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Morphological aspects of *Angiostrongylus costaricensis* by light and scanning electron microscopy

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ABSTRACT

Angiostrongylus costaricensis is a parasitic nematode that can cause severe gastrointestinal disease, known as abdominal angiostrongiliasis, in humans. This paper presents the characterization of first- and third-stage larvae and male and female adult worms of *A. costaricensis* by scanning electron and light microscopy. Several novel anatomical structures were identified by scanning electron microscopy, including details of the cuticular striations of the spicules in male worms and a protective flap of the cuticle covering the vulvar aperture in female worms. Other taxonomic features revealed by light microscopy include the gubernaculum and the esophageal–intestinal valve. The use of two microscopy techniques allowed a detailed characterization of the morphology of this nematode. A number of previously identified taxonomic features, such as the striated nature of the spicules and the lateral alae were confirmed; however, the use of scanning electron microscopy resulted in a reassessment of the correct number of papillae distributed around the oral opening and behind the cloacal opening. These observations, in combination with light microscopy-based characterization of the gubernaculum and esophageal valves, have allowed a more detailed description of this nematode taxonomy.

1. Introduction

The taxonomic position of the *Angiostrongylus* genus is based both on morphological characteristics, such as the rays of the copulatory bursa, and on its host group specificity and the sites where the adult worms reside within the host. However, the best parameters for characterizing helminth species are morphological and morphometric characteristics. *Angiostrongylus* genus was separated into two subgenera: *Angiostrongylus* and *Parastrongylus*. The major taxonomic parameter used in this stratification was the morphology of the lateral rays of the caudal bursa. *Angiostrongylus* has a ventrolateral ray that arises independently from the mediolateral and posterolateral rays, which emerge as a single trunk (Drozdz, 1970).

Angiostrongylus costaricensis is an intestinal parasitic nematode of wild rodents, with the adult worm residing within mesenteric arteries or their branches. The parasite may accidentally infect humans, causing abdominal angiostrongyliasis (Morera, 1988), a widespread and poorly studied human disease in Latin America (Hulbert et al., 1992; Incani et al., 2007; Palominos et al., 2008; Ubelaker and Hall, 1979). The life cycle of *A. costaricensis* is characterized as heteroxenic and requires two hosts: a vertebrate (the definitive hosts are rodents) and an invertebrate (the intermediate hosts are slugs and terrestrial mollusks). The first stage larvae (L1) are expelled in rodent feces, infecting mollusks orally (Morera, 1973) and/or percutaneously (Mendonça et al., 1999; Thiengo, 1996). After two molting events, the larvae (L3) become infective. When rodents ingest mollusks infected with L3, the larvae penetrate the intestinal epithelium, closing the parasite life cycle.

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Humans can also be infected by the ingestion of L3 contaminated fruits, vegetables, raw food or water. Larvae migrate from the blood and lymphatic vessels to the heart, reaching the arterial circulation and subsequently the mesenteric arteries, where the parasites mature into the adult stage. The eggs are then deposited in the mesenteric arteries and are carried through the blood to the capillaries of the intestinal wall, where L1 will emerge to be expelled in feces.

A. costaricensis was first described in Costa Rica (Morera and Céspedes, 1971), and the details of its morphology during the life cycle were redescribed using light microscopy by Morera (1973) and Thiengo et al. (1997). Additional characteristics of the external surface architecture of the different developmental stages of this parasite were subsequently revealed by electron scanning microscopy (SEM) (Ishih et al., 1990). In the present work, we employed light microscopy and SEM adding novel morphological features of *A. costaricensis*. In this study, the use of SEM, complements descriptions of *A. costaricensis* by allowing better characterization of taxonomically significant structures, including the number and distribution pattern of papillae, spicules and accessory structures supporting the cuticular layer. The combination of data obtained using both light and scanning electron microscopy is important for generating a more precise species identification and the confirmation, description and/or re-description of species of nematodes.

2. Materials and methods

2.1. Parasites

A. costaricensis specimens in different developmental stages were maintained at the Laboratório de Patologia of Instituto Oswaldo Cruz (FIOCRUZ) through successive passage in snails (*Biomphalaria glabrata*) and rodents (*Sigmodon hispidus*). Adult worms were recovered by dissecting the mesenteric arteries of rats 40 days post-infection. The worms were then separated by gender.

