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Study of Helminth Parasites of Amphibians by Scanning Electron Microscopy

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1. Introduction

Amphibians, like all other animals, are subject to a variety of parasites and diseases, including viral, bacterial and fungal infections as well as some forms of cancer and tuberculosis (Hoff et al., 1984). Various viruses and bacteria such as *Pseudomonas* or *Salmonella*, and fungi such as *Candida*, are recorded as common infectious agents in amphibians, but currently the focus of studies are the fungi of genus *Batrachochytrium*, agents of the disease known as chytridiomycosis, which is considered as one of the factors responsible for the decline of amphibian populations in many parts of the world (Berger and Speare, 1998). In addition, protozoans of the genera *Opalina* and *Entamoeba* in the digestive tract, and trypanosomes in the circulatory system, as well as coccidian protozoa have been recorded in amphibians (Duellman and Trueb, 1986; Duszynski et al., 2007).

However, helminths are the most common invertebrate parasites of amphibians. One well known example among trematodes is the monogenean genus *Polystoma*, which infects the urinary bladder of adult amphibians around the world. Parasitic digenean trematodes include both larval stages (metacercariae) and adults. Cestodes are not common parasites in the gastrointestinal tract of amphibians, but when present may persist for a long time. Adult acanthocephalans adhere to the mucosa of the stomach or intestine. Finally, nematodes are particularly abundant in the digestive tract, lungs and blood vessels of these vertebrates (Pough et al., 2001). Amphibians are also hosts to other groups of less common parasitic invertebrates, such as annelids, pentastomids and arthropods (copepods, ticks, insects) (Tinsley, 1995).

Of these, analyses using scanning electron microscopy techniques have been mainly applied to digenean trematodes (flatworms), nematodes (roundworms) and acanthocephalans (thorny or spiny headed), particularly to their adult stages (Fig. 1-3).

The study of parasitic nematodes and trematodes by scanning electron microscopy began in the 1970s and involved mainly those organisms that produced diseases in humans and livestock, as well as parasites of crops (Halton, 2004). In particular, studies in amphibian hosts were first made by Nollen and Nadakavukaren (1974) and Nadakavukaren and

Nollen (1975) who provided details of the tegument of the trematodes *Megalodiscus temperatus* (Paramphistomatidae) and *Gorgoderina attenuata* (Gorgoderidae) from *Rana pipiens*. Regarding nematodes, Navarro et al. (1988) provided details of the cuticle of the species *Cosmocerca ornata*, *Oxysomatium brevicaudatum* (Cosmocercidae) and *Seuratascaris numidica* (Ascarididae) collected in ranid hosts from different geographical areas of the Iberian Peninsula.

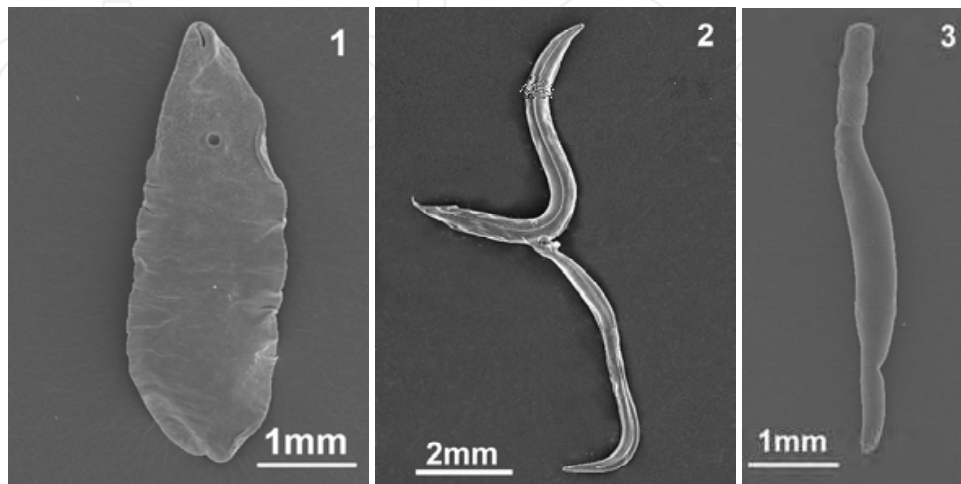


Fig. 1-3. Helminth parasites found in amphibian hosts. 1. Flatworms (Trematoda), general view. 2. Roundworms (Nematoda) male and female mating. 3. Thorny or spine headed (Acanthocephala), general view.

This chapter presents scanning electron micrographs taken during diverse studies undertaken to determine the helminth fauna of Argentinean anurans, especially those living in Northeastern Argentina. The survey includes the classes Trematoda (specifically subclass Digenea) and Nematoda (specifically subclass Secernentea) and the phylum Acanthocephala. A total of five families of amphibians (Bufonidae, Cycloramphidae, Hylidae, Leptodactylidae, Leiuperidae) were analyzed, both at larval (tadpole) and adult stages, to study their helminth parasites. At the end of the chapter we present a summary of the present-day advances in this topic, including new contributions presented in this work; finally, we discuss possible future lines of research in this field of Parasitology.

The classification of helminths follows Anderson et al. (2009) and Gibbons (2010) for class Nematoda; Gibson et al. (2002) and Jones et al. (2005) for class Trematoda and Amin (1985) for Acanthocephala.

2. Preparation of helminth parasites of amphibians for observation by scanning electron microscopy

2.1 Collection of hosts and obtaining of helminth parasites

Adult frogs were hand captured, mainly at night, using the sampling technique defined as visual encounter survey (Crump and Scott, 1994). The individuals were transported live to the laboratory and killed in a chloroform solution (CHCl_3). The abdominal cavity of each frog was opened and the oesophagus, stomach, gut, lungs, liver, urinary bladder, kidneys, body cavity, musculature, integument and brain examined for parasites under a dissecting

microscope (Fig. 4). Tadpoles were captured with a 45-cm-diameter dip net and kept alive in the laboratory until their dissection. They were killed using a chloroform solution, and subsequently all organs, musculature and body cavity were examined for parasites.



Fig. 4. Ventral view of adult amphibian showing all organ systems.

The analyzed amphibian species were: *Rhinella bergi*, *R. fernandezae*, *R. granulosa*, *R. schneideri* (Bufonidae), *Odontophrynus americanus* (Cycloramphidae), *Dendropsophus nanus*, *D. sanborni*, *Hypsiboas raniceps*, *Pseudis limellum*, *Phyllomedusa hypochondrialis*, *Scinax acuminatus*, *S. nasicus*, *Trachycephalus venulosus*, *Pseudis paradoxa* (Hylidae), *Physalaemus albonotatus*, *P. santafecinus*, *Pseudopaludicola boliviana*, *P. falcipes* (Leiuperidae), *Leptodactylus bufonius*, *L. chaquensis*, *L. elenae*, *L. latinasus*, *L. latrans*, *L. podicipinus* (Leptodactylidae), *Lepidobatrachus laevis* (Ceratophryidae). Anurans were identified using different guides and keys (Faivovich et al., 2005; Frost et al., 2004).

2.2 Procedure applied to helminth. Complications

In 1972, Allison et al. proposed a simplified four-step procedure that resulted in excellent preservation, support in the high vacuum and dissipation of surface charging of nematodes. The procedure involved: fixation, dehydration, treatment with an antistatic agent and gold-palladium coating. These authors obtained best results using fixation with 4% paraformaldehyde (phosphate-buffered) and AFA (acetic acid-formalin-alcohol); specimens were dehydrated in an ascending series of ethanol solutions to 70%, then transferred to 5% glycerine-95% ethanol from which the alcohol was allowed to evaporate, and cleared in 96.6% glycerol-0.05% potassium chloride-3.35% distilled water, 24 to 48 hours prior to examination. Subsequently, specimens were mounted on metal specimen stubs with Duco cement, outgassed in a vacuum evaporator for 1 hour or more and rotary-coated with gold-palladium.

In this study we basically followed the aforementioned procedure, with some modifications. Helminths were observed *in vivo*, counted, fixed and stored. Adult nematodes and trematodes were fixed in hot 4% formaldehyde; larval nematodes and trematodes were

removed from cysts with the aid of preparation needles and fixed in hot 4% formaldehyde; acanthocephalan larvae were placed in distilled water for 24 hours at 4° C for the proboscis to evert, and then fixed in hot 4% formaldehyde.

Another technique used for the study of nematodes and acanthocephalans by SEM consists of transferring specimens for 2 hours into 1% osmium tetroxide and dehydrate them in an ethanol series for 2 hours in each bath (Mafra and Lanfredi, 1998). In the case of trematodes, fixation can be made with paraformaldehyde, glutaraldehyde or a formaldehyde-glutaraldehyde mix such as Karnovsky's fixative in phosphate or cacodylate buffer. Postfixation is usually done for 2 to 3 hours at 4°C with cacodylate- or phosphate- buffered 1% osmium tetroxide (Karnovsky, 1965).

For processing of helminths, it should be taken into account that a hypertonic solution will cause shrinkage and almost complete disappearance of inflation in specimens, whereas a hypotonic solution may produce artificial inflations. In some cases, in spite of careful processing of samples for SEM study, good results are not achieved. Very frequently, the samples contain bacteria or debris, or tears of the cuticle of nematodes or tegument of trematodes (Fig. 5-9).

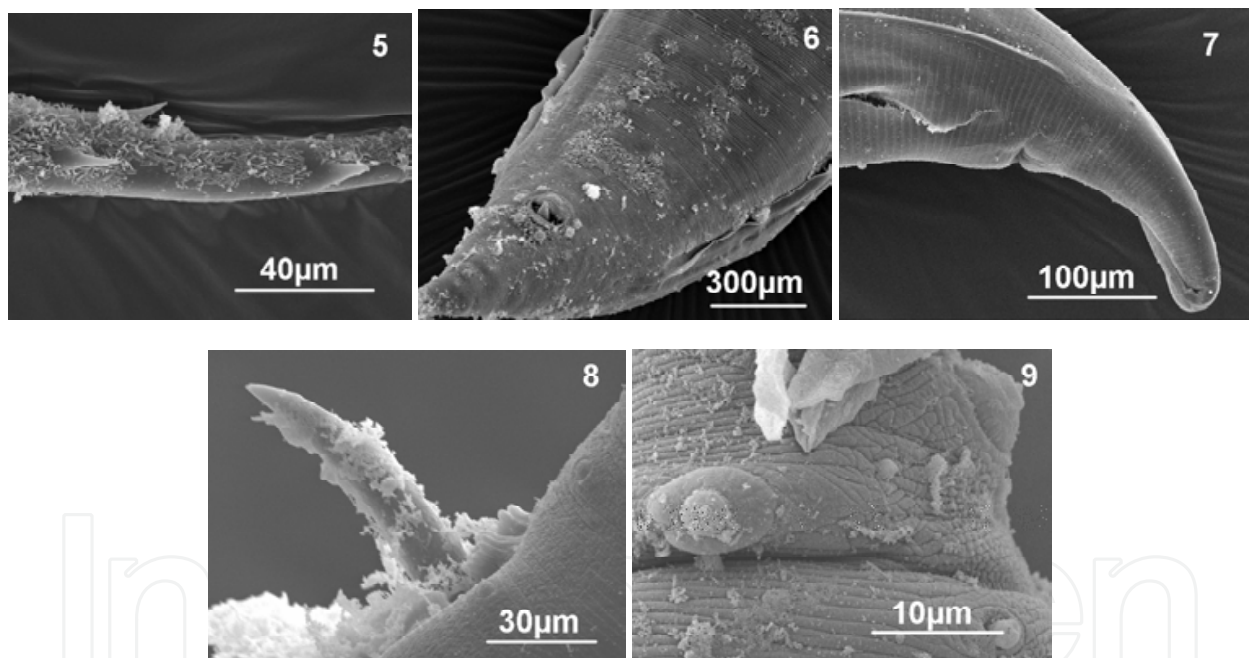


Fig. 5-9. Some complications in samples for SEM studies. 5. 6. Bacteria in posterior end of *Skrjabinodon* sp. (5) and in ventral surface of *Cosmocerca parva* (6). 7. Broken cuticle in posterior end of female of *Aplectana* sp. 8. 9. Debris in spicules and adanal papillae of *Cosmocerca podicipinus* (8) and in posterior end of *Falcaustra* sp. (9).

