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Original Article

Morphological effects on helminth parasites caused by herbicide under experimental conditions

Efeitos morfológicos em helminto parasito causado por herbicida em condições experimentais

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Abstract

Helminth parasites have been studied as potential accumulators for different pollutants. *Echinostoma paraensei* is a foodborne trematode whose vertebrate host, the rodent *Nectomys squamipes*, is naturally exposed to environmental pesticides. However, little information exists regarding the pesticide's effects on helminths. This study investigated the morphological effects on the trematode, *E. paraensei*, after experimental Roundup® herbicide exposure, in concentrations below those recommended for agricultural use. After two hours of exposure, scanning electron microscopy (SEM) showed changes to the tegument, such as furrowing, shrinkage, peeling, spines loss on the peristomic collar, and histopathological evidence of altered cells in the cecum and acinus vitelline glands with vacuoles and structural changes to the muscular layers. Glycidic content was decreased, primarily in the connective tissue. As *E. paraensei* is an intestinal parasite of the semi-aquatic wild rodent, *N. squamipes*, it is predisposed to pesticide exposure resulting from agricultural practices. Therefore, we emphasize the need to evaluate its impact on helminth parasites, due to their pivotal role in regulating host populations.

Keywords: Experimental model, herbicide, *in vitro* assays, morphology, trematode.

Resumo

Helmintos parasitos tem sido estudados como acumuladores potenciais para diferentes poluentes. O trematódeo *E. paraensei* tem como hospedeiro vertebrado o roedor *Nectomys squamipes* naturalmente exposto a pesticidas no meio ambiente. No entanto, pouca informação está disponível sobre os efeitos dos pesticidas em helmintos parasitos. O presente estudo investigou, em condições experimentais, os efeitos morfológicos no trematódeo *E. paraensei* após a exposição ao herbicida Roundup®, em concentrações abaixo das recomendadas para a utilização agrícola. A microscopia eletrônica de varredura (MEV) mostrou após duas horas de exposição, alterações no tegumento, como enrugamento, contração e descamação com perda de espinhos no colar peristômico e análise histopatológica evidenciou células do ceco alteradas, as glândulas vitelínicas com vacúolos e mudanças estruturais nas camadas musculares. Diminuição do conteúdo glicídico, principalmente no tecido conjuntivo, também foi observado. Considerando a predisposição à exposição a pesticidas agrícolas de *N. squamipes* infectado por *E. paraensei,* são necessários estudos para avaliar o impacto de tais resíduos frente aos helmintos e seus hospedeiros.

Palavras-chave: Modelo experimental, herbicida, ensaios *in vitro*, morfologia, trematódeo.

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Introduction

Helminth parasites have been studied as potential accumulators of different pollutants resulting from modern industrial processes, particularly gastrointestinal helminths, as they acquire nutrients from their vertebrate host's intestinal lumen contents (BRÁZOVÁ et al., 2015; MCGREW et al., 2015; TELLEZ & MERCHANT, 2015; SURES et al., 2017). Pesticides impact the environment and biodiversity and have been related to human pathologies linked to occupational activity and consumption of horticulturally exposed products (AKTAR et al., 2009).

Recently, we demonstrated that exposure to the herbicide, Roundup®, can affect the life cycle and water dependent larval stages of the trematode, *Echinostoma paraensei*, Lie & Basch, 1967, causing miracidia and cercariae mortality and reducing egg development to concentrations below those recommended for agricultural use (MONTE et al., 2016). *E. paraensei* is a foodborne trematode whose vertebrate host is the semi-aquatic rodent, *Nectomys squamipes* Brants, 1827 (LIE & BASCH, 1967; MALDONADO et al., 2001; BONVICINO et al., 2008). Because it is semi-aquatic, these rodents are naturally exposed to pesticides and pesticide degradation products in the environment (ERNEST & MARES, 1986; BONVICINO et al., 2008). However, little is known about the pesticide's effects on helminth parasites (MONTE & MALDONADO, 2017).