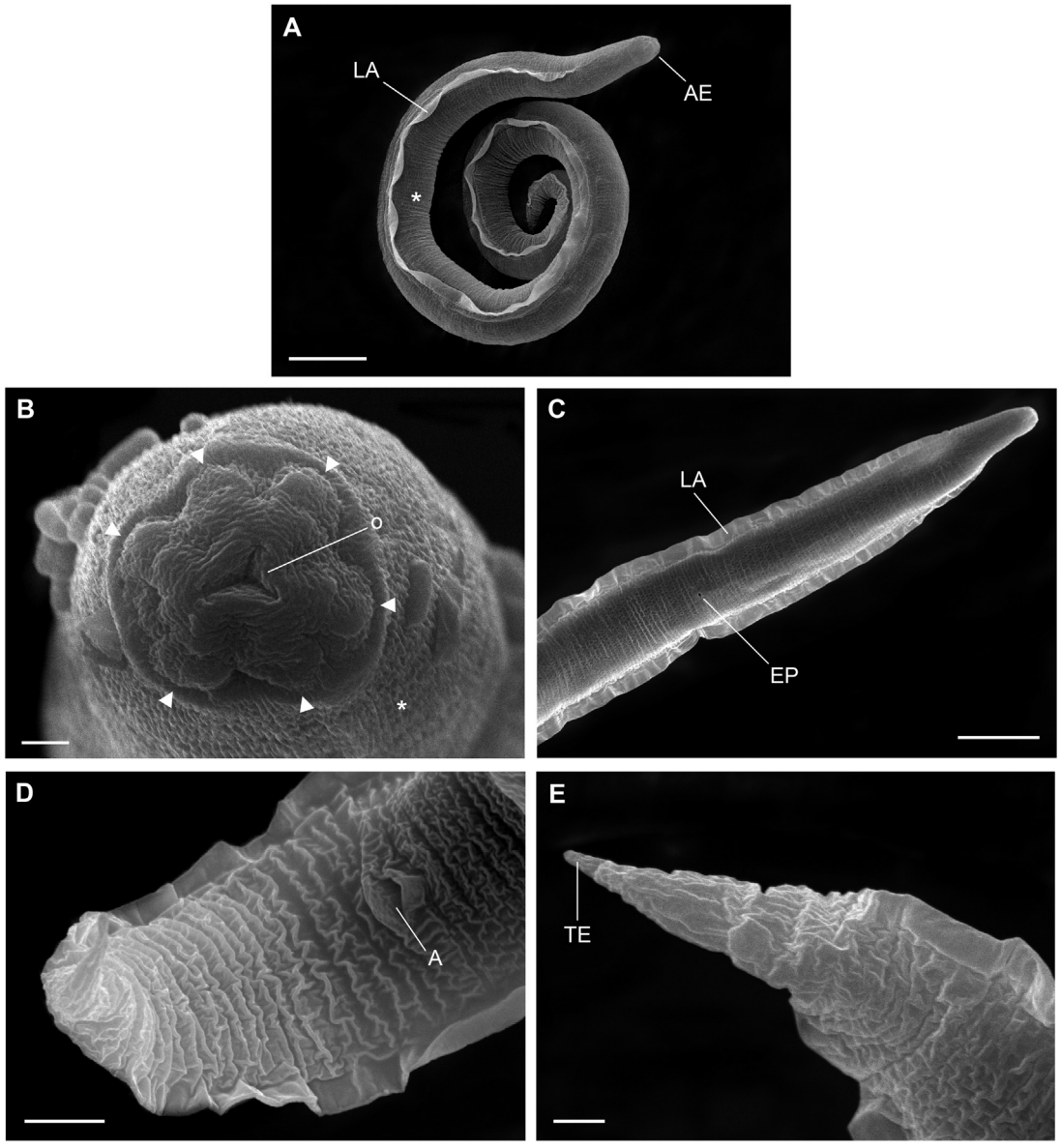

Fig. 1. SEM analysis of the L1 stage of *A. costaricensis*. (A) The whole body of the larva showing the anterior extremity (AE), the lateral alae (LA) and striations (*). Scale bar: 10 μm; (B) details of the anterior extremity showing the six cephalic papillae (head arrow) around the oral opening (O). Scale bar: 1 μm. (C) Ventral view of the larva showing the excretory pore (EP) and the lateral alae (LA) on both sides. Scale bar: 10 μm. (D) Ventral view of the posterior end showing the anus (A). Scale bar: 2 μm; (E) details of the extremity of the tail (TE). Scale bar: 1 μm.

L1 larvae were obtained from the feces of infected rodents and were passaged through a discontinuous Percoll gradient (Graeff-Teixeira et al., 1999) to separate L1 larvae from small debris and bacteria. L3 larvae were collected from mollusks previously infected with L1 larvae.