2.3 Characteristic of the scanning electron microscope used for this study

The Microscopy department at Universidad Nacional del Nordeste possesses a Jeol 5800LV scanning electron microscopy. Specimens are critical-point dried using a Denton Vacuum DCP-1 critical point drying apparatus, and sputter-coating is made using a Denton Vacuum Desk II sputter-coating unit.

For examination by scanning electron microscopy (SEM), specimens were dehydrated through an alcohol 70° and acetone series (70%, 85% and 100%; 15 minutes in each solution) and then subjected to critical point drying using CO₂; in the case of larval digenean trematodes, critical point drying time is shortened because these individuals are more fragile than adults. Samples were mounted on a metal sheet of copper or aluminum using double-sided tape. Then the specimens were sputter-coated with gold or gold-palladium for three minutes. Helminth parasites were observed in high vacuum in all cases.

Adequate fixation contributes to specimen preservation and stability within the microscope; dehydration allows outgassing and drying of specimens in the vacuum evaporator prior to coating without subsequent "bubbling" or shrinkage artifacts; glyceration provides an antistatic surface coating over all surface irregularities, even those which might not be adequately coated with 200 Å of gold-palladium. The thin metal coating does not obscure fine structural detail but, in combination with the glycerol-KCl coating, ensures that the specimen can be exposed for extended periods of time to the electron beam without surface changes (Allison et al., 1972).

3. What can SEM studies tell us about helminth parasites of amphibians?

3.1 Nematoda

The body surface of nematodes is covered by a truly inert cuticle of extracellular material in the form of cross-linked collagens and insoluble proteins that are synthesised and secreted by the underlying epidermis (= hypodermis). In addition to proteins and collagen, the cuticle possesses glycoproteins, fibrin and keratin. It consists of three main layers and the epicuticle. The external layer is divided into internal and external cortex; the middle layer varies from having a granular uniform structure to presenting skeletal rods, fibers or channels; the internal basal layer can be laminated or grooved. The thin epicuticle may have a coating of quinone (Lee, 2002).

The above discussion refers specifically to the cuticle of the external body surface. In other body parts, such as the buccal cavity, excretory pore, vagina, cloaca and rectum, as well as the eggs and spicules in males, the cuticle is of a different nature.

The particular composition of the cuticle along the whole body of nematodes, and especially the ornamentations that they present in the anterior and posterior extremity, are the focus of primary study of these helminths by scanning electron microscopy.

The nomenclature for the structures detailed here is based on Chitwood and Chitwood (1975), Gibbons (1986) and Willmott (2009). We describe the modifications of the cuticle along the body surface, at the anterior and posterior body ends, and finally the cuticle of the eggs, vulva and spicules of these helminths.

3.1.1 Cuticle of body

The features analyzed along the body surface are the morphology and disposition of somatic papillae as well as the striation of the cuticle -longitudinal, transverse or oblique-, and the presence of annulations, punctuations, longitudinal ridges, alae -lateral, cervical or caudal-, inflation and spination.

There are several types of gross cuticular markings, namely, *transverse*, *longitudinal* and *oblique* markings. Of these, *transverse* and *longitudinal* markings are very common in nematode parasites of amphibians.

Transverse markings: the superficial markings are grooves or ridges. *Striations* are defined as fine transverse grooves occurring at regular intervals; the distance between two striae is the *intestrial* region (*Aplectana hylambatis*, *Cosmocerca* spp.) (Figs. 10, 11). Deep striae are very commonly and are known as *annulations*; the distances between them are termed *annules* (*Aplectana delirae*, *Falcaustra* sp.) (Figs. 12, 13). In nematode parasites of the family Pharyngodonidae, annulations are much broader and more prominent (Figs. 14, 15, 34).

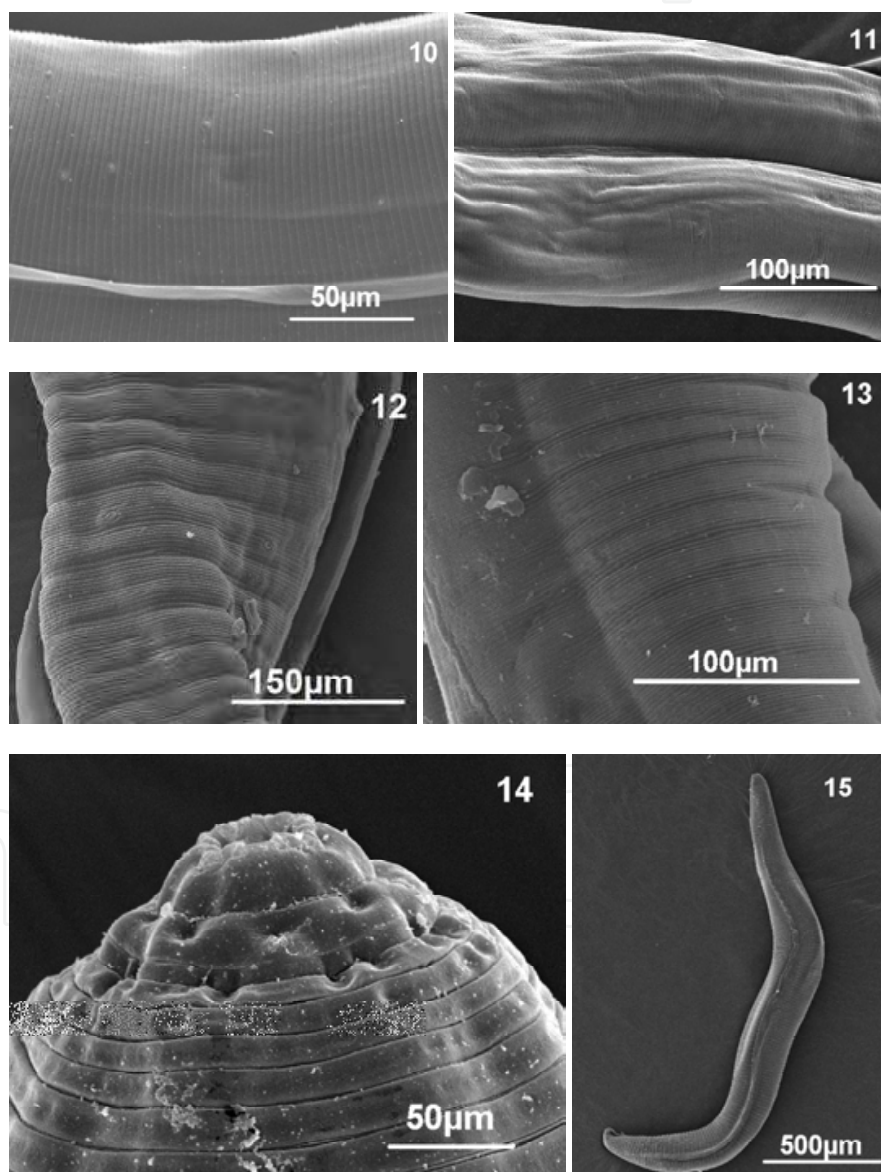


Fig. 10-15. Transverse marking in cuticle of nematode parasites of amphibians. 10. 11. Striations (*Aplectana hylambatis*, *Cosmocerca podicipinus*, respectively). 12. 13. Striations and annules (*A. delirae*, *Falcaustra* sp., respectively). 14. 15. Annulations (*Skrjabinodon* sp.).

Striations can be distributed uniformly along the whole body of the parasite (*Cosmocerca* spp.), or they may become less evident (*A. delirae*) or wider (*A. hylambatis*) at the body ends, or more marked, as for example, in the posterior body (*Skrjabinodon*) (Figs. 10-15).

Longitudinal markings: these may take the form of ridges or alae, or they may be merely the result of gaps in transverse markings.

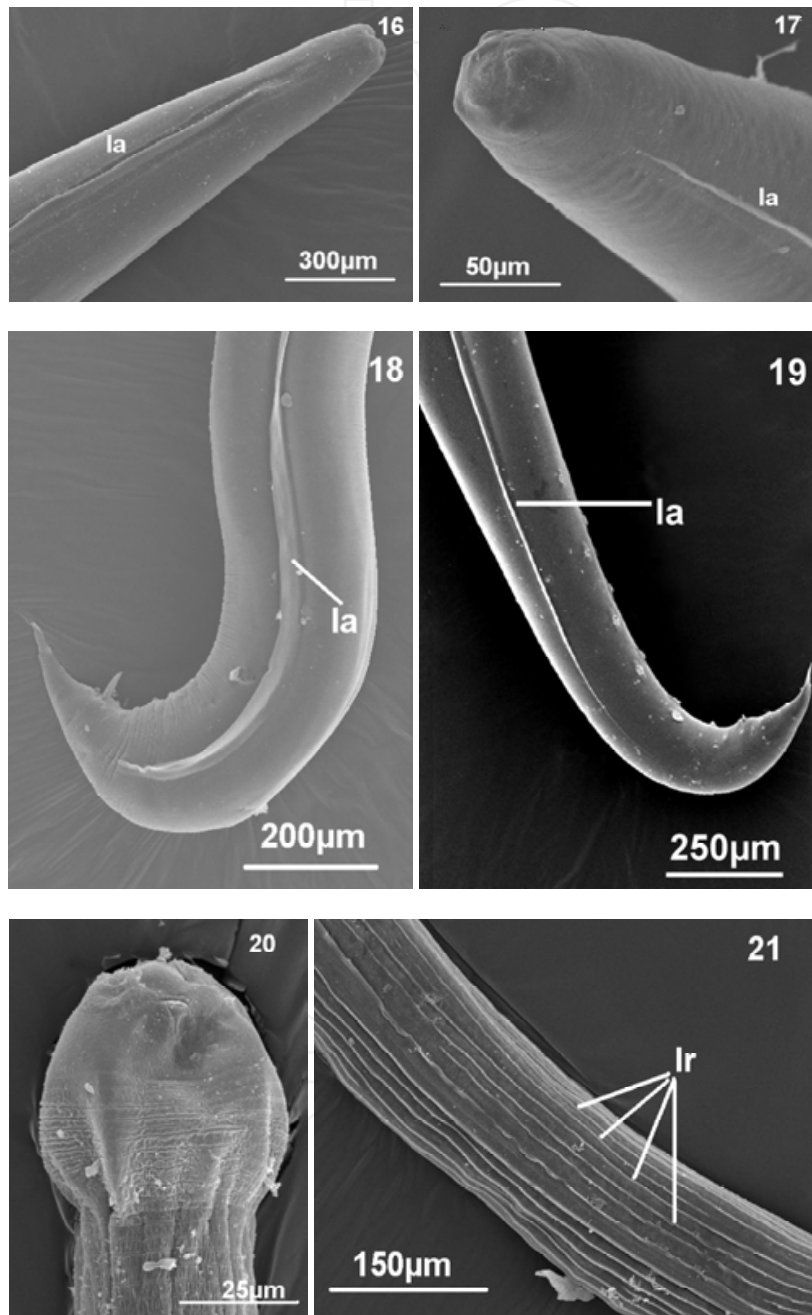


Fig. 16-21. Longitudinal markings in nematode parasites of amphibians. 16. 17. Lateral alae in anterior end of body in *Paraoxyascaris* sp. (16) and in *Porrocaecum* sp. (larvae) (17). 18. 19. Lateral alae in posterior end of body in *Cosmocerca* spp. 20. Cephalic cuticular vesicle in *Oswaldocruzia* sp. 21. Longitudinal ridges in *Oswaldocruzia* sp. la: lateral alae; lr: longitudinal ridges.