Therefore, this study investigated the morphological effects on the newly excysted larvae (NEL) and adult stages of *E. paraensei* after herbicide exposure.

Materials and Methods

Parasites and experimental infection

Helminth isolates were obtained from the wild rodents, *N. squamipes* (MALDONADO et al., 2001). The *E. paraensei* life cycle was maintained in the Laboratory of Biology and Parasitology of Wild Mammal Reservoirs (Oswaldo Cruz Institute, Rio de Janeiro, Brazil) through passages in *Mesocricetus auratus* Waterhouse, 1839 (hamsters) and *Biomphalaria glabrata* Say, 1818 (GARCIA et al., 2011). All animal experiments were conducted per the rules of the Oswaldo Cruz Foundation's Ethical Committee on Animal Use (License LW-51/14). To obtain newly excysted larvae (NEL), metacercarial cysts were recovered by stereomicroscopic dissection of the pericardial region of *B. glabrata* experimentally infected with *E. paraensei*.

Metacercariae were placed in alkaline trypsin bile salts medium (TB medium) containing 0.5% trypsin (1:250, C, GIBCO) and 0.5% bile salts (Sigma-Aldrich) with Earle's balanced salt solution (Earle's BSS), for the *in vitro* excystation (SOUZA et al., 2013).

Four female hamsters, aged three weeks, were experimentally infected. Each animal was administered 50 *E. paraensei* metacercariae by gavage obtained as described above, to obtain helminths at seven and fourteen days post-infection. The hamsters received commercial pellet food (Nuvilab) and water *ad libitum* and were maintained under a 12 h light/dark cycle, at 22 °C and 50% humidity. Two animals were euthanized in each period (7 and 14 days) after being anesthetized using ketamine (5 mg/kg, body weight) and xylazine (0.5 mg/100 g, body weight) and subsequently necropsied using a CO_2 chamber, to recover the helminths from the small intestine (FERRAZ et al., 2012).

Roundup® concentrations and in vitro exposure

The pesticide, Roundup® Original (480 g/L isopropylamine salt *N*-(phosphonomethyl) glycine; 360 g/L equivalent acid *N*-(phosphonomethyl) glycine; 684 g/L inert ingredients; Monsanto do Brasil Ltda), was purchased from a commercial source. Successive dilutions were obtained using RPMI 1640 culture medium (Sigma Aldrich), supplemented with 100 U/ml of penicillin, 100 mg/L of streptomycin, 0.25 mg/mL of amphotericin and 20% fetal bovine serum, to obtain the experimental concentrations. A total of 60 NEL, 20 7-day-old helminths and 20 14-day-old helminths, were washed twice in Locke's solution and transferred to the supplemented RPMI 1640 culture medium containing the following concentrations of Roundup®: 225 mg/L, 450 mg/L or 900 mg/L (exposed group), or to the culture medium alone (control group), for two hours at 37 °C and 5% CO_2 (PANIC et al., 2013). These concentrations are in accordance with those recommended for use in agricultural fields by the manufacturer (1-2%) (MONTE et al., 2016). The assays were performed in duplicate.

For histological analysis, 14-day-old helminths were exposed only to the highest concentration of Roundup® (900 mg/L) for 2 hours.

Morphological analysis

For scanning electron microscopy (SEM), the specimens (NEL, 7-day-old and 14-day-old helminths) were removed from the culture medium after 2 hours of exposure, transferred to petri dishes with Locke's solution and gently washed. After that, the samples were fixed for 1 hour in 2.5% glutaraldehyde diluted in 0.1 M sodium cacodylate buffer containing 3.5% sucrose at 4 °C, washed in the same buffer at pH 7.2, and post-fixed for 2 hours at 4 °C in a solution of 1% O_SO_4 in 0.1 M sodium cacodylate buffer. The specimens were then dehydrated through an ascending ethanol series (30-100%), for 1 hour at each step, with three passages in absolute ethanol, and dried using the critical point method with CO_2 (GONÇALVES et al., 2013). The material was mounted on aluminum stubs, coated with an approximately 20-nm layer of gold, and analyzed using a Jeol JSM 6390LV scanning electron microscope, in the Rudolf Barth Electron Microscopy Platform of IOC/Fiocruz.