2.2. Ethics

All procedures with animals were approved by the Animal Ethics Committee at Fiocruz (CEUA license # P0246/05) and conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences.

2.3. Preparation for scanning electron microscopy and light microscopy

For scanning electron microscopy, the parasites were fixed for 24 h at 60 °C in AFA (2% glacial acetic acid, 3% formaldehyde, and 95% ethanol) or at 25 °C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Samples were post-fixed for 1 h with 1% OsO_4, 0.8% potassium ferricyanide and 2.5 mM $CaCl_2$. The parasites were dehydrated in a crescent ethanol series (30–100%), dried using the critical point method with CO_2, mounted on aluminum stubs coated with a 20 nm gold layer, and examined with a Jeol JSM6390LV scanning electron microscope (Tokyo, Japan).

In addition, some adult worms were fixed at 25 °C overnight in Milloning's solution and stained with alcoholic chloride carmine at the same temperature for 24 h. Samples were dehydrated in a crescent ethanol series, clarified in Faia's creosote, mounted in Damar resin and analyzed by light microscopy using a Zeiss Axio Observer Z1 (Oberkochen, Germany).

3. Results

3.1. Larval stages

L1 larvae have a filiform body and are covered by a transversely striated cuticle (Fig. 1A). At the anterior end, we observed a triangular oral opening surrounded by six cephalic papillae (Fig. 1B). Broad, lateral double alae extended nearly the entire length of the larval body, from the cephalic extremity to the posterior region, terminating just before the tail (Fig. 1C). An excretory opening was located on the ventral side of the body (Fig. 1C). The anus was located on the ventral surface near the tip of the tail (Fig. 1D). The posterior end was ventrally curved and slender, and the tail ends were sharply pointed (Fig. 1E).

L3 larvae had slightly larger diameters than L1 larvae. Overall body shape was similar (Fig. 2A), but the tails were conical (Fig. 2C), and the lateral alae were thicker and shorter (Fig. 2A, C and D). The six L3 larval cephalic papillae were more widely separated than those of L1 larvae (Fig. 2B). The excretory pore (data not shown) and the anus were observed on the ventral surface, with the latter adjacent to the tip of the tail (Fig. 2D).

3.2. Adult worms

Adult worms exhibited distinct sexual dimorphisms, but both had elongated and slender bodies. Female specimens were larger and more robust than males. The oral aperture was directly connected to the claviform-shaped esophagus (Fig. 3A). We observed cylindrical valves at the esophageal–intestinal junction (Fig. 3B). Furthermore, an excretory pore was observed on the ventral surface near the anterior end (data not shown). In both sexes, the anterior end was round with a circular