Alae: these are usually lateral or sublateral cuticular thickenings or projections. There are three types of alae: longitudinal, cervical and caudal.

Longitudinal alae: these are usually lateral or sublateral and occur in both sexes; extending along the length of the body. They occur in the families Cosmocercidae (*Aplectana*, *Cosmocerca*, *Cosmocercella*, *Paraoxyascaris*) (Figs. 16, 18, 19), Ascarididae (*Ortleppascaris*, *Porrocaecum*) (Fig. 17); in *Gyrinicola* sp. (Family Pharyngodonidae) lateral alae are present in males only.

Cervical alae: these structures are confined to the anterior part of the body. This modification of the cuticle does not occur in nematode species that parasitize amphibians.

In some parasitic nematodes, the cervical alae is modified as a *cephalic cuticular vesicle* as in the genus *Oswaldocruzia*; in some cases this cuticular vesicle is divided into a larger anterior part and a smaller posterior part (Fig. 20).

Caudal alae: these alae are confined to the caudal region of the body and limited to the males only; apparently they serve as clasping organs during copulation. Among nematodes that parasitize amphibians, they occur in the family Pharyngodonidae (*Parapharyngodon*). On the other hand, modified caudal alae occur in the males of some families such as the Molineidae, where they are called *bursa copulatrix* or *copulatory bursa* (see below).

Longitudinal ridges: these are raised areas that extend along the length of the body and are present on the submedian as well as on the lateral surfaces. *Oswaldocruzia* (Family: Molineidae) has longitudinal ridges throughout the body length that can disappear or appear along its body (Fig. 21). The system of longitudinal cuticular ridges or *synlophe* in this genus is very important as a taxonomic character.

Inflation: when the cuticle is swollen in a blister-like manner, this condition may be termed inflation (eg. *Rhabdias*; Figs. 22-24). Besides being inflated, the cuticle can be also striated. Inflation of the cuticle may be generalized over the whole body surface instead of restricted to certain areas.

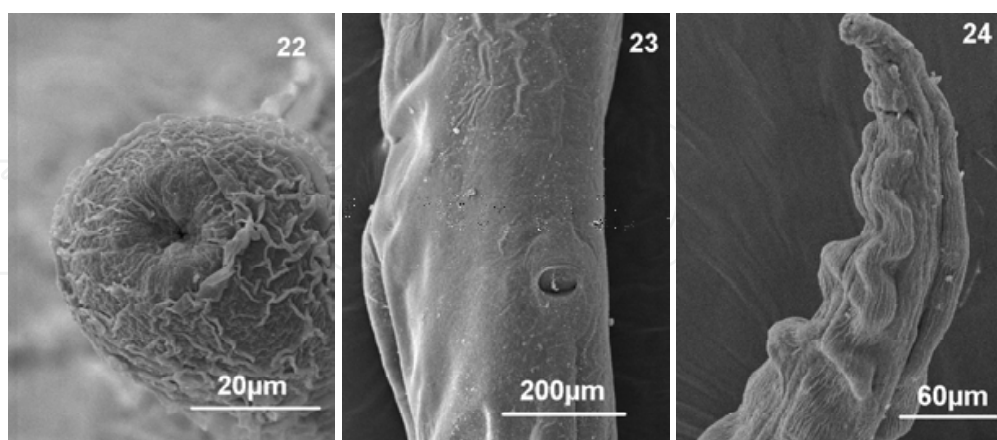


Fig. 22-24. Inflation in the cuticle of nematode parasites of genus *Rhabdias*. 22. Cephalic end. 23. Vulvar region. 24. Anterior end of body.

Papillae: these structures are nerve endings, some of which have a tactile function and others are chemoreceptors; they appear as cuticular elevations of different shapes and sizes (Figs. 25-27). According to their position, they are divided into labial, cephalic, cervical and genital

papillae. In this section, we deal with the distribution and structure of somatic papillae, i.e. those that are distributed throughout the body of the nematode. These papillae are generally arranged in two subdorsal and two subventral rows along the body of the nematode.

Finally, in some genera of nematode parasites of amphibians, the excretory pore has conspicuous cuticularized walls, surrounded by a rough cuticular area (Pharyngodonidae); in others, the excretory pore is located in a depression of the cuticula (*Falcaustra*) (Figs. 28, 29).

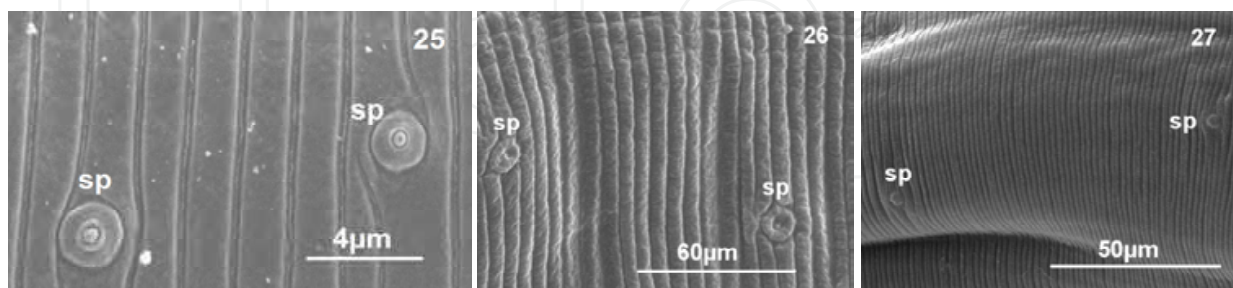


Fig. 25-27. Somatic papillae in nematode parasites of amphibians. 25. *Aplectana hylambatis*. 26. *Aplectana delirae*. 27. *Cosmocerca podicipinus*. sp: somatic papillae.

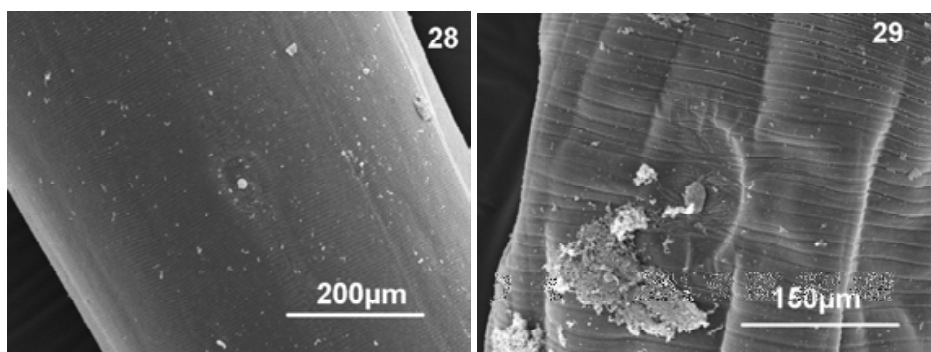


Fig. 28-29. Excretory pore in nematode parasites of amphibian. 28. *Falcaustra* sp. 29. *Skrjabinodon* sp.

Punctuations: this type of marking is frequent and appears as minute round areas of the cuticle which are arranged in definite patterns for each species. In nematode parasites of amphibians, this type of marking appears in males of the family Cosmocercidae, specifically in the genus *Cosmocerca* as part of the rosette papillae (see below; Figs. 38-44) and in the genus *Cosmocercella* (Figs. 46, 48).

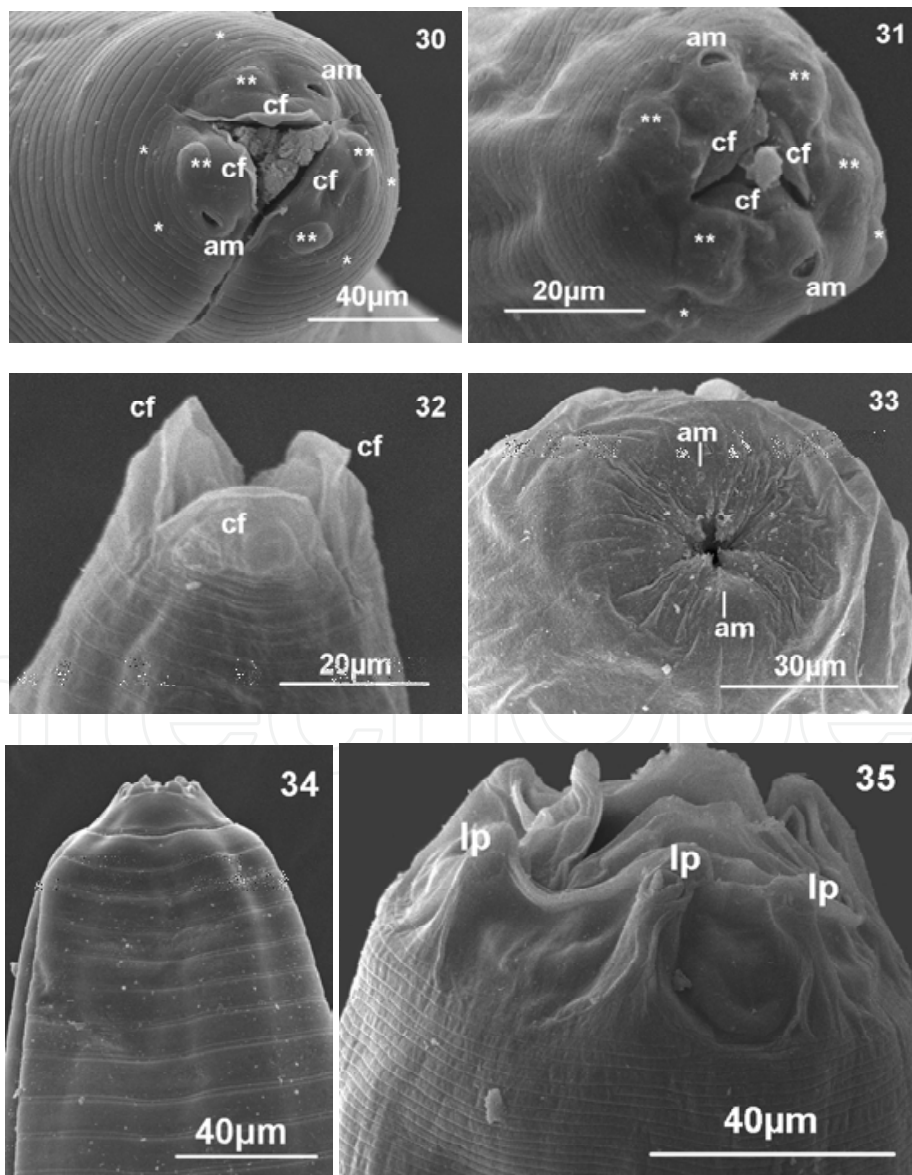
3.1.2 Cuticle of anterior end of body

The cuticular modifications that occur in the anterior end of the body of nematode parasites of amphibians are: head papillae - cephalic papillae, externo-labial papillae, interno-labial papillae-, interlabia, deirids and amphids. These two latter structures are not modifications of the cuticle itself, but their opening in the cuticle can present diverse shape and structure.

