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ORIGINAL PAPER

Development of *Angiostrongylus costaricensis* Morera and Céspedes 1971 (Nematoda: Angiostrongylidae) larvae in the intermediate host *Sarasinula marginata* (Semper 1885) (Mollusca: Soleolifera)

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Abstract In life cycle of *Angiostrongylus costaricensis*, veronicellidae mollusks participate as the invertebrate host while rodents as the main vertebrate host. The current work shows a sequential larval development of *A. costaricensis* in *Sarasinula marginata*, individually killed and digested from day 1 to 43, post infection. Some larvae, recovered from sedimentation, were submitted to selective staining after paraffin embedded or inclusion in JB-4 to study inner structures. As control, four slugs were used, two killed at the beginning of infection and the others at the end of the experiment. At day 2 post infection, larvae were motionless and thick, presenting initial retention of granules. At day 4, L_2 were detected, persisting until 43 days post infection. Larvae L_2 displayed a large amount of granules rich in lipids and carbohydrates through its overall body, with more accumulation at the medial third corresponding to the esophagus–intestine transition site. Lipid granules, the main energetic source, were located at the basal and apical regions of intestinal cells. Both L_1 and L_3 presented bilateral alae, which is also common in other nematodes. Transition forms between L_2 to L_3 molts were also observed.

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Introduction

The Metastrongilidae *Angiostrongylus costaricensis* Morera and Céspedes 1971, order Strongylida and family Angiostrongylidae, is a heteroxenic parasite. Throughout its life cycle, adult worms are located into rodent mesenteric arteries, requiring veronicellidae mollusks as intermediate hosts. The first description on its evolutionary cycle was performed by Morera (1973) and further revisions have added extensive data to the cycle both in vertebrates (Mota and Lenzi 1995, 2005) and invertebrates (Mendonça et al. 1999, 2003).

Studies have demonstrated that the nematode larval development in mollusks may be influenced by environmental factors such as humidity, temperature, estivation, intern factors such as infection intensity, and specific characteristics of the host like age, size, and susceptibility (Cheng and Alicata 1965; Gerichter 1948; Guilhon and Gaalon 1969; Halvorsen and Skorping 1982; Hori et al. 1985; Ishii 1984; Kutz et al. 1999; Rachford 1976; Rose 1957; Samson and Holmen 1984; Solomon et al. 1996; Yousif and Lammler 1975; Wallace and Rosen 1969; Kutz et al. 2001; Lv et al. 2006; Jenkins 2006).

The current work detailed sequential morphological aspects of *A. costaricensis* larval development in the intermediate host, characterizing the main chemical composition of the larvae body, the cells responsible for lipid secretion and the sequential structural modifications in the molting process from L_1 to L_3 stages.

Materials and methods

A total of 185 slugs, weighting around 1.0 g, were infected with 5,000 L_1 during 24 h. Five slugs were killed daily, for

each period of time, as follows: from the first to 30th day and at 33, 34, 35, 36, 40, 41 and 43 days post infection. They were individually digested (HCl + pepsin) and the sediment was recovered in Petri dishes and examined with inverted microscope for L_1, L_2, and L_3 identification, according to Morera (1973).

Purified larvae were stained with Sudan Black and Oil Red (Pearse 1968) to verify the presence of lipids. For negative control, larvae were previously treated with xylol and alcohol for grease dissolution, followed by another Sudan staining.

Search for glycoprotein was performed with periodic acid–Schiff (PAS) staining and fluorescence assay using *Glycine max* (soybean) and *Lens culinaris* (Lentil)-fluorescein isothiocyanate (FITC) labeled. Amylase digestion was used as control of PAS reaction to glycogen.

The L_1, L_2 and L_3 were also fixed in Carson's formalin (Carson et al. 1973) and washed and embedded in hydrophilic resin JB-4 (Junqueira et al. 1979); this procedure excluded the alcoholic dehydration as a requirement for resin infiltration. Semi-thin sections obtained with ultracut microtome (Leica) were stained with Lee's methylene blue-basic fuchsine (Bennett et al. 1976).