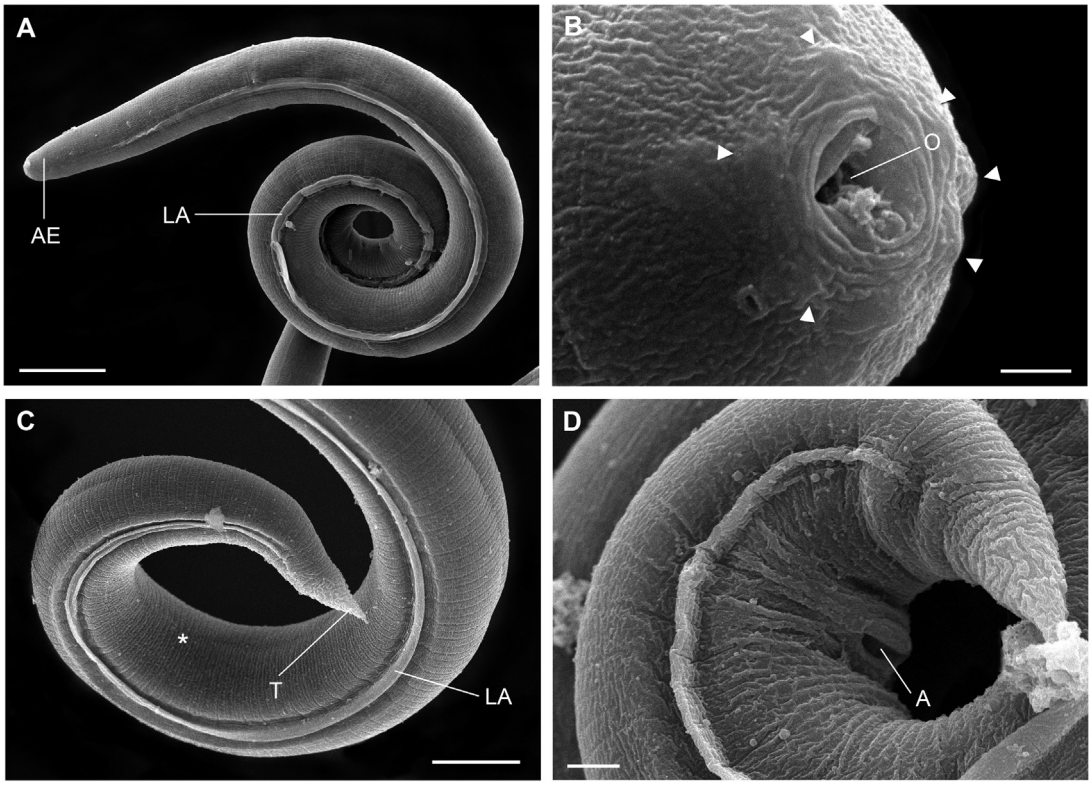

Fig. 2. SEM analysis of the L3 stage of *A. costaricensis*. (A) The whole body of the larva showing the anterior portion (AE) and the lateral alae (LA). Scale bar: 20 μm. (B) Details of the anterior extremity showing the six cephalic papillae (head arrow) surrounding the oral opening (O). Scale bar: 1 μm. (C) Posterior portion showing striations (asterisk), the lateral alae (LA) and the tail (T). Scale bar: 10 μm. (D) Posterior end showing the anus (A) next to the tail. Scale bar: 2 μm.