Head papillae: these are tactile sensory organs usually located on the lips or labial region, including two circles of six labial papillae and one circle of four cephalic papillae. This arrangement has been proposed for ancestral nematodes considering a radial type of symmetry (De Coninck, 1965). The head papillae are divided into: *cephalic papillae*: outer

circle of four head papillae (or latero-ventral and latero-dorsal papillae); *externo-labial papillae*: median circle of six head papillae, and *interno-labial papillae*: inner circle of six head papillae.

The basic structure proposed by De Coninck (1965) shows modifications in the nematodes that parasitize amphibians. For example, cosmocercid nematodes present three lips. The genus *Aplectana* has a circle of four internal labial papillae, 1 on each subventral lip and 2 on the dorsal lip, and a circle of six external labial papillae; the amphids are large, one on each subventral lip. The anterior end of the oesophagus presents three tooth-like projections covered with a thick cuticle, also called cuticular flap (Fig. 30). The genus *Cosmocerca* presents the same arrangement in the internal and external labial papillae and, as in the previous genus, the anterior end of the oesophagus bears three tooth-like projections (cuticular flap); the amphids are very prominent in some cases (Fig. 31). In the nematodes of family Atractidae, specifically in the genus *Schrankiana*, each lip has a cuticular flange overhanging the mouth opening (Fig. 32).



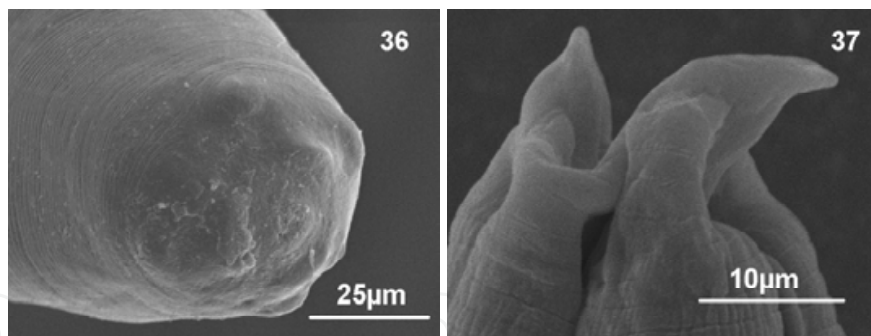


Fig. 30-37. Modifications of the cuticle in the anterior end of body. 30. *Aplectana* sp. 31. *Cosmocerca* sp. 32. *Schrankiana* sp. 33. *Rhabdias* sp. 34. *Pharyngodon* sp. 35. *Falcaustra* sp. 36. *Porrocaecum* sp. (larvae). 37. *Brevimulticaecum* sp. (larvae). am: amphids; cf: cuticular flap; lp: labial papillae; one asterisk: external labial papillae; two asterisk: internal labial papillae.

In the lung nematodes of genus *Rhabdias*, which have an inflated cuticle, the cephalic structures are in most cases very difficult to observe (Fig. 33). In oxyurid nematodes, eg. *Parapharyngodon*, there are three lips, each one bilobed with one small papillae (Fig. 34). In genus *Falcaustra* the mouth is surrounded by 3 large lips, each with 2 forked papillae, and one amphid on each ventrolateral lip (Fig. 35)

On the other hand, in nematode parasites found in larval stage, the cephalic structures of adult forms are generally not present. For example, *Porrocaecum*, a genus found in the liver of the host, has a very little developed lip anlagen (Fig. 36); *Brevimulticaecum*, found encapsulated in various organs of the hosts, presents two toothlike prominences, 1 dorsal and 1 ventral (Fig. 37).

Interlabia: these are cuticular outgrowths (neoformations) originating at the base of the lips or pseudolabia and extending between them, occurring in some ascarids and spirurids. These modifications of the cuticle are present in the larval stage of genus *Physaloptera*; this genus is characterized by the presence of four teeth on the internolateral face of each pseudolabium, an internal group of three teeth, two sublateral and one lateral, and a single externolateral tooth.

Deirids: a pair of sensory organs found laterally in the cervical region and usually protruding above the surface of the cuticle. That may be simple or claw-like and forked, and are situated laterally in the vicinity of the nerve ring in most species (Rhabdochoniidae).

Amphids: a pair of glandular sensory organs situated laterally in the cephalic region and opening through the cuticle; according to some authors, they are chemoreceptors. They have various shapes and sizes but usually occur as two lateral pores (Figs. 30, 31).

3.1.3 Cuticle of posterior end of body

In the case of nematode parasites of amphibians, the special modifications of the cuticle in the posterior end of males, which are generally associated with the copula, are particularly important. These comprise: plectanes, rosettes papillae, vesiculated rosette, papillae, bursa, phasmids, spines suckers and caudal lateral alae. Phasmids are not modifications of the cuticle such as the deirids and the amphids, but their opening onto the cuticle can present different shapes and structures.

Plectanes: these are cross striated cuticular plates functioning as supports for the genital papillae in some males (Fig. 38). This structure is very common in the genus *Cosmocerca*, which is widely distributed in amphibians. In some species, these supports are very marked, as in *C. podicipinus*, in this case, the plectanes of each row are even fused to each other (Fig. 39). In other species, this support is not so developed (eg. *C. parva*); on the other hand, the same species in different hosts can present different degree of development of these structures (Figs. 40-43); indeed, in some immature specimens, the plectanes are imperceptible (Fig. 44).

These structures are arranged in two longitudinal rows on the ventral surface of the males; the number of pairs of plectanes varies among species (*C. podicipinus*, *C. cruzi*, *C. travassosi*: 5 pairs; *C. chilensis* and *C. rara*: 6 pairs; *C. longispicula*, *C. uruguayensis*, *C. vrcibradici*: 7 pairs). Furthermore, some species show wide intraspecific variation regarding the number of pairs of plectanes, even in the same host species (*C. parva*: 5-7 pairs; *C. brasiliensis*: 8-11 pairs; *C. paraguayensis*: 4-5 pairs) (Figs. 18, 19) (González and Hamann, 2010b).

Table 1 shows characteristics of the posterior end of males for species of *Cosmocerca* that parasitize amphibians, observed under SEM.

<i>Cosmocerca</i> spp.	Plectanes + rosette papillae	Adanal papillae*	Caudal papillae	Puncatations of rosette papillae	References
<i>C. ornata</i>	5 pairs	Not stablished	Not stablished	Not stablished	Navarro et al. (1988)
<i>C. ornata</i>	5 pairs	Not stablished	Not stablished	6-7 pad-like protuberances around the posterior border only	Grabda-Kazubska and Tenora (1991)
<i>C. commutata</i>	7 pairs	Not stablished	Several pairs	Interior and exterior rosette: 15	Grabda-Kazubska and Tenora (1991)
<i>C. parva</i>	5-7 pairs + 1 unpaired	1 + 2-4	3 pairs	Interior and exterior rosette: 12-16	Mordeglia and Digiani (1998)
<i>C. podicipinus</i>	5 pairs	3 pairs	Not stablished	Interior rosette: 11-12; exterior rosette: 12-15	González and Hamann (2010b)
<i>C. parva</i>	5-7 pairs	1 + 3 pairs	Not stablished	Interior rosette: 10-11; exterior rosette: 12-14	González and Hamann (2010b)
<i>C. parva</i>	4-5 pairs	1 + 3 pairs	Not stablished	Interior rosette: 12-15; exterior rosette: 12-15	González and Hamann (2008)

Table 1. Characteristics of the caudal region of males of *Cosmocerca* spp. that parasitize amphibian hosts, studied under SEM. *Arrangement of adanal papillae: unpaired papillae anterior to anus + pairs of adanal papillae.