Stained sections were analyzed by bright-field microscopy and entire larvae were studied by laser scanning confocal microscopy (LSM-410, Zeiss), applying reflected mode to fluorescein labeled material, while unstained larvae were seen under Nomarski mode.

Results

At day 2 post infection, L_1 were thick and motionless with initial expression of lipid granules. At day 4, L_2 were detected, persisting until day 43 post infection. Throughout the molting process, larval thickness varied from thin with few granules, when young, to thick with a great amount of granules, after maturation (Figs. 1a,b and 2).

Together with mature L_2, young L_3 were found with residual cuticle (Fig. 1c) at days 11, 15, 18, 20 and 28 post infection. Concomitant mature L_3 and L_2 and young L_3 were observed at days 11, 14, 15, 16, 18, 21, 22, 26, 28 and 43 post infection.

Larvae L_2 displayed a great amount of lipid granules throughout the whole body, with more density in the medial third, corresponding to the esophagus–intestine transition site (Fig. 2a–c). Granules varied in size and initially were more homogeneously distributed and, throughout molting from L_2 to L_3, they gradually moved to the subnuclear region of the intestinal epithelium (Fig. 3a,b), disappearing in mature L_3 (Fig. 3c). Lipid nature of granules was confirmed by a previous treatment with xylol and alcohol, turning Sudan staining completely negative.

Larvae L_2 were PAS positive, indicating the presence of neutral glycoproteins which did not vanish after amylase digestion, precluding the glycogen presence. Larvae L_1 and L_3 were not stained by Sudan and PAS.

G. max-FITC and *L. culinaris*-FITC lectins, with affinity for *N*-acetyl-D-galactosamine, α-*d*-manosyl and α-*d*-glycosyl, respectively, developed granules strongly stained and located predominantly in the medial third of L_2 (Fig. 2d). The density of carbohydrate granules was lower when compared with the lipid ones, mainly at the ends of larval body. Lectins also developed, besides large granules, a diffuse and thin granular pattern, more evident in the medial and proximal thirds of the larval body.

Larvae L_1 and L_3 presented typical bilateral alae (Fig. 3c,d).