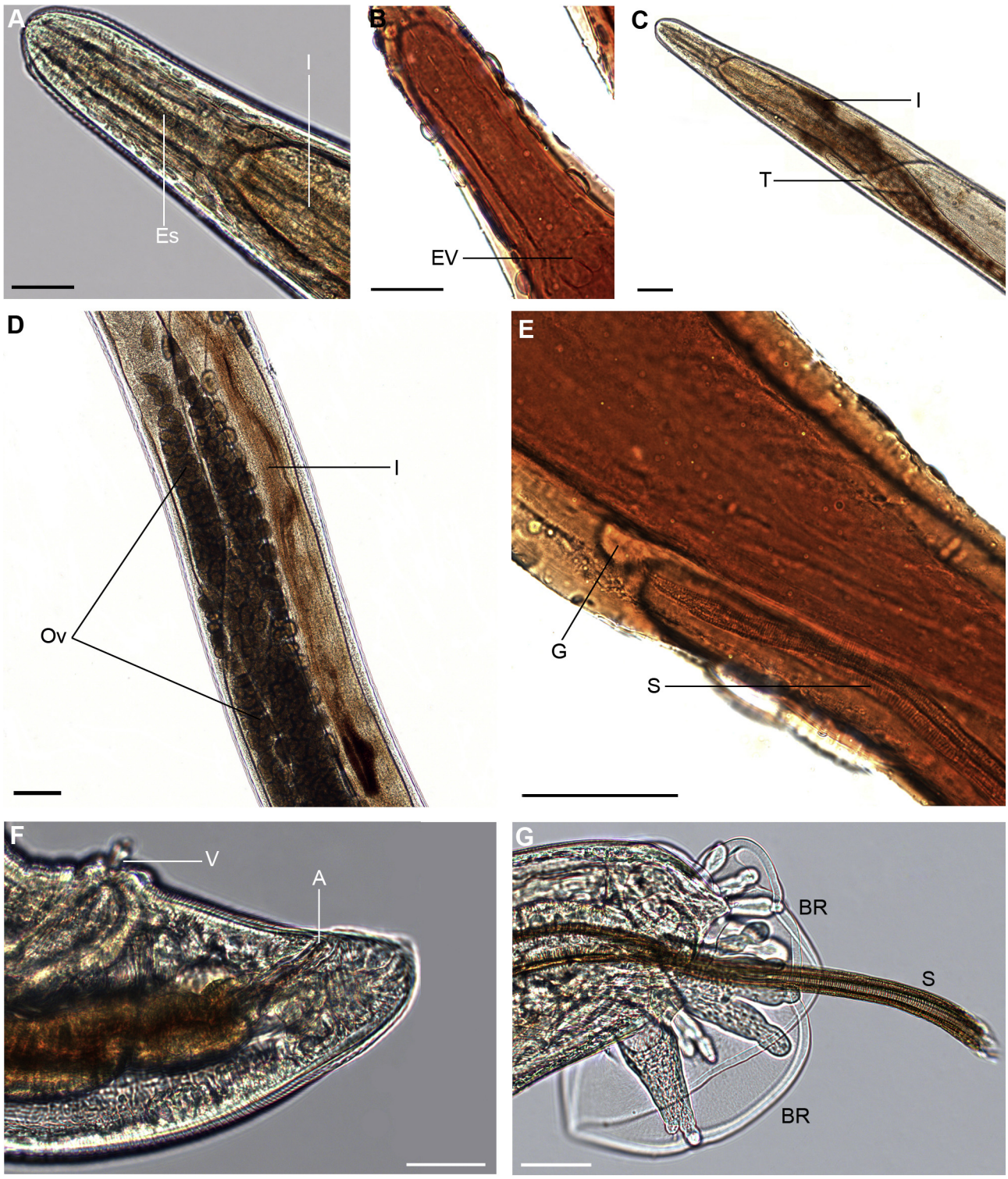

Fig. 3. Light microscopy analysis of *A. costaricensis*. (A) Anterior portion showing the esophagus (Es) and intestine (I) of the adult worm. Scale bar 500 μm. (B) Anterior portion showing the esophageal valves (EV) between the esophagus and the intestine of the adult worm. Scale bar 500 μm. (C) Anterior portion of a male adult worm showing the intestine (I) and the beginning of the testis (T). Scale bar 500 μm. (D) Lateral view of the female showing the two tubular ovaries (Ov) spiraling around the intestine (I). Scale bar 500 μm. (E) Male posterior portion showing the gubernaculum (G) and the beginning of the spicules (S). Scale bar 500 μm. (F) Lateral view of the posterior portion of the female adult worm showing the vulva with the flap (V) and the anus (A). Scale bar 500 μm. (G) Male posterior end with the copulatory bursa showing the bursal rays (BR) and a visible spicule (S). Scale bar 500 μm.

oral opening (Fig. 4A), and the three lips around the mouth were surrounded by six sensory papillae (Fig. 4B). Additionally, two amphidial pores were located on either side of the oral aperture (Fig. 4B). The cuticle along the body was transversally striated, and longitudinal lateral alae were absent (Fig. 4A and C).

The female reproductive system was tubular and consisted of two ovaries, each of which connected to an oviduct and a uterus (Fig. 3D). The two uteri joined to form the vagina, which opened to the exterior by a cuticle-covered vulva (Figs. 3F and 4C). The posterior end was ventrally bent, roughly conical, and had a terminal projection (Fig. 4C and D).

