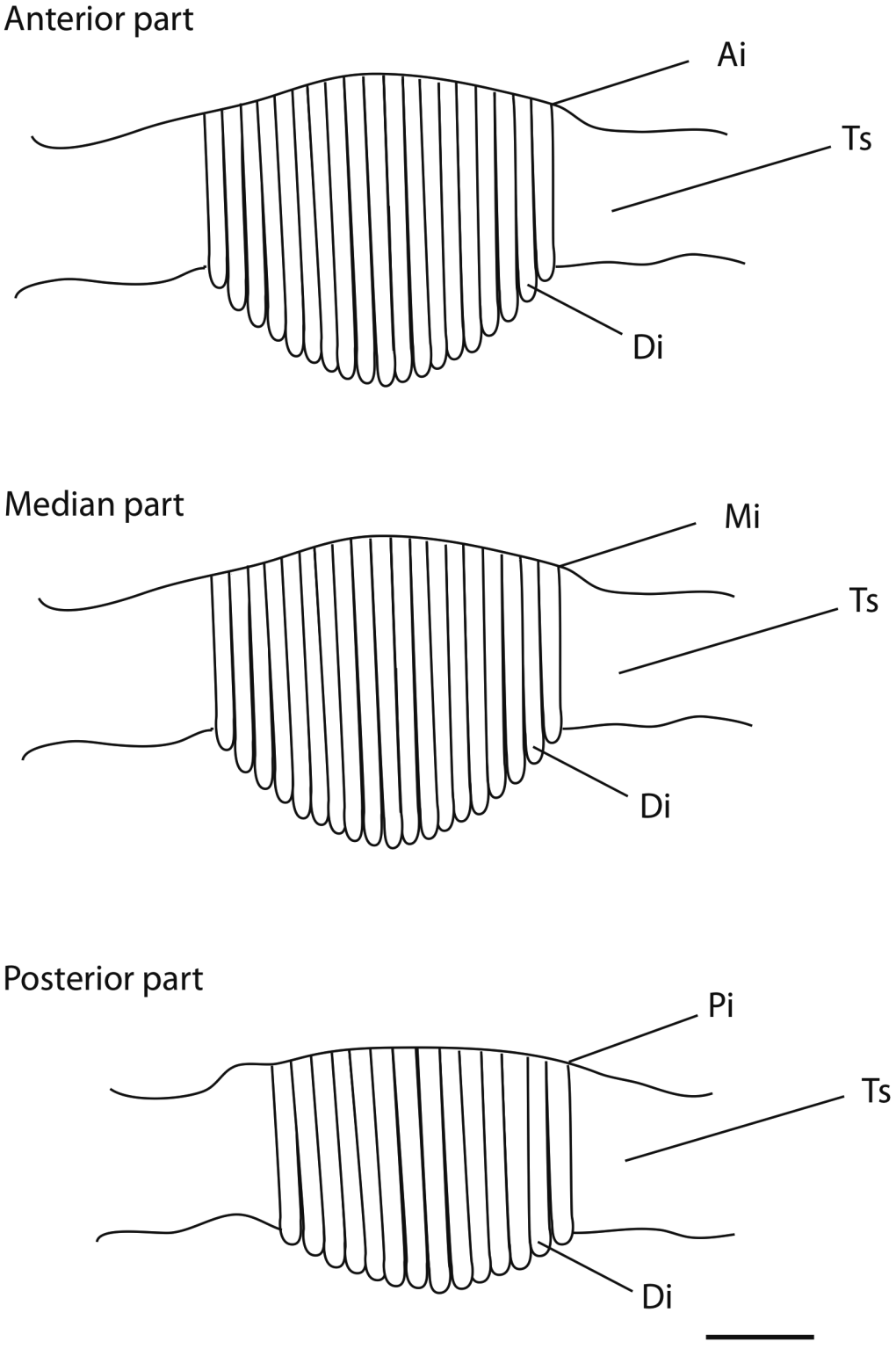

Fig. 3. Diagram of anterior, median and posterior spines of *Aphallus tubarium* showing digitations (*Di*), anterior insertion area (*Ai*), median insertion area (*Mi*), posterior insertion area (*Pi*) and tegument surface (*Ts*). Scale bar = 1 μm.