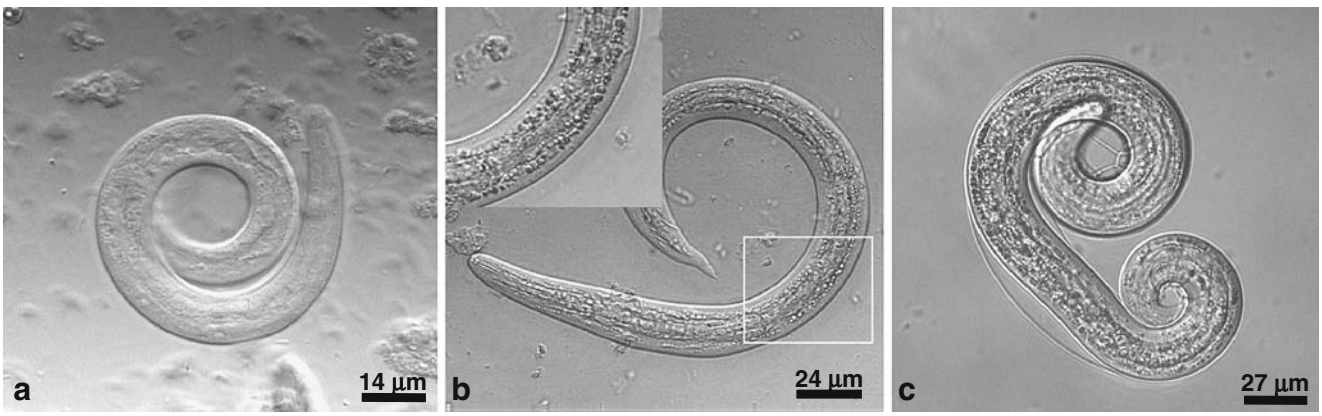

Fig. 1 Larvae of *Angiostrongylus costaricensis* in different stages of development. **a** External aspect of L_1 (DIC, LSCM, *Bar*=14 µm). **b** L_2 undergoing L_3 with lipids lining up close to the cuticle. The *inset* details the lipid granules display (DIC, LSCM, *Bar*=24 µm). **c** Young L_3 with residual L_2 cuticle (DIC, LSCM, *Bar*=27 µm). DIC=Differential interference contrast, LSCM=laser scanning confocal microscopy

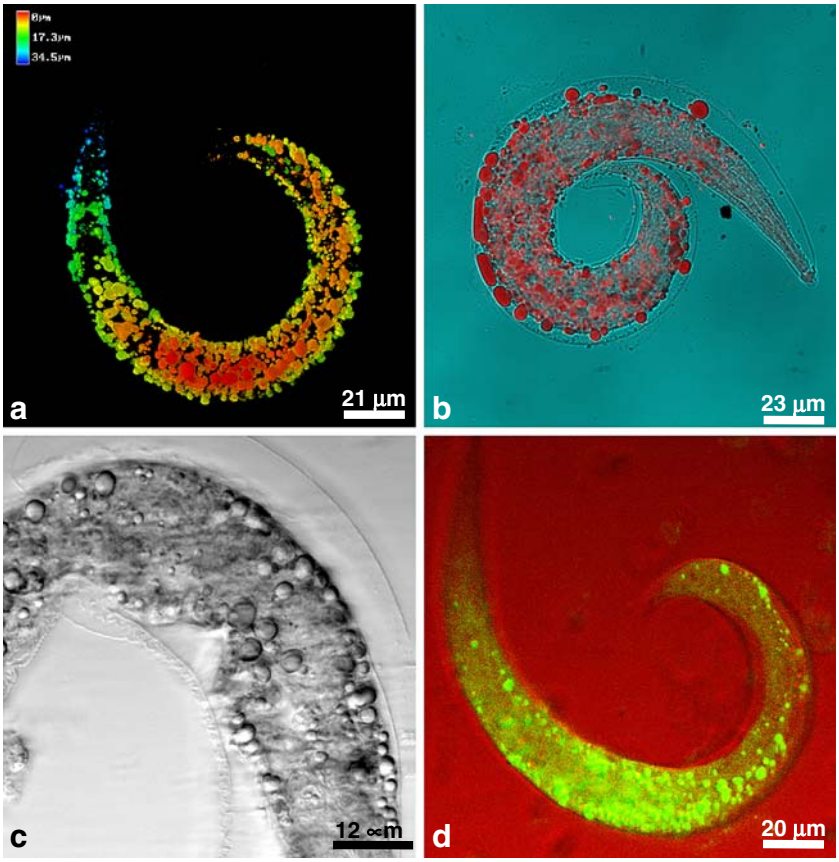

Fig. 2 **a** Larvae L_2 with many lipid granules, diminishing in the anterior end, seen in color code, from tomography sections (LSCM). **b** Larvae L_2 with Oil-Red-stained lipid granules (LSCM, reflection mode). **c** Molting larvae L_2 showing residual cuticle and lipid granules in peripheral location (LSCM). **d** Larvae L_2 with *Glycine max*-FITC-positive dense granules at the medial third, with a shortage at the extremities, mainly in the anterior end (*Glicine max*-FITC, LSCM). FITC=Fluorescein isothiocyanate, LSCM=laser scanning confocal microscopy

Discussion

The present work focused on morphological and biochemical characteristics of *A. costaricensis* larvae in the invertebrate host *Sarasinula marginata*, using diverse methodological analysis. It is demonstrated that the second molt ($L_2 \rightarrow L_3$) occurred from day 4 to 43 post infection, and the main energetic source of L_2 consisted of lipids and carbohydrates, which were predominantly located in the medial third of the larvae, corresponding to the esophagus–intestine transition site and primordial genital system.