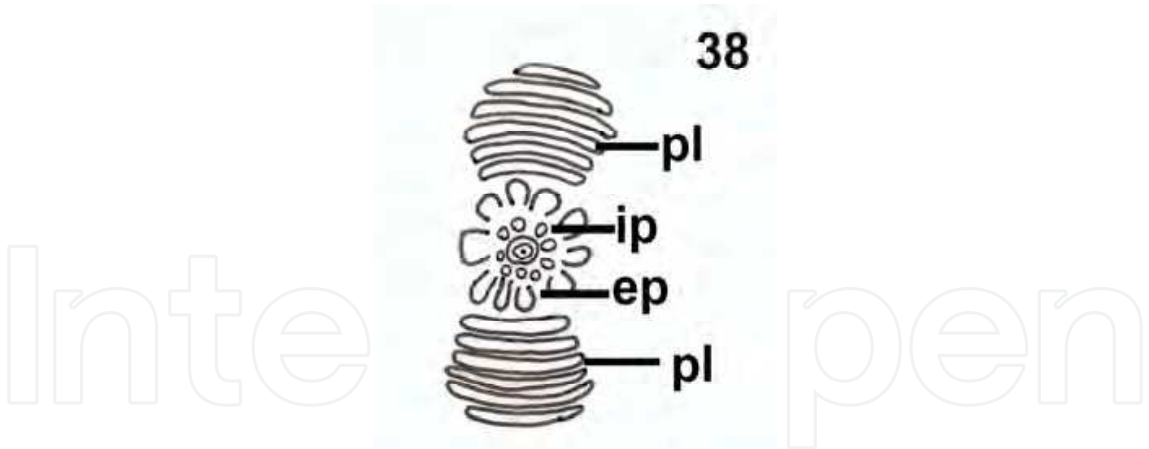
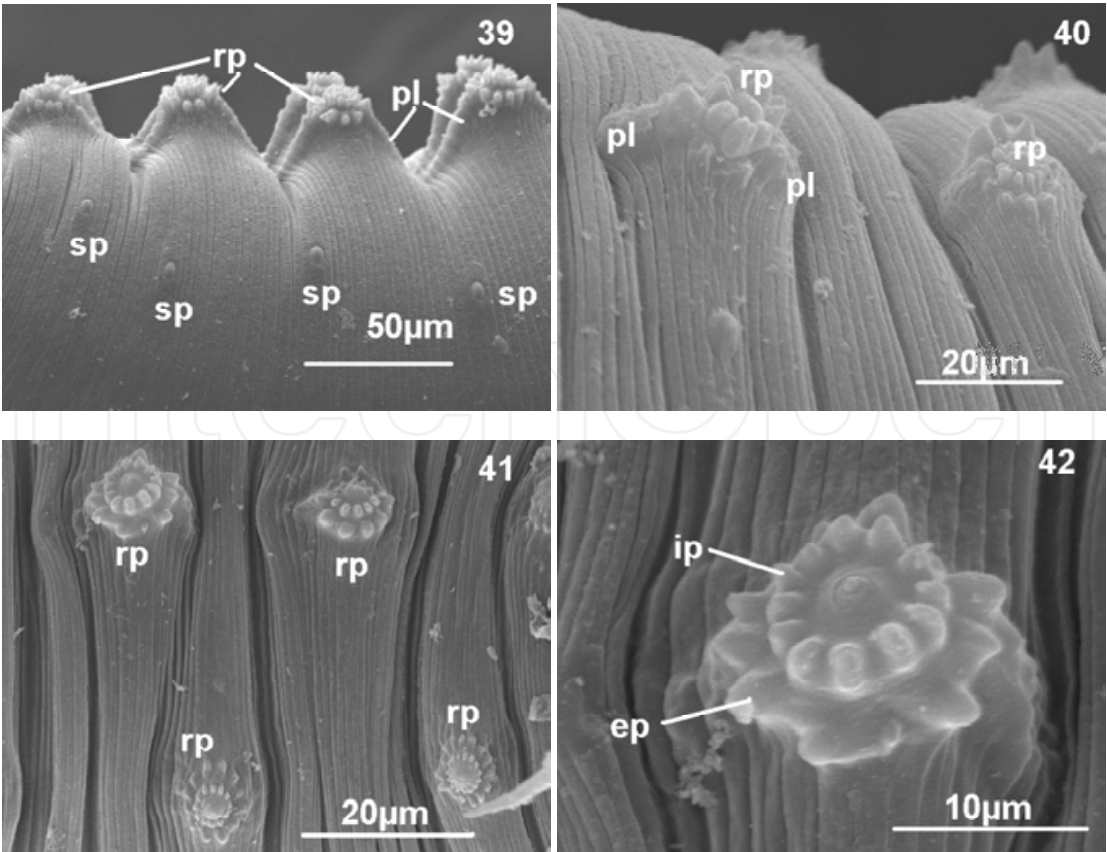


Fig. 38. Plectanes and rosette papillae in nematode parasites of genus *Cosmocerca*. pl: plectanes; ip: internal circle of punctations; ep: external circle of punctations.

Rosette papillae: these structures consist of papillae surrounded by punctuations. This modification is present in the genus *Cosmocerca*. In this genus, the plectanes are located outside the rosette papillae and are directed toward the anterior and posterior end (Fig. 38).

These rosette papillae are formed by two circles of punctuations, one internal and one external. The number of punctuations in each circle varies among species and within the same species for individuals collected from different hosts (González and Hamann, 2008; 2010b) (Figs. 38-44). In the genus *Cosmocercoides*, the caudal rosette papillae are not raised above the cuticular surface.



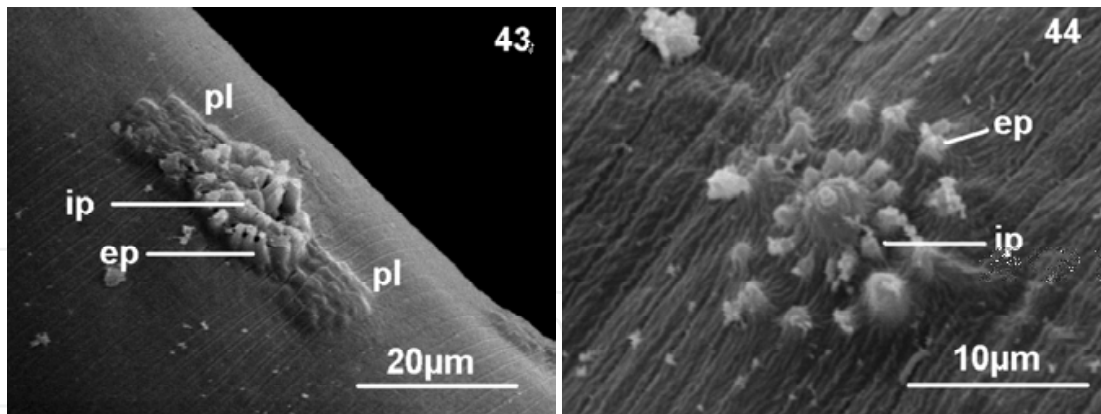


Fig. 39-44. Plectanes and rosette papillae in nematode parasites of genus *Cosmocerca*. 39. *Cosmocerca podicipinus* collected in *Pseudopaludicola falcipes* with fused plectanes. 40. *Cosmocerca parva* collected in *Rhinella granulosa* with very marked plectanes. 41. 42. *Cosmocerca parva* collected in *Rhinella schneideri*. 43. *Cosmocerca parva* collected in *Leptodactylus bufonius*. 44. *Cosmocerca parva*, immature specimen, collected in *Rhinella fernandezae* with imperceptible plectanes. sp: somatic papillae; pl: plectanes; rs: rosette papillae; ip: internal circle of punctations; ep: external circle of punctations.

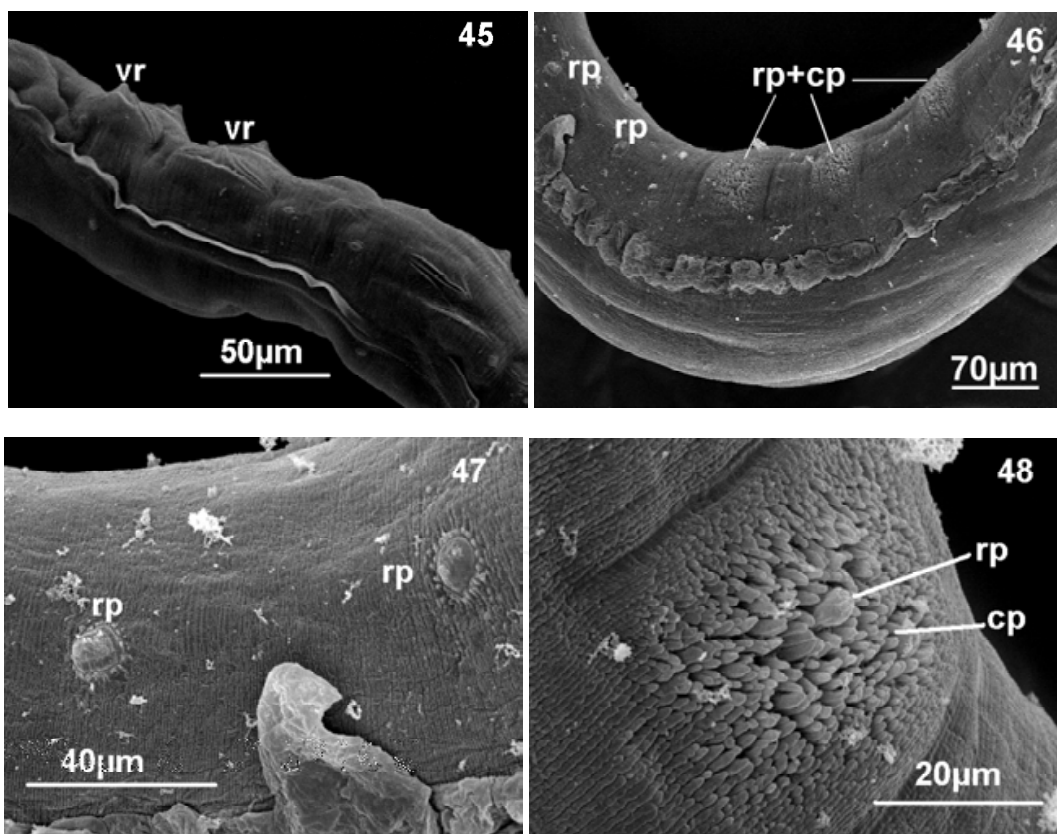
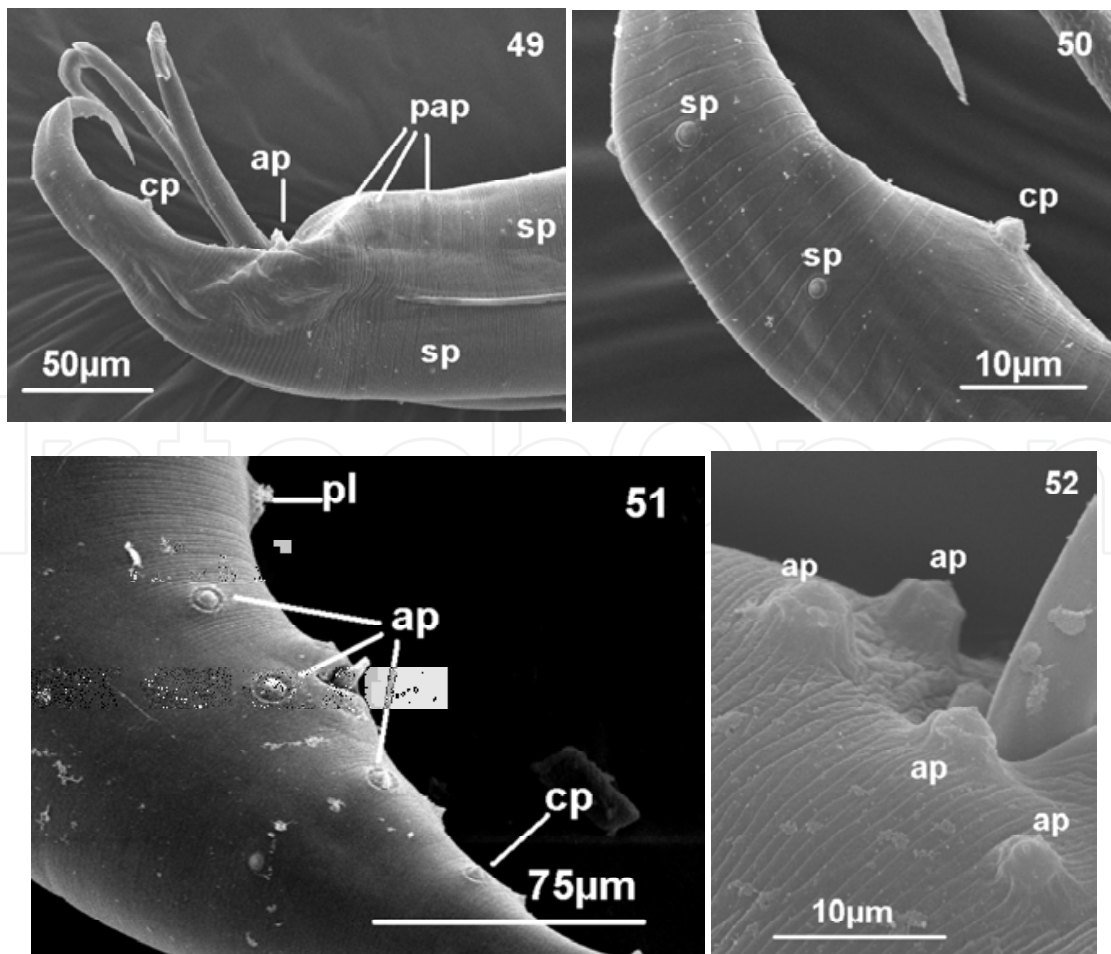


Fig. 45-48. Modifications of the cuticle in the posterior end of males of the genus *Cosmocercella*. 45. Vesiculated rosette in *C. minor*. 46. Combination of different structures in *C. phyllomedusae*. 47. Detail of small rosette papillae. 48. Detail of rosette papillae surrounded by wide areas of cuticular punctations. vr: vesiculated rosette; rp: rosette papillae; cp: cuticular punctation; rp+cp: rosette papillae surrounded by areas of cuticular punctations.