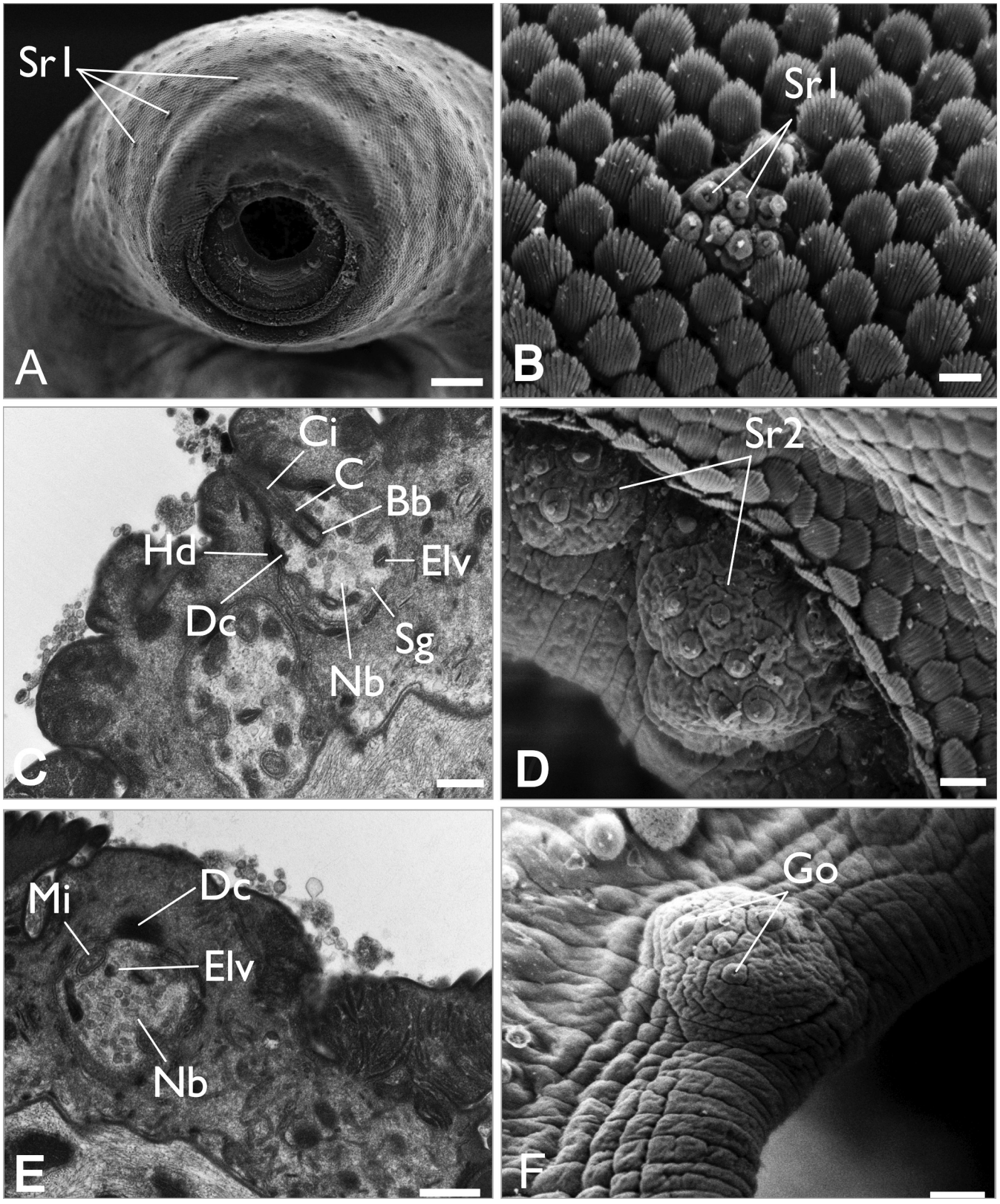

Fig. 4. Sensory receptors of *Aphallus tubarium*. **A –** Location of Type 1 receptor (*Sr1*); **B –** View of type 1 receptor (*Sr1*); **C –** Ultrastructure of type 1 sensory receptor; basal body (*Bb*), centriole (*C*), cilium (*Ci*), dense-collar (*Dc*), electron-lucent vesicle (*Elv*), hemidesmosome (*Hd*), nerve bulb (*Nb*), spherical granular (*Sg*); **D –** Type 2 sensory receptor (*Sr2*); **E –** Ultrastructure of type 2 sensory receptor; dense collar (*Dc*), electron-lucent vesicle (*Elv*), mitochondria (*Mi*), nerve bulb (*Nb*); **F –** Gland opening (*Go*). Scale bars: A = 40 μm; B = 1 μm; C = 250 nm; D = 20 μm; E = 500 nm; F = 20 μm.

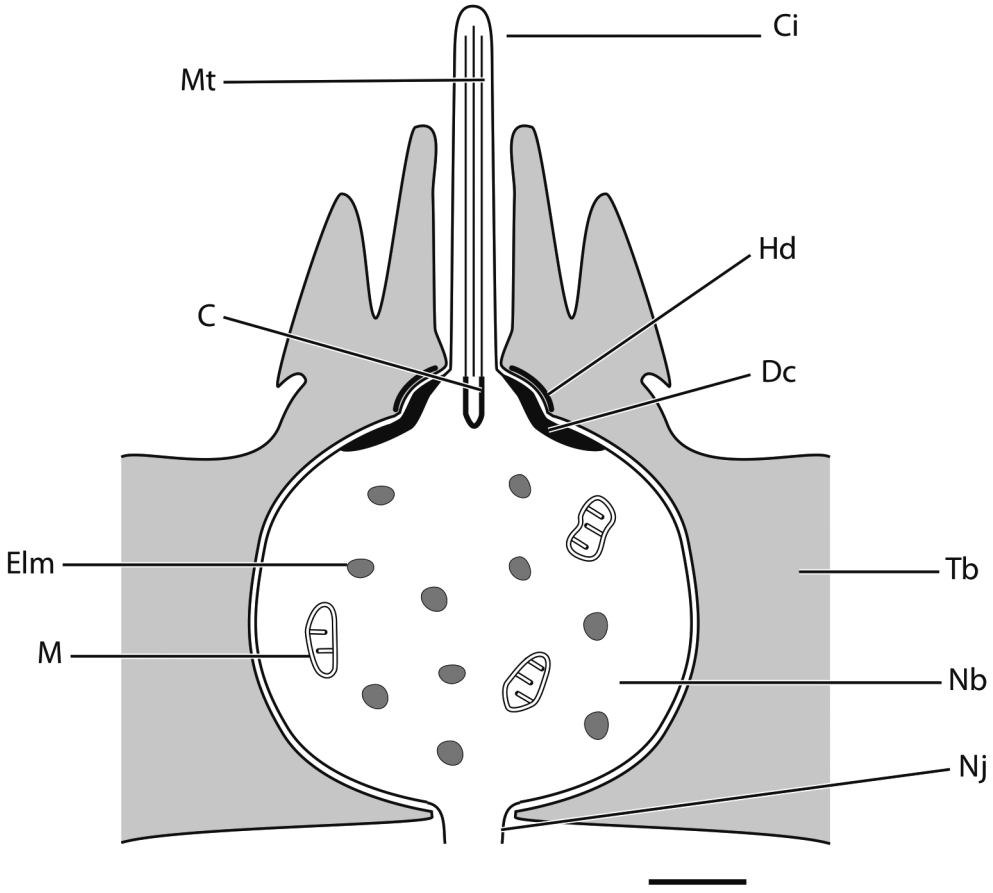

Fig. 5. Diagram of type 1 sensory receptor of *Aphallus tubarium*. *C* centriole, *Ci* cilium, *Dc* dense collar, *Elm* electron-lucent material, *Hd* circular hemidesmosome, *M* mitochondria, *Mt* microtubules, *Nb* nerve bulb, *Nj* nerve junction. Scale bar = 1 μm.