For the histopathological analysis, the 14-day-old helminths were exposed for 2 hours to 900 mg/L of Roundup®. The helminths were then gently washed with Locke's solution and placed in Carson's Millonig formalin for 24 h. Next, the material was dehydrated through increasing concentrations of ethanol, clarified with xylene and embedded in liquid paraffin at 60 °C (TOLOSA et al., 2003). The inclusion was orientated to observe transverse sections of the helminth body structure. Subsequently, 5-µm thick serial sections were cut using a Leica RM2125 microtome. The sections were stained with hematoxylin and eosin (HE), Gomori's reticulin

and Periodic Acid-Schiff (PAS) reagents (CAPUTO et al., 2010). Histological sections from six helminths were measured to evaluate the damage caused by the herbicide. Measurements of the cecum, the distance between the inner border and the lumen of the cecum (cell hypotrophy) (4 measurements performed in each cecum) and the number of cecum epithelial cells were inferred from the number of nuclei observed. The tegument thickness from the external membrane to basal lamina and the number of acinus vitelline glands were also measured. Sections were viewed through a Zeiss Axio Scope.A1 and the images were captured by a Zeiss Axio Cam MRc camera. Measurements were processed using the Axio Zen 2 lite software.

For confocal scanning laser microscopy (CSLM), the 14-day-old helminths were exposed for 2 hours to 900 mg/L of Roundup®. The specimens were stained with hydrochloric carmine, dehydrated through a graded ethanol series, cleared with methyl salicylate and mounted in Canada balsam (SOUZA et al., 2011). The material was then analyzed using a Leica TCS SP8 confocal microscope and a 488-nm argon laser.

Statistical analysis

The paired t-test was used to compare the mean differences among measures performed on the cecum, the tegument thickness to the basal lamina and the acinus vitelline glands for the control and experimental groups. All data were tested for normal distribution using the Kolmogorov-Smirnov test. Values with $p \leq 0.05$ were considered significant (InStat, GraphPad, v.4.00, Prism, GraphPad, v.3.02, Prism, Inc.).

Results

Scanning electron microscopy (SEM)

The NEL showed preserved oral and ventral suckers with presence of papillae, as well as the peristomic collar, with slightly longer lateral and corner spines (Figures 1A, B). In the group exposed to 225 mg/L of Roundup®, an intense musculature contraction of the posterior end was observed, forming a node (Figure 1C). The oral and ventral suckers of the 7- and 14-day-old helminths were well-defined and preserved as was the excretory pore (Figures 1D-F). In the 14-day-old helminths, vesicle scattering was evident in some areas of the body, including in the excretory pore (Figure 1G), and swollen tegument was noted with the spines submerged by the surrounding tegument (Figure 1H). In the group exposed to 450 mg/L, the 7-day-old helminths lost their peristomic collar spines (Figure 1I), and the 14-old-day helminths showed dorsal surface peeling (Figure 1J). The group exposed to 900 mg/L also presented swelling and furrowing along with spine loss in the peristomic collar (Figure 1L) as well as muscular contraction in the post-acetabular region (Figure 1M).