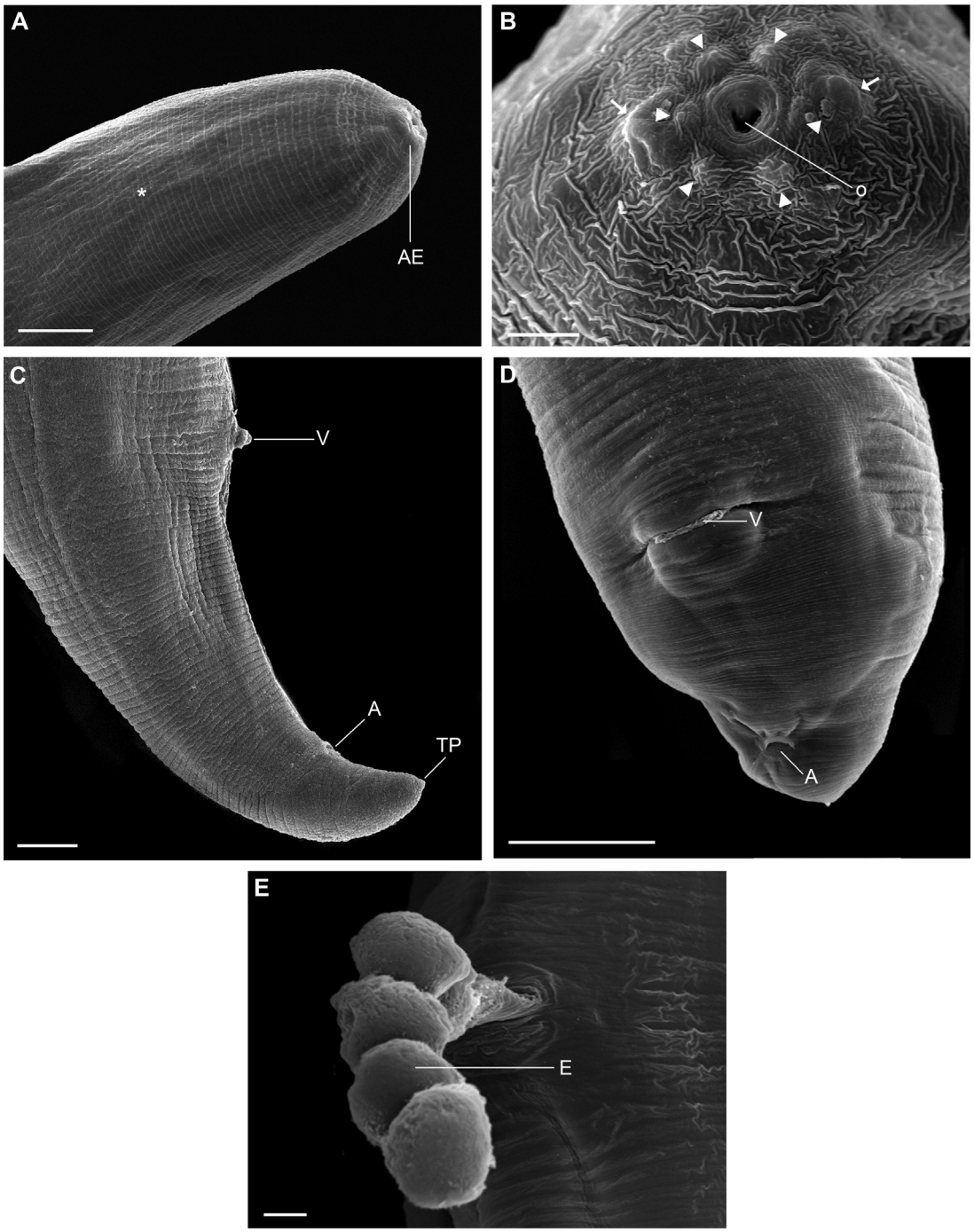

Fig. 4. SEM analysis of the adult female *A. costaricensis*. (A) Anterior portion showing the anterior extremity (AE) and the cuticle with transversal striations (*). Scale bar: 20 μm. (B) Anterior extremity showing six cephalic papillae (head arrow) and two amphidial pores (arrow) surrounding the oral opening (O). Scale bar: 2 μm. (C) Lateral view showing the vulva (V), anus (A) and a projection at the end of the tail (TP). Scale bar: 20 μm. (D) Ventral view of the posterior portion showing the vulva (V) and the anus (A). Scale bar: 50 μm. (E) Vulva opening with eggs. Scale bar: 10 μm.

The male reproductive system consisted of a single tube differentiated into testis (Fig. 3C), which lay at the free end of a convoluted or re-curved tube. This tube led into the seminal vesicle and vas deferens and terminated in the muscular ejaculatory duct that emptied into the cloaca (Fig. 5B). Behind the cloacal opening, there were three papillae (Fig. 5A and B). A pair of spicules guided by gubernaculum (Fig. 3E) protruded through the cloacal opening (Fig. 5C). The two spicules were slender and striated, had sharply pointed distal ends, and were not projected (Fig. 5E). Muscular movements and body contractions exposed the spicules (Fig. 5D). The posterior end of the male *A. costaricensis* worm presented a copulatory bursa (Fig. 5A–D). The bursa cuticle was supported by finger-like structures, referred to as bursal rays, which can be associated with the muscle layer. In this nematode, the ventral bursal rays were fused (Fig. 5F) except at the tips. Ventrolateral rays were slightly longer than ventroventral rays. The posterolateral and mediolateral rays were fused in their proximal half and were separated from the externolateral rays after their emergence, forming a common trunk. The dorsal bursal ray was short and terminated in tips.