and filled with a granular material more or less electron lucent (Fig 1C). Type 2 secretory vesicles are round, electron-lucent, and variable in diameter (Fig. 1C). Type 1 and type 2 secretory vesicles are regularly distributed in the distal cytoplasm. The musculature consists of bundles of circular and longitudinally disposed muscle fibres (Figs 1B,D). Eccrine gland cells containing electron-lucent granular material and opening to exterior were present in the distal cytoplasm of *A. tubarium* (Fig. 1E). The secretory product is concentrated in the broadened ducts, opening over the plasma membrane, while a septate junction connects the gland cell with the distal cytoplasm (Fig. 1E). Gland cells enclose secretory granules in a membrane, which is surrounded by numerous cortical microtubules (Fig. 1F).

Table I. Main scales characteristics of *Aphallus tubarium*

	Anterior part	Median part	Posterior part
Length (in μm)	1.95	1.91	1.47
Width (in μm)	2.52	2.46	1.69
N digitations	18.84	19.57	36.89
D digitations	36.78	36.89	27.44

Spines

Our observations reveal rows of pectinate spines arranged quincuncially, and distributed throughout body surfaces as wide as long. Spines are dorsoventrally flattened, curved anterior–posteriorly, and multipointed (Fig. 2A). They are observed immediately near the crown of oral sucker (Fig. 2B). These spines are digitated and inserted in the lower part of the distal cytoplasm (Fig. 2C). At the spine base there is an increase in the density of the spine matrix (Fig. 2D). They have a refined crystalline structure, with units arranged in parallel, alternating dense and light lines in sections. Digitations are covered with a thin layer of dense material (Fig. 2E). Size of spines varies along body. Results for length spines measurements are reported in Table I. Statistically significant differences between the sizes of spines on body parts ($p<0,05$) are highlighted, but values are relatively close between the anterior and median part, 2,52 and 2,46 respectively. Width measurements of spines for two parts of body are similar; there is no significant difference between values of anterior and median part. The Fischer test shows a statistically significant difference with posterior part ($p<0,005$). The posterior part of body surface is covered with spines slightly smaller that ante-

rior and median spines. The anterior and median parts of are densely spines, and values are similar (D = 36,78–36,89), but there is a statistically significant difference with the posterior part ($p<0,005$, D = 27,4). Our results showed also variations in the number of digitations. Values showed a statistically significant difference between body surfaces ($p<0,05$). Spines appear to be reduced in size, density and number of digitations towards posterior part of body (Fig. 3). Lining and central apical depression of rhynchus and tegument of ventral sucker are devoid of spines. The genital pore possessed a wrinkled lip. The morphology and distribution of spines on the ventral surface were similar to those on the dorsal surface.

Sensory Structures

Several types of presumed sensory structures were identified on the distal cytoplasm of *A. tubarium*. Two types of sensory structures were highlighted. These receptors show variation in sizes and morphology. Type 1 sensory receptors are predominant and encircling the anterior part of the worm (Fig. 4A). These ciliated receptors are outgrowths bearing groups of papillae with shorter and rigid apical seta (Fig. 4B). They consist of a nerve bulb attached to the tegumental membrane by a ring-like hemidesmosome at the level of the dense collar, and a short cilium that extends from a centriole (Figs 4C,5). The nerve bulb contains spherical granular, electron-lucent vesicles, and mitochondria. Cilia are anchored in the nerve bulb by a dense basal body without rootlets. Hemispherical electron-dense collar surrounding the centriole were observed at the top of the nerve bulbs (Fig. 4C,5). Type 2 sensory receptors are protuberances like-dome shaped papillae devoid of seta (Fig. 4D). They are present on surface of oral sucker and distributed bilaterally and symmetrically around the outer rim of the oral sucker. They were observed as single and grouped forms of two to three papillae (Fig. 4D). Receptors of type 2 present a more prominent contour with highly swollen dome. These structures seemed to be nerve bulbs filled with electron-lucent vesicles and mitochondria. Hemispherical electron-dense collars were observed at the top of the nerve bulbs (Fig. 4E). Gland-duct openings perforating tegument are mainly observed on dome shaped papillae around the oral sucker (Fig. 4F).