Vesiculated rosette: caudal rosette papillae raised on the surface of a clear vesicle. These modifications are present in the genus *Cosmocercella*. In *C. minor*, for example, there are 4 pairs of vesiculated papillae (Fig. 45) and in *C. phyllomedusae* there is a combination of different structures; this species has small rosette papillae in the preanal subventral surface (Figs. 46, 47), rosette papillae surrounded by wide areas of cuticular punctuations (Fig. 46, 48), and large unpaired vesiculated papillae extending almost to the level of the oesophagus.

Papillae: some genera of nematode parasites, such as *Aplectana*, *Raillietnema*, *Falcaustra* or *Paraoxyascaris* do not possess conspicuous structures in the posterior end such as plectanes, rosette papillae or vesiculated papillae; these genera have simple papillae with variable number and arrangement (Figs. 49-56). These structures can be divided into caudal papillae (located in the tail, posteriorly to the anus) and cloacal papillae (surrounding the cloaca; these can be precloacal, postcloacal and adcloacal). These papillae may be sessile (*Aplectana*, *Schrankiana*, *Falcaustra*) (Figs. 49-52, 54-56) or pedunculate (*Parapharyngodon*) (Fig. 53). On the other hand, other genera such as *Cosmocercella* and *Cosmocerca* have this type of papillae in addition to plectanes, rosettes and vesiculated rosette. These papillae are commonly surrounded by one or two small rosettes of punctuations (Fig. 51). In some cases they protrude above the surface of the cuticle (Fig. 52).



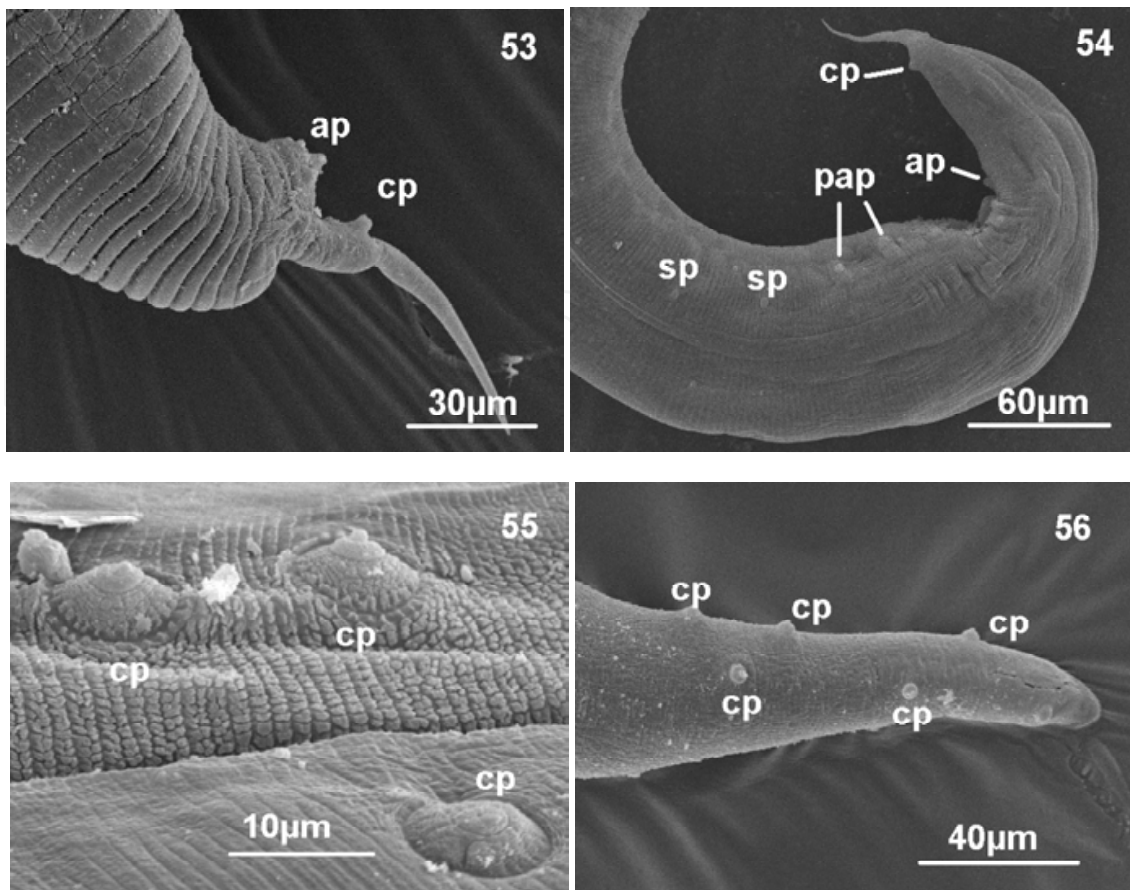


Fig. 49-56. Papillae in the posterior end of body of males nematode parasites of amphibians. 49. 50. Arrangement of caudal papillae in *Aplectana hylambatis*. 51. Arrangement of precloacal, adcloacal and postcloacal papillae in genus *Cosmocerca*. 52. Detail of precloacal, adcloacal and postcloacal papillae in *Cosmocerca*. 53. Pedunculate papillae in tail of *Parapharyngodon*. 54-56. Sessile papillae in *Schrankiana* (54), *Falcaustra* (55) and *Cosmocerca* (56). sp: somatic papillae; pl: plectane; pap: preanal papillae; ap: adanal papillae; cp: caudal papillae.

Bursa: this structure is present in nematodes of the genus *Oswaldocruzia*. This structure may be circular or oval, often divided into two symmetrical or asymmetrical lateral lobes, separated by a dorsal lobe and supported by rays or papillae. The rays of the bursa are visualized well under light microscope because they are not a part of the cuticle but embedded in the lobes.

Caudal lateral alae: these are sublateral or lateral longitudinal wings of the cuticle that occur on the male tail. Among nematode parasites of amphibians, they occur in the genus *Physaloptera*; however, these caudal alae develop in the adult stage, while it is typically the larval stage of *Physaloptera* that occurs as a parasite of amphibians.

Spines: some male and female nematode parasites, such as those of the genus *Skrjabinodon*, have cuticular spines on the tail filament (Fig. 57).

Suckers: this is a sucker-like pre-cloacal structure. A series of stages in sucker development occurs in some kathlaniids, indicating that there is first a concentration of copulatory

muscles in this area which later becomes a sucker through modification of the cuticle. This modification of the cuticle is found in some species of genus *Falcaustra*.

Phasmids: these are paired glandular sensory organs situated laterally in the caudal region and opening to the surface by a slit or pore (Fig. 58).

As in the previous case, the structures and modifications of the cuticle that are observed in the posterior body end of adult specimens do not occur in larvae (Fig. 59).

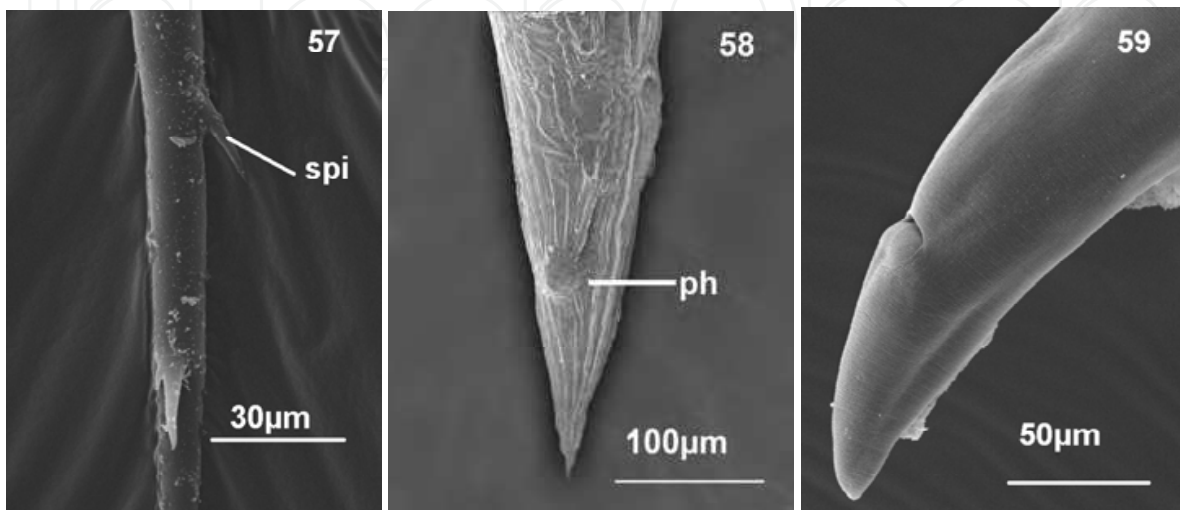


Fig. 57-59. Modifications of the cuticle in posterior end of body of nematode parasites. 57. Spines in the tail of *Skrjabinodon* sp. 58. Phasmid in *Rhabdias* sp. 59. Posterior end of *Physaloptera* sp. spi: spines; ph: phasmid.

3.1.4 Eggs

The eggs are variable in size, shape and structure; they usually have a many-layered shell with either smooth or rough, sometimes sculptured, external surface, and their poles may bear a characteristic operculum or plug. Among the nematode parasites of amphibians, eggs may present punctuations (*Pharyngodonidae*) (Figs. 60, 61), an operculum (*Gyrinicola*) or a thin membrane that has no special features, as in the *Cosmocercidae*.