Histopathological analysis

The control group had cecum formed by cylindrical cells with basal nuclei and cells with one or two nucleoli (Figure 2A), while the exposed group presented vacuolated cecal epithelial cells (Figure 2B). The testes contained rosette-shaped structures with sperm bundles appearing normal in the control group (Figure 2C), but after two hours of exposure, spermatogenesis was evident in the testes within the gonad due to disruption of the testicular parenchyma (Figure 2D). The control group tegument presented well-defined structures with syncytium, basal lamina, circular and longitudinal muscle layers and loose connective tissue with parenchyma cells inside the trematode body (Figure 2E). After two hours of exposure, the tegument changed, with reduced and damaged structures and disrupted and disorganized muscle layers. Parenchymal cells associated with the tegument were disordered (Figure 2F). A significant difference was observed between the control and exposed groups in comparing the means for cecal cell hypotrophy, the distance between the cecal border and the lumen and the number of parenchyma cells. However, no significant difference was observed in the cecal area of between the two groups. Significant differences were also observed for the thickness of tegument to basal lamina and the number of acinus vitelline glands compared to the control and exposed groups (Table 1).

Additionally, after 2 hours of exposure, the PAS stained histological sections, presented decreased glycidic content compared to the control group, mainly in the connective tissue around the cecum and the vitelline glands, as well as in the acinus vitelline glands themselves. Tegument loss was observed in the basal lamina (Figures 3A, B). Spermatogenesis was not altered upon histological observation (Figure 3B). The Gomori's reticulin stain revealed

Table 1. Measurements from histological sections from six *Echinostoma paraensei* specimens to evaluate the damages caused by the herbicide Roundup®, performed in duplicate.

	14-day-old Helminths				
	Control group	Exposed group (900 mg/L)			
	Mean±SD	Mean±SD	t	p	df
Cecum					
Area (μm²)	6216.7 ± 2243.3	6950.3 ± 1294.8	0.69	0.5036	5
Cell hypotrofy area (μm²)	134.3 ± 77.7	2716.2 ± 1231.8	5.00	<0.01	5
Border - lumen (μm)	28.8 ± 5.6	14.2 ± 2.9	4.54	<0.01	5
N° parenchyma cells	23.3 ± 3.5	14.6 ± 2.6	4.24	<0.01	5
Tegument - Basal lamina (μm)	8.7 ± 1.9	3.7 ± 0.8	4.84	<0.01	5
N° acinus vitelline glands	17.2 ± 3.3	12.8 ± 3.3	5.70	<0.01	5