Discussion

General morphology

This paper describes, for the first time using SEM and TEM, most of the surface features of adult *A. tubarium* collected from naturally infected dentex. Our results revealed that the tegument of *A. tubarium* is similar in ultrastructural appearance from those of other Cryptogonimidae species (Bartoli and Gibson, 2001; Ostrowski de Nũnez *et al*., 2011). Hong (2009) mentioned that the tegument of trematodes is a functional syncytium that covers the whole body and function to maintain physiologic homeostasis in the host-parasite interphase. The tegument is a metabolically active layer and one that appears to be morphologically specialized to function in absorption of nutrients, the synthesis and secretion of materials whose functions are not fully known, osmoregulation and excretion, and a sensory role (Antonelli, 2010). Exocytosis of the contents of electron-dense granules surface suggests a possible involvement in the provision of a protective layer of mucus to minimize mechanical, osmotic and immunological damage to the tegument surface (Dalton *et al*., 2004). The secretory products opening to exterior may be involved in the immunological relationship between parasites and host.

Spines

Pectinate spines with digitations have been described for species of several digenean families, associated to the gastrointestinal tract, e.g. *Bucephalus* Baer 1826, *Himasthla* Dietz 1909, *Pygidiopsis* Koie, 1990 or *Prosorhynchus* Odhner 1905 (Køie, 1992; Cohen *et al*., 1995; João Santos and Gibson, 2002; Han *et al*., 2003). Tegument morphology is seldom used for differentiating between genera (Miller and Cribb, 2008), but it may be useful for separating species. The basic surface topography of *A. tubarium* resembles what was established for other Cryptogonimidae, but studies have shown that, as the fluke matures, the tegumental spines exhibit gradual changes in shape and the number of points, and may be used for taxonomic differentiation (Russell-Smith and Wells, 1982; Lee *et al*., 1985; Mouahid, 1989).

In *A. tubarium*, these structures differ from other Cryptogonimidae species in size, shape, number of digitations and distribution on the body surface (Bartoli and Gibson, 2001; Tkach and Snyder, 2003; Ostrowski de Nũnez *et al*., 2011). Examples include *Metadena depressa* (Stossich, 1883), *Metadena phoceae* Bartoli and Gibson 2001, *Parspina argentinensis* (Szidat, 1954) or *Parspina bagre* Pearse, 1920 (Bartoli and Gibson, 2001; Ostrowski de Nũnez *et al*., 2011). The pattern of digenean tegumental spines is a commonly quoted taxonomic feature, and differences in structures and distribution over the body may be significant in host parasites relationship (Bundy, 1982; Chai *et al*., 2000). Investigations with electron microscopy have permitted to deepen and complete results mentioned by Bartoli and Bray (1987). Authors mentioned that spine density and length were constant in pretesticular region, decreasing gradually behind posterior testis, whereas our results revealed that pectinate spines of *A. tubarium* possess the same distribution on the anterior and median part of body. These spines have a largest length and highest density whereas the posterior part possesses smaller spines with a lower density. The posterior spine measurements of our material are clearly smaller than those from the rest of body. Our results contrast also with those reported for the *P. argentinensis* Szidat 1954, *M. depressa* (Stossich, 1883) *or M. phoceae* Bartoli and Gibson 2001, which mentioned that spines are largest and densely