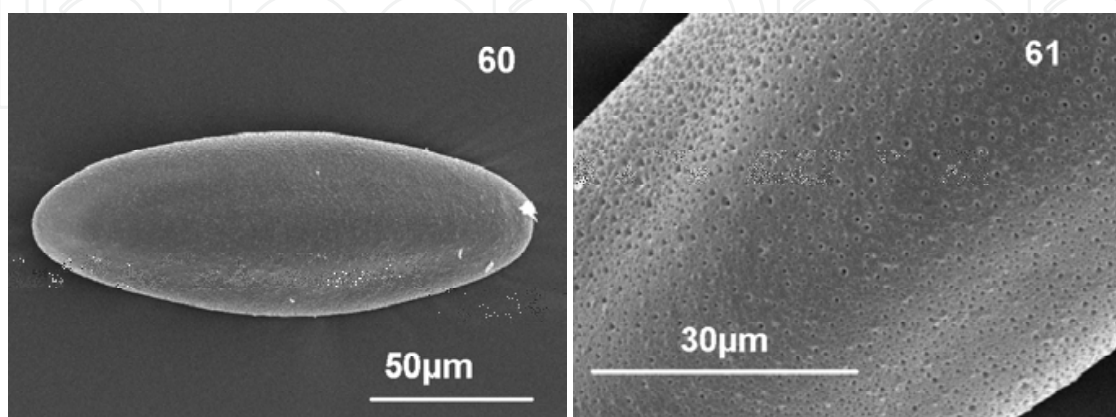


Fig. 60-61. Egg cuticle of nematode parasites of amphibians. 60. General view. 61. Detail of punctations.

3.1.5 Vulva

The area of the body immediately anterior and posterior to the vulvar opening is called vulvar region. In most females of nematode parasites of amphibians, this may be simply an opening transversal to the longitudinal body axis without any special striation, as in the genus *Rhabdias* (Fig. 62), or with a striation that differs slightly from that of the rest of the body as in the genus *Cosmocercella* (Fig. 63); or it may present more complex structures as in *Aplectana* (Fig. 64); in this latter case, the cuticle forms an extension in the anterior side of the vulvar opening that can be observed as a vulvar flap (Gibbons, 1986).

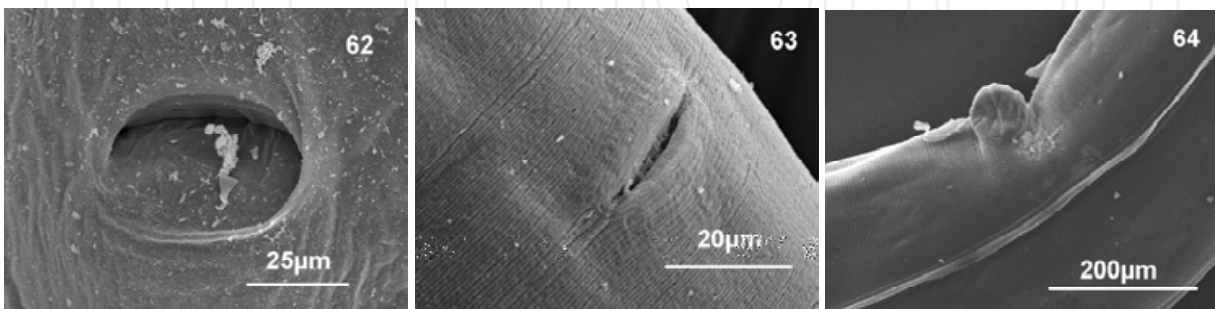


Fig. 62-64. Vulvar cuticle in nematode parasites of amphibians. 62. *Rhabdias* sp. 63. *Cosmocercella* sp. 64. *Aplectana* sp.

3.1.6 Spicules

Nematodes usually have two spicules; each one is essentially a tube covered by a sclerotized cuticle and containing a central protoplasmic core. In terms of the taxonomy of nematode parasites of amphibians, the importance of the spicules lies in their morphology and size, and not in the presence of ornamentation on the cuticle of these structures. In this case the spicules can be studied with SEM only when they are outside the individual, i.e., when protruding from the cloaca (Figs. 65-67).

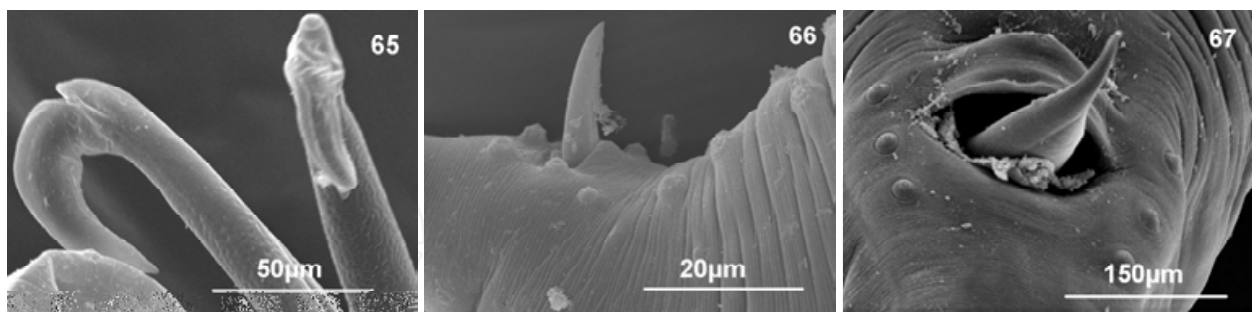


Fig. 65-67. Spicules in nematode parasites of amphibians. 65. *Aplectana hylambatis*. 66. *Cosmocerca* sp. 67. *Falcaustra* sp.

3.2 Trematoda

The tegument of trematodes is syncytial and consists of a tegumental outer membrane (trilaminar), a matrix (with discoid bodies, membranous bodies and usually mitochondria) and a basal tegumental membrane. The tegument is variously interrupted by cytoplasmic projections of gland cells and by openings of excretory pores. The tegumental surface often contains ornamentations such as spines between the outer and basal membranes; these are

often present in different areas of the body; there are also numerous sensory papillae, pits and ridges of various configurations (Fried, 1997; Schmidt and Roberts, 2000). The surface topography of the cirrus of digenetic trematodes also shows spine-shape protrusions and papillae (Bušta and Našincová, 1987).

3.2.1 Tegument of suckers

The rim of the oral and ventral suckers in some species of digenean parasites of amphibians (both larval and adult) shows sensory papillae with variable morphology and distribution. Thus, papillae may appear as button-like structures and can be distributed as single and double papillae on the oral and ventral surfaces of the sucker (Hamann and González, 2009; Mata-López, 2006; Nadakavukaren and Nollen, 1975). In other digeneans, the oral sucker has a spongy surface with numerous pores (Whitehouse, 2002). Figures 68-70 show some characteristics of the tegument of the oral and ventral suckers of digenean trematodes found in Argentinean amphibians.

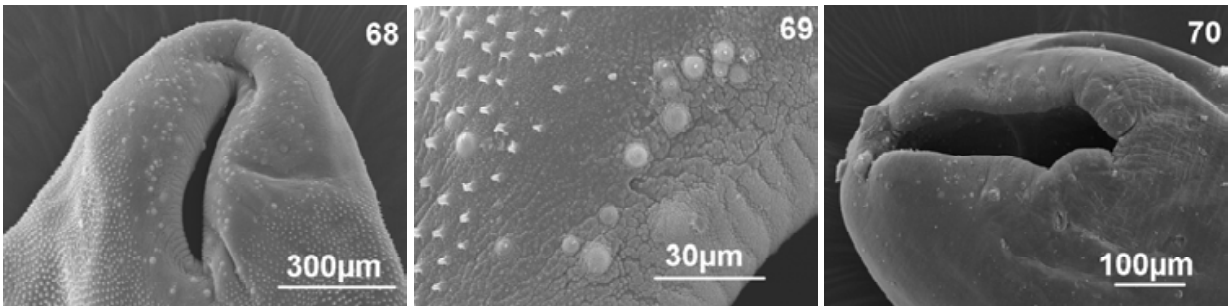


Fig. 68-70. Tegument of suckers of digenean parasites of amphibians. 68. Papillae on oral sucker of Macroderoididae. 69. Detail of papillae on oral sucker. 70. Detail of papillae on ventral sucker of Paramphistomatidae.

3.2.2 Tegument of the ventral surface

The tegument of digenean trematodes (larval and adults) that occur in amphibian hosts shows spines with varied morphology (e.g. scale-like spines) and variable distribution; they generally extend from the anterior end to variable levels of the posterior region (Hamann and González, 2009; Razo-Mendivil et al., 2006). In other digeneans, the surface of the tegument possesses regular ridges and interspersed protuberances (Nadakavukaren and Nollen, 1975). Figures 71-72 show the shape and distribution of spines found in the tegument of some digenean parasites of Argentinean amphibian.

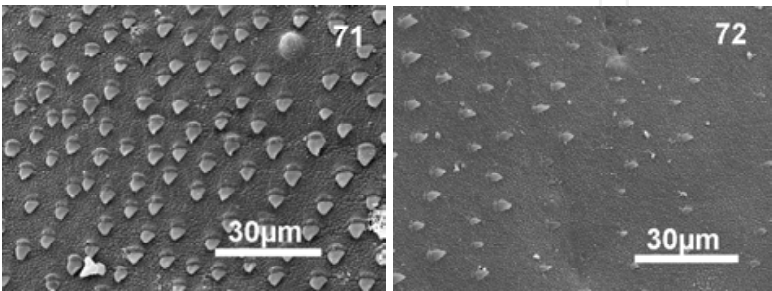


Fig. 71-72. Tegument of the ventral surface of Macroderoididae. 71. Spines of the anterior third of the body. 72. Spines of the posterior third of body.

3.3 Acanthocephalan

The body surface of acanthocephalans has 5 layers. The outermost layer is the epicuticle, followed by the cuticle which is composed mainly of lipoproteins; the third layer has a homogeneous nature; the fourth layer possesses fibrous bands besides mitochondria, bladders and lacunar channels, and the fifth layer contains scarce fibres but larger and more abundant lacunar channels than in the previous layer (Olsen, 1974).

Regarding this group of helminth parasites, most SEM studies are focused on the hooks that they possess in the proboscis, as well as their body spines. Likewise, they present sensory structures with diverse ornamentations in the posterior part of the bursa.

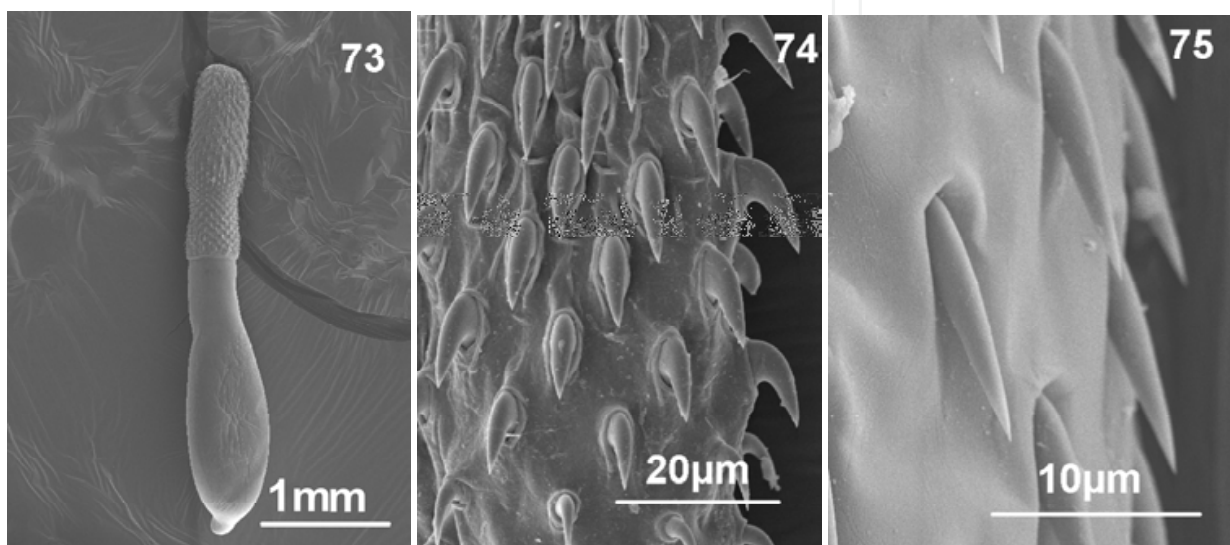


Fig. 73-75. Acanthocephalan parasites of amphibians. *Centrorhynchus* sp. 73. General view. 74. Detail of hooks of the proboscis. 75. Detail of spines of the proboscis.