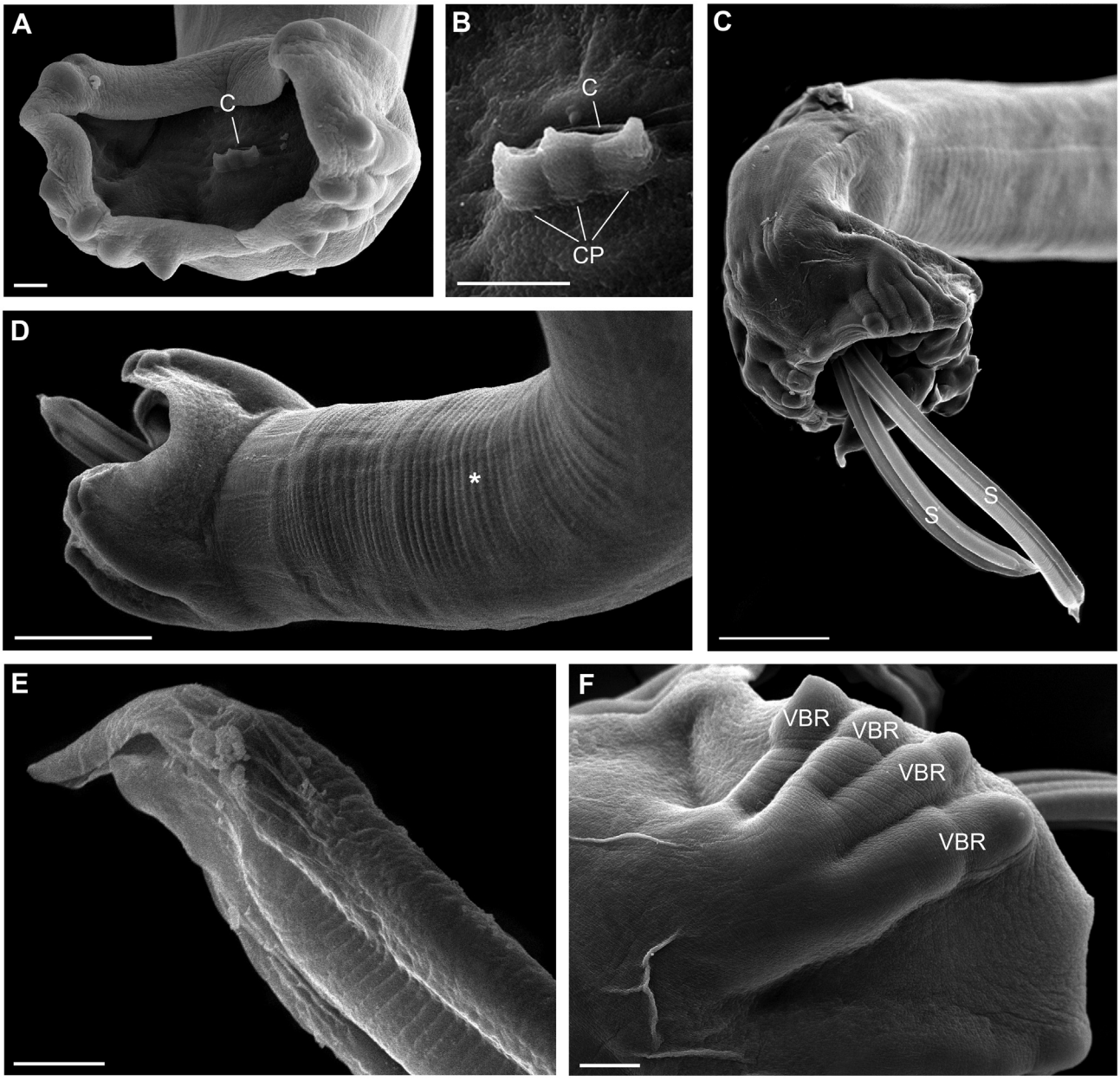

Fig. 5. SEM analysis of the adult male *A. costaricensis*. (A) Copulatory bursa showing the cloaca (C). Scale bar: 10 μm. (B) Details of the cloacal opening (C) with three papillae (CP). Scale bar: 5 μm. (C) Copulatory bursa with the projection of two spicules (S). Scale bar: 50 μm. (D) Details of the anterior end showing body contractions (asterisk). Scale bar: 5 μm. (E) Details of the spicule with a striated flange and a pointed end. Scale bar: 5 μm. (F) Details of the copulatory bursa of the ventral bursal rays (VBR). Scale bar: 10 μm.

4. Discussion

Morphological studies of helminths are important tools for taxonomic analyses. The present study reports novel relevant taxonomic features of *A. costaricensis*, a lungworm parasite of the mesenteric veins of rodents and humans (Morera and Cespedes, 1971a).

Variability in the number and distribution of cephalic sensory organs in the vast majority of nematodes species is useful for taxonomic classification. Ishih et al. (1990) used SEM techniques to describe six cephalic papillae lying in two rows around the oral opening in both adult worms and larval stages. In the present study, SEM revealed that there were only six sensory papillae around the mouth and two amphidial pores in adult worms. The number and arrangement of cephalic papillae in *A. costaricensis* adult worms are similar to those detected in *A. cantonensis* (Lian-Yin et al., 1984). Our results analyzing larval stages, only the presence of six cephalic papillae surrounding the mouth was observed. Lian-Yin et al. (1984) reported that *A. cantonensis* larval stages have two rows of six cephalic papillae.