The first description of *A. costaricensis* life cycle in the intermediate host, reported by Morera (1973), referred the rapid changes that occurred in L_1, which became thicker due to the accumulation of numerous granules. Such transformations were more evident at day 3 and 4, when the first molt ($L_1 \rightarrow L_2$) took place. Larvae L_2 grew bigger from day 4 to 10, presenting a gradual increase in the amount of granules, making the visualization of inner organs very difficult. The second molt ($L_2 \rightarrow L_3$) was observed from day 11 to 14. Between 16 and 19 days, larvae reached maturity, the moment at which they became infective to the vertebrate host.

In the current study, the second molting period ($L_2 \rightarrow L_3$) was longer and extended up to day 43. This event could be related to the low temperature during the cold weather (May, June, and July) in Brazil, a natural factor able to delay a larval development in its intermediate host. Indeed, Graeff-Teixeira et al. (1991) observed that a seasonal transmission of *A. costaricensis* in the south of Brazil (late spring and early winter) might be due to ecological factors associated with the parasite–host interaction. At low temperatures, larvae evolution in the mollusk may be delayed and in the presence of heat and humidity they keep evolving, which coincides with their highest activity and the mollusks reproduction, enhancing chances to reach man.

A similar pattern was also observed in *Angiostrongylus cantonensis* (Yousif and Lammler 1975; Ishii 1984; Hori et al. 1985; Lv et al. 2006), *Angiostrongylus vasorum* (Guilhon and Gaalon 1969), *Umingmastrongylus pallikuukensis* (Kutz et al. 1999; Kutz et al. 2001), *Protostrongylus* spp (Samson and Holmen 1984), *Elaphostrongylus rangiferi* (Skorping 1984; Schjetlein and Skorping 1995), *Cystocaulus ocreatus* (Gerichter 1948), *Muellerius capillaris* (Gerichter 1948; Rose 1957) and *Parelaphostrongylus odocoilei* (Jenkins 2006) for which a strong association between temperature, larval development and infection rates in invertebrate hosts was shown. Other factors have also been mentioned as possible interference agents in