In our study we found larval stages belonging to the genus *Centrorhynchus*. This genus is characterized for possessing an unarmed trunk, the proboscis divided into two regions (an anterior portion with hooks and a posterior portion with spines), and for having subterminal genital pores. Because the genital complex was not fully developed, specific determination was not possible. The proboscis presented 28 to 30 longitudinal rows of 20 to 23 hooks, 8 to 11 rooted hooks and 10 to 13 rootless spines (Figs. 73-75).

4. Helminth parasites of Argentinean amphibian studied with SEM: Synthesis and new contributions. Research perspectives

Up to the present, studies performed with scanning electron microscopy techniques on helminth parasites of Argentinean amphibians have included three families of nematodes: Rhabdiasidae, *Rhabdias fülleborni* (González and Hamann, 2008), Cosmocercidae, *Cosmocerca parva*, *C. podicipinus*, *Aplectana hylambatis*, *A. adaechevarriae* (González and Hamann, 2010b; Mordegia and Digiani, 1998; Ramallo et al., 2008) and Physalopteridae, *Physaloptera* sp. (González and Hamann, 2010b), and one family of trematodes: Diplostomidae, *Lophosicyadiplostomum* aff. *nephrocystis* and *Bursotrema tetracotyloides* (Hamann and González, 2009).

New contributions presented in this work include, for the Class Nematoda, the families Molineoidae (*Oswaldocruzia* spp.), Pharyngodonidae (*Parapharyngodon*, *Pharyngodon*, *Skryabinodon*), Cosmocercidae (*Aplectana* spp., *Cosmocerca* spp., *Cosmocercella* spp., *Paraoxyascaris* sp.), Kathlaniidae (*Falcaustra* sp.), Atractidae (*Schkrankiana* sp.) and Ascarididae (*Porrocaecum* sp., *Brevimulticaecum* sp.), and for the Phylum Acanthocephala, family Centrorhynchidae (*Centrorhynchus* sp.).

Future research in this topic should focus on extending the geographical areas studied while at the same time, expanding the examination to other possible amphibian hosts.

Reports about helminth parasites of Argentinean amphibians studied under SEM refer mainly to specimens collected in host from the Northeast region, specifically Corrientes province, and the Northwest region, with only one record for Salta province so; thus, there is still a vast portion of the Argentinean territory that has not yet been studied (Fig. 76).

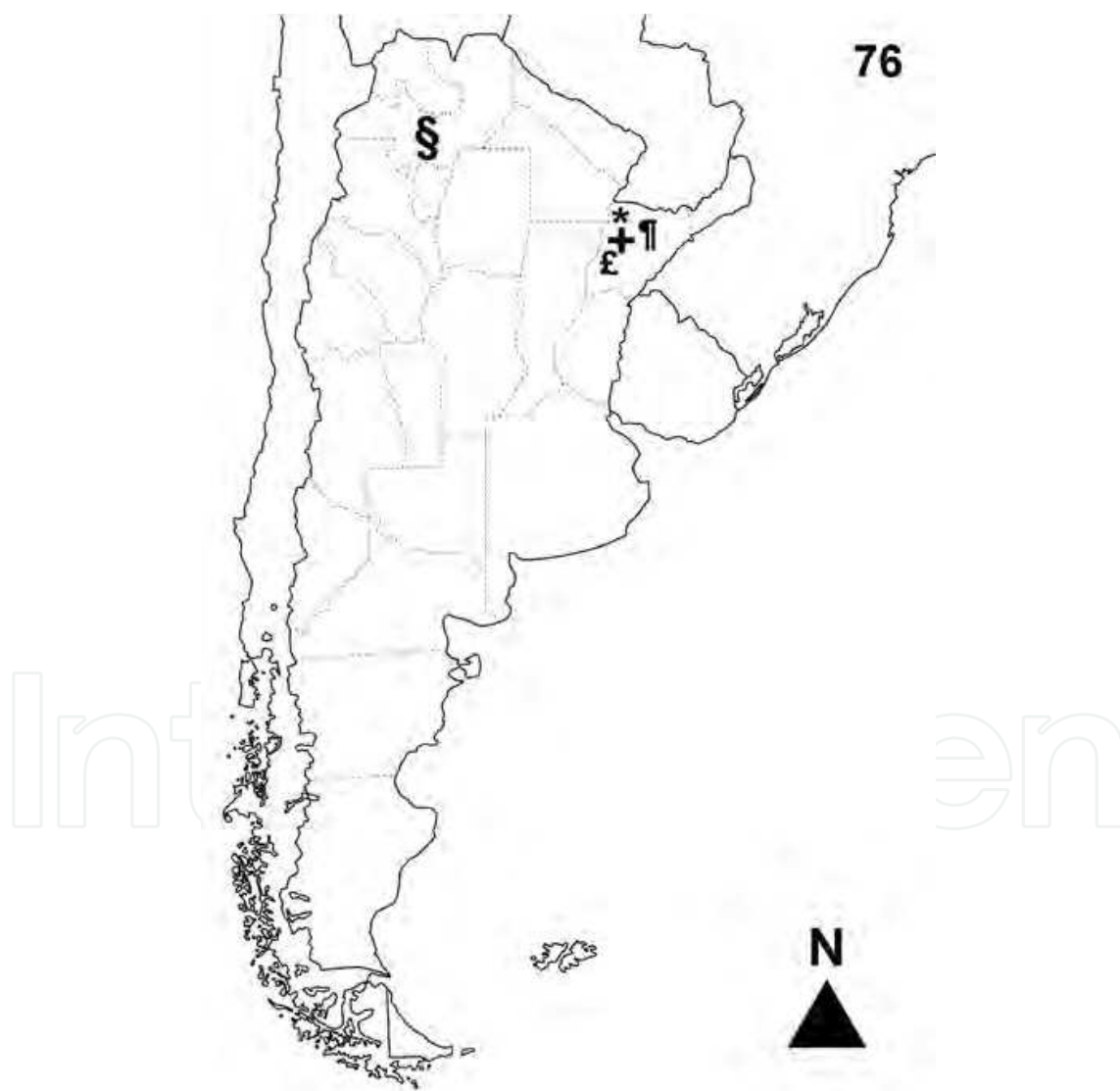


Fig. 76. Reports of helminth parasites of amphibians studied using SEM in Argentina. ¶: González and Hamann (2008); *: González and Hamann (2010b); +: Hamann and González (2009); £: Mordeglia and Digiani (1998); §: Ramallo et al. (2008).

Lavilla et al. (2000) reported a total of 271 amphibian species for Argentina (167 anurans and 4 gymnophions); of these, only 32 (11.8%) have been cited as hosts for helminth parasites (González and Hamann, 2004, 2005, 2006a, 2006b, 2007a, 2007b, 2008, 2009, 2010a, 2010b, 2011; Hamann and Pérez, 1999; Hamann and González, 2009; Hamann et al., 2006a, 2006b, 2009a, 2009b, 2010; Lajmanovich and Martinez de Ferrato, 1995; Lunaschi and Drago, 2007; Ramallo et al., 2007a, 2007b, 2008). Of all the anuran families, the most studied for helminth parasites are Hylidae, Bufonidae, Leiuperidae and Leptodactylidae. Nine species of hylids have been studied for helminth parasites, but only one of these studies included SEM: *Scinax nasicus* (González and Hamann, 2008; Hamann and González, 2009); similarly, seven species of bufonids have been studied for helminth parasites, but SEM was employed in only two cases: *Rhinella schneideri* and *R. granulosa* (González and Hamann, 2008; Mordeglia and Digiani, 1998; Ramallo et al., 2008), finally, six species of leiuperids and leptodactylids have been studied for helminth parasites, but only one of them was analyzed with SEM, the leiuperid *Physalaemus santafecinus* (González and Hamann, 2010b).

The SEM study of the tegument of helminth species (e.g. morphology of spines) collected in different localities could detect possible intraspecific variation related to geographical location; this phenomenon has been highly documented in both trematode (Grabda-Kazubska and Combes, 1981; Kennedy 1980a) and nematode (Chitwood, 1957) parasites of amphibians. Similarly, variations related to occurrence in a wide range of phylogenetically unrelated hosts, i.e. the cases of generalist helminths could detect possible intraspecific variation related with the host age or diet, previous exposure to the parasite, presence of another parasite and number of specimens present (Chitwood, 1957; Haley, 1962; Kennedy, 1980b; Watertor, 1967).

5. Importance of the use of scanning electron microscope for the study of helminth parasites of amphibians

In helminth parasites, all morphological aspects must be studied under light microscope, because these structures are very important in the context of their systematic classification. Some examples of these traits include, in the case of parasitic nematodes, the type of esophagus (oxyuroid, rhabditoid, strongyloid), presence of ventriculus and its shape, and the caecum and its shape; in the females, the arrangement of ovaries (prodelphic, amphidelphic or opisthodelphic), number of uteri (monodelphic or didelphic), structure of the ovovector and the vagina and, in the males, the structure and measurements of the gubernaculum. In the case of trematodes, the distribution of vitelline follicles, the position of the testes and ovary, size of the eggs, position of oral and ventral suckers, reproductive structures, among others, are characteristics of taxonomic importance. Finally, regarding the internal anatomy of acanthocephalans, some particularly relevant structures are the proboscis receptacle, lemnisci, retractor muscle, testis, seminal vesicle, cement gland, Saeftigen's pouch, etc. Thus, the scanning electron microscope represents an additional tool for the study of this group of organisms. The importance of SEM lies in its ability to provide three-dimensional images with high magnification that allow understanding the spatial relationships among surface structures. It could be used to separate species that appear morphologically identical when examined under light microscope, validate species and demonstrate differences between populations or races (Gibbons, 1986; Hirschmann, 1983).

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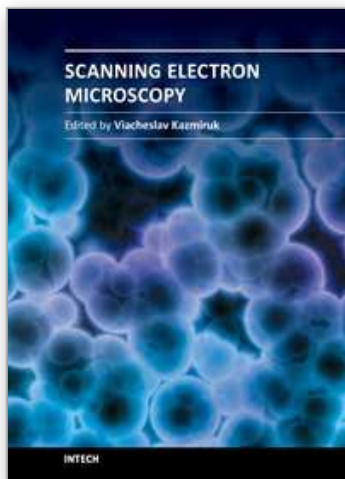
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