distributed around the oral cavity, decreasing posteriorly. In some species, e.g. *P. argentinensis* Szidat 1954 or *M. depressa* (Stossich, 1883), there are absent at the posterior tip of body (Bartoli and Gibson, 2001; Ostrowski de Nũnez *et al*., 2011). The number of digitations differs from other digenean species according parts of body. Cohen *et al*. (1995) described spines on the anterior part tegument of *P. arcuatus*, with up to 30 digitations. Pandey and Tewari (1984) found spines with 3 to 25 digitations in the anterior part of *Bucephalopsis karvei* and a posterior part of body unspined, whereas *A. tubarium* posses the highest number of digitations on the posterior part (N = 36.89). The number of digitations differs also from other Cryptogonimid species studied, e.g. *P. argentinensis* Szidat 1954 possesses highest number of digitations in the anterior body surface (N = 24). Of the various morphological features, the scaly tegument is particularly important. It plays a major role in the process of attachment of the parasite to the host and in maintaining its position in the intestine (Matthews, 1973). The tegumental spines are probably involved in abrasion of host tissues for feeding and to a lesser extent for parasite anchorage after establishment in his final habitat (Køie, 1992). The crystalline structure of the spines was also observed in larval and adult trematodes (Burton, 1964; Zhou and Podesta, 1989). Authors suggested that the spines consist of hexagonally packed actin filaments and this suggestion was supported by other studies. According to Zhou and Podesta (1989), the spines may play an important role in the movement.

Sensory structures

Different sensory endings were identified on the surface of *A. tubarium*. Sensory receptors are common and varied among digeneans. They have been observed singly or grouped, bearing short or long cilia or devoid of cilia (Cohen *et al*., 1995; Han *et al*., 2003; Ostrowski de Nũnez *et al*., 2011). Our results highlighted 2 types of sensory structures already identified in other Cryptogonimidae species such as *Parspina argentinensis* Szidat 1954 or *Metadena phoceae* Bartoli and Gibson 2001. Theses findings are thought to be a common feature of the surface ultrastructure of cryptogonimids trematodes; although the form and distribution varies according to other species. The ultrastructural configuration of type 1 receptor is similar to those observed in some digeneans, even though small modifications appear from other species. Some digenean species such as *Diplostomum pseudospathaecum* Niewiadomska, 1984 or *Prosorhynchoides arcuatus* (Linton, 1900) Bray, 1984 posses a conical dense structure and rootlets on basal body of cilium not observed on *A. tubarium* (Cohen *et al*., 1995; Czubaj and Niewiadomska, 1996). Our study revealed also a difference in mean cilia lengths between *A. tubarium* and other species (Ostrowski de Nũnez *et al*., 2011). Bennett (1975) mentioned that the cilium might be capable to recording the direction and degree of any pressure acting on spines, as they are protruding to the same height as the surrounding spines. The mechanism of attachment is probably mediated by tactile sensory papillae (type 2 receptors) abundant around the oral sucker of *A. tubarium*. These papillae probably act as rheoreceptors/tangoreceptors involved in mechanisms of feeding and attachment, detecting contact with host-tissue and manipulating the adhesive pressure of the sucker-like organ (Ramasamy *et al*., 1987; Cohen *et al*., 1995; Abdul-Salam and Sreelatha, 2000). On the other hand, type 1 receptor could possibly be chemoreceptors used in recognition of host chemical stimuli serving to orient the body during feeding and mating (Whittington *et al*., 2000; Buchmann and Lindenstrøm, 2002). Gland-duct openings perforating tegument observed on dome shaped papillae around the oral sucker may be involved in absorption of organic nutrients, excretion and osmoregulation as in monogeneans (Oliver, 1981; Williams and McKenzie, 1995; Antonelli *et al*. 2010). Various types of sensory receptors found on *A. tubarium* indicate the complex sources of stimuli to which the parasite must react.

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