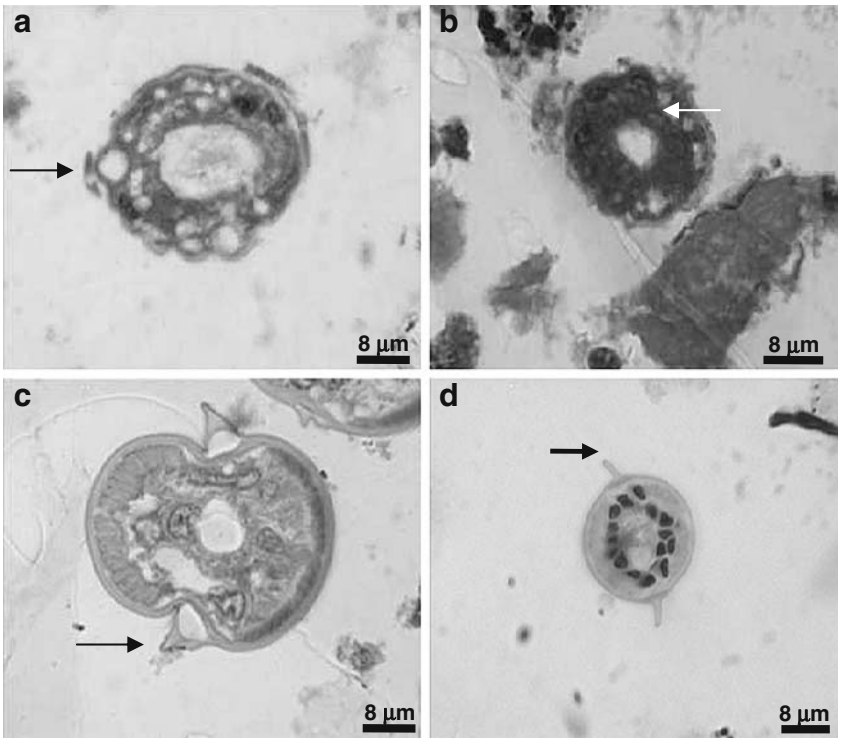

Fig. 3 **a** Transversal section of L_2 showing lipid drops in intestinal epithelial cells, which are located at a supra and subnuclear positions in the cytoplasm (*arrow*; JB-4 resin, MBBF). **b** Molting L_2–L_3, with a decrease in lipid vacuoles, displayed in a line position tending to the basal region of intestinal epithelial cells (*arrow*; JB-4 resin, MBBF). **c** Transversal section of L3: intestinal lumen and epithelium; cells located between intestinal epithelium and cuticle, muscular layer and prominent bilateral alae (*arrow*; JB-4 resin, MBBF). **d** Transversal section of L_1 at the esophagus level showing bilateral alae (semi-thin section, JB-4 resin, MFB, *Bar*=8 µm). MBBF=Lee's methylene blue-basic fuchsine

larval cycles of nematodes in their snail hosts such as age (Cabaret 1987; Cheng and Alicata 1965; Wallace and Rosen 1969), parasitic load (Gerichter 1948), susceptibility (Gerichter 1948; Halvorsen and Skorping 1982), lack of nutrients - aestivation (Solomon et al. 1996), and the host constitution (Rachford 1976). Therefore, we also believe that temperature may be the main environmental factor that directly influences larval development of Metastrongilidae, which constitutes important data in epidemiology and cycle maintenance in laboratory.

The main energetic source of parasite nematodes is glycogen, but during free-living life cycle lipid is the main one. Such reserves may be kept in hypoderm, into muscular cells and epithelial cells of intestine and reproductive organs.

During the larval period, lipid is kept in intestine and during encapsulating phase, in intestine lume (Rey 2001). Other studies have shown that the stored granules in intestinal cells used as energetic source consisted of glycogen and fat. Herein, Sudan Black and Oil red staining substantiated the predominant lipid composition of the granules during L_2 phase (Fig. 2a,b), which additionally contain also carbohydrate and/or glycoprotein, without evidence of glycogen. The carbohydrate content was partially constituted of *N*-acetyl-D-galactosamine and α-manosyl and α-*d*-glycosyl (Fig. 2d). Therefore, lipid and carbohydrate (glycolipid, glycoproteins…) were the main energetic source, not glycogen, implicated in the following larval development phase (L_3).

Analysis of the semi-thin sections (JB-4) of L_2 demonstrated that lipid granules were secreted by intestinal cells, which are located at a supra and subnuclear positions in the cytoplasm (Fig. 3a). During the transition of $L_2 \rightarrow L_3$, lipid moved preferentially to the subnuclear region of such cells (Fig. 3b), which probably, also synthesizes the glicídios.

The metabolic process which is triggered in the consumption of a large amount of synthesized lipid and glycoprotein during L_2 stage is still unknown. Some nematode use acetyl CoA, derived from β-oxidation of fatty acid, in order to make use of it during the Krebs cycle (Roberts and Janovy 2000). Probably, as in *Ancylostoma tubaeforme* Dubini 1843 and *Haemonchus contortus* Rud 1803 (Onwuliri 1985), larvae L_1 and L_2 of *A. costaricensis* are anaerobic, while infective L_3 are aerobic and able to perform β-oxidation of a great quantity of lipid synthesized by L_2.

This is the first report in the literature that emphasizes some internal morphological and biochemical characteristics of L_2.

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