We additionally demonstrated in this study that the mouth of the *A. costaricensis* adult worm opens into a buccal capsule, where food is moved into the esophagus. Unlike most other nematodes, the buccal capsule is cylindrical and lacks a bulb at its posterior end (Roberts and Janovy, 2000). The esophagus is connected to the intestine via an esophageal–intestinal valve, a muscular structure usually referred to as the cardia. These structures exhibit different functions, such as regulating the rate or direction of food intake to the intestine, providing secretory material for extra- or intra-corporeal digestion, or possibly for lubrication,

as has been previously described in others nematodes including *Ostertagia bison* and *Pseudomarshallagia enlongata* (Hoberg et al., 2010).

The female posterior end was ventrally bent, roughly conical, and had a terminal projection (Fig. 4C and D). Morphometric variation is quite common among nematodes and may occur due to the number of specimens parasitizing the same niche in host tissues or organs or even as a result of climatic and environmental conditions where the host lives. In some species of *Angiostrongylus*, variation in the distance between the vulva and anus has been reported. Dias et al. (2008) reported that this distance is 68 μm in non-gravid females and 144 μm in gravid females. This discrepancy in distance is visible in drawings and micrographs and in the present work, we used an experimental model of infection that allows us to ensure that only a single species of nematode is examined. A similarly conspicuous spine at the tip of the tail has been previously observed only in *A. mackerrasae* (Morera and Cespedes, 2002).

Our SEM results also revealed cuticular membranes situated at both ends of the vulva. This structure is very common in some nematode groups as Desmodoroidea, Mermithida, Oxyuroidea, Tylenchida, Rhabditida, and Trichostrongyloidea (Chitwood and Chitwood, 1974), and it is named vulvar flap. It is thought that the flap functions to ensure fertilization once the vulvar aperture has closed, similar to the cementum found in other nematodes. Among plant or insect parasites, vulvar flaps have primarily been described in *Tylenchida* and *Aphelenchida* (Nickle, 1970), and among animal parasites, they have been described in *Ostertagiinae* (Durette-Desset et al., 1999; Hoberg and Lichtenfels, 1994). This study represents the first description of vulvar flaps in *Angiostrongylus* species.

As previously mentioned, the position, number, and morphological characteristics of the rays of the copulatory bursa are important taxonomic parameters for identifying *Angiostrongylus* species. Our light microscopy data are in agreement with initial descriptions (Morera and Cespedes, 1971a). The well-developed copulatory bursa is slightly asymmetric, and the dorsal ray is short and bifurcated into arms that terminate in sharp tips. On the ventral side behind the bifurcation, there is a conspicuous papilla. The lateral rays emerge from a common trunk and are widely separated from the ventral rays. The mediolateral and posterolateral rays are fused at the proximal half. The anterolateral ray is thicker and separates from the common trunk immediately after its emergence from the trunk. The externodorsal ray arises adjacent to the lateral trunk and is well separated from the dorsal ray. Its distal end is knoblike. The ventral rays are fused except at the tips, and the ventrolateral ray is slightly longer than the ventrolateral ray (Morera and Céspedes, 1971).

Using light microscopy, previous reports described three papillae behind the cloacal opening (Morera and Céspedes, 1971; Thiengo et al., 1997). However, this is the first report of these structures in SEM. The gubernaculum is a sclerotized accessory element of the male reproductive system. Male specimens of *Angiostrongylus* spp. display two branches that come together just prior to termination in the cloaca, and their function is to guide spicules during copulation. The presence of the gubernaculum was previously detected in males of *A. costaricensis* (Morera and Cespedes, 1971a; Thiengo et al., 1997) and was confirmed here by SEM.

In the present work, *A. costaricensis* spicules were shown to be slender and similar in size to those previously described (Morera and Cespedes, 1971b; Thiengo et al., 1997). By SEM, we confirmed the striated nature of the spicules and additionally raise a novel hypothesis regarding the projection of the spicules during copulation: the spicules are associated with muscle attached to the body of the worm, and muscle contraction leads to spicule extrusion.

The use of both light microscopy and SEM allowed for a detailed analysis of the morphology of this nematode. A number of taxonomic features were confirmed, and some additional characteristics were documented for the first time by SEM, including a more accurate count of papillae distributed around the oral opening and identification of papillae behind the cloacal opening. The light microscopy observation of esophageal valves is a novel and relevant feature for the taxonomy of this species.

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