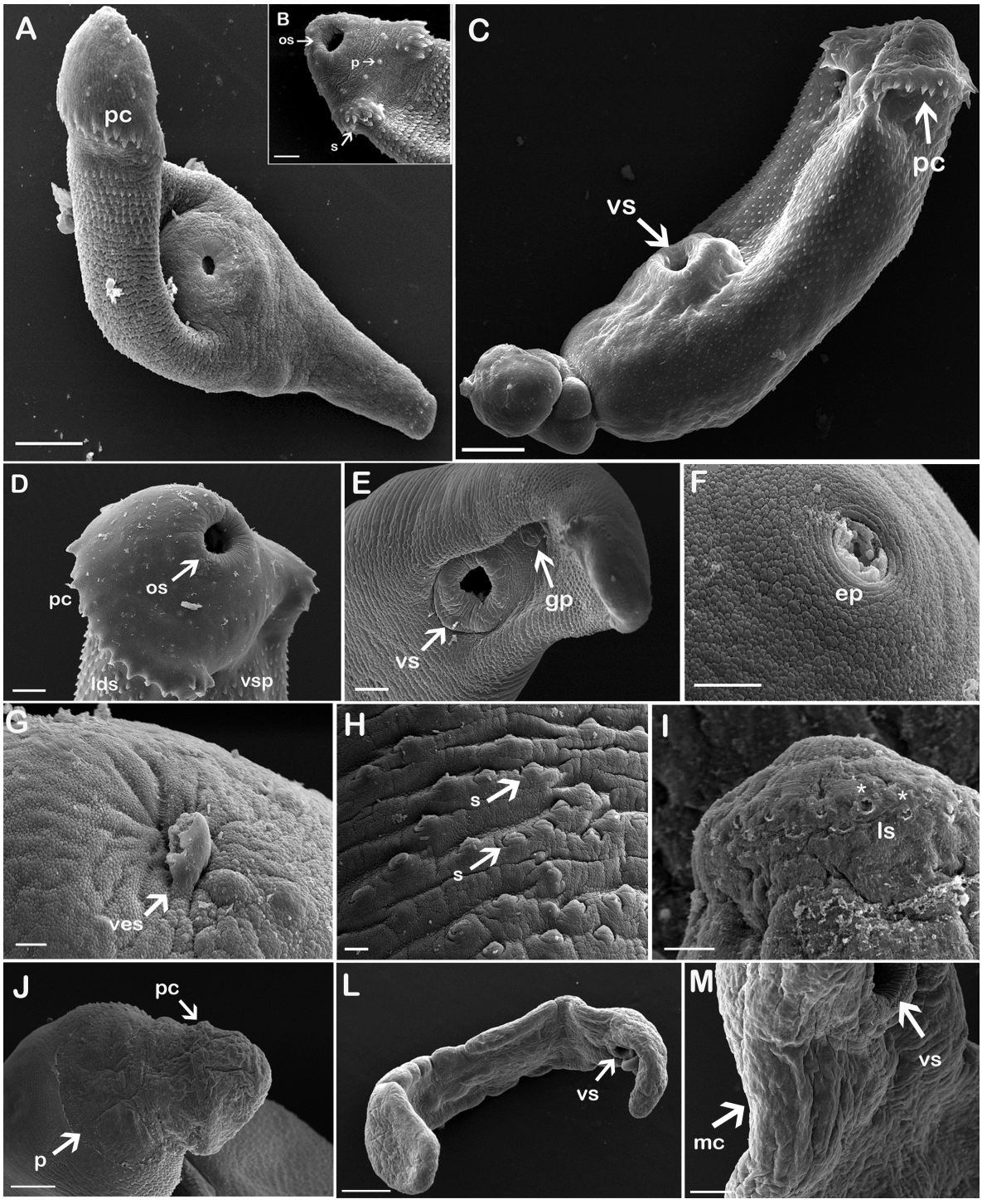

Figure 1. Scanning electron micrograph of *Echinostoma paraensei*. (A, B) Control group: NEL - Preserved peristomic collar (pc) (A). Bar: 20 µm. Detail of preserved oral sucker (os) surrounded by peristomic collar with spines (s) and presence of papillae (p) (B). Bar: 10 µm; (C) Group exposed for two hours to 225 mg/L of Roundup®: NEL - Furrowing tegumental surface, including the ventral sucker (vs) and peristomic collar region (pc), and a posterior end node due to intense musculature contraction. Bar: 20 µm; (D-F) Control group: 7-old-day helminths - Peristomic collar (pc) and oral sucker (os) with preserved morphology. Preserved latero-dorsal spines (lds) and ventral spines (vsp) (D). Bar: 20 µm. 14-old-day helminths - Preserved ventral sucker (vs) and prominent genital pore (gp) (E). Bar: 100 µm. Fourteen-old-day helminths - Excretory pore (ep) with preserved aspect (F). Bar: 10 µm; (G, H) Group exposed for two hours to 225 mg/L of Roundup®: 14-old-day helminths - Excretory pore tegument with vesicles (ves) (G). Bar: 10 µm. Swollen tegument with spines (s) submerged by the surrounding tegument (H). Bar: 10 µm; (I-M) Group exposed for two hours to 450 mg/L and 900 mg/L of Roundup®: 7-old-day helminths - Peristomic collar region with loss of spines (*ls) (I). Bar: 20 µm. 14-old-day helminths - Peeling of the dorsal region of the body (p) and shrinkage of the peristomic collar with no spines (pc) (J). Bar: 100 µm. Body with swollen and furrowed tegument. Ventral sucker (vs) (L). Bar: 500 µm. Muscle contraction (mc) in the post acetabular region (vs) (M). Bar: 100 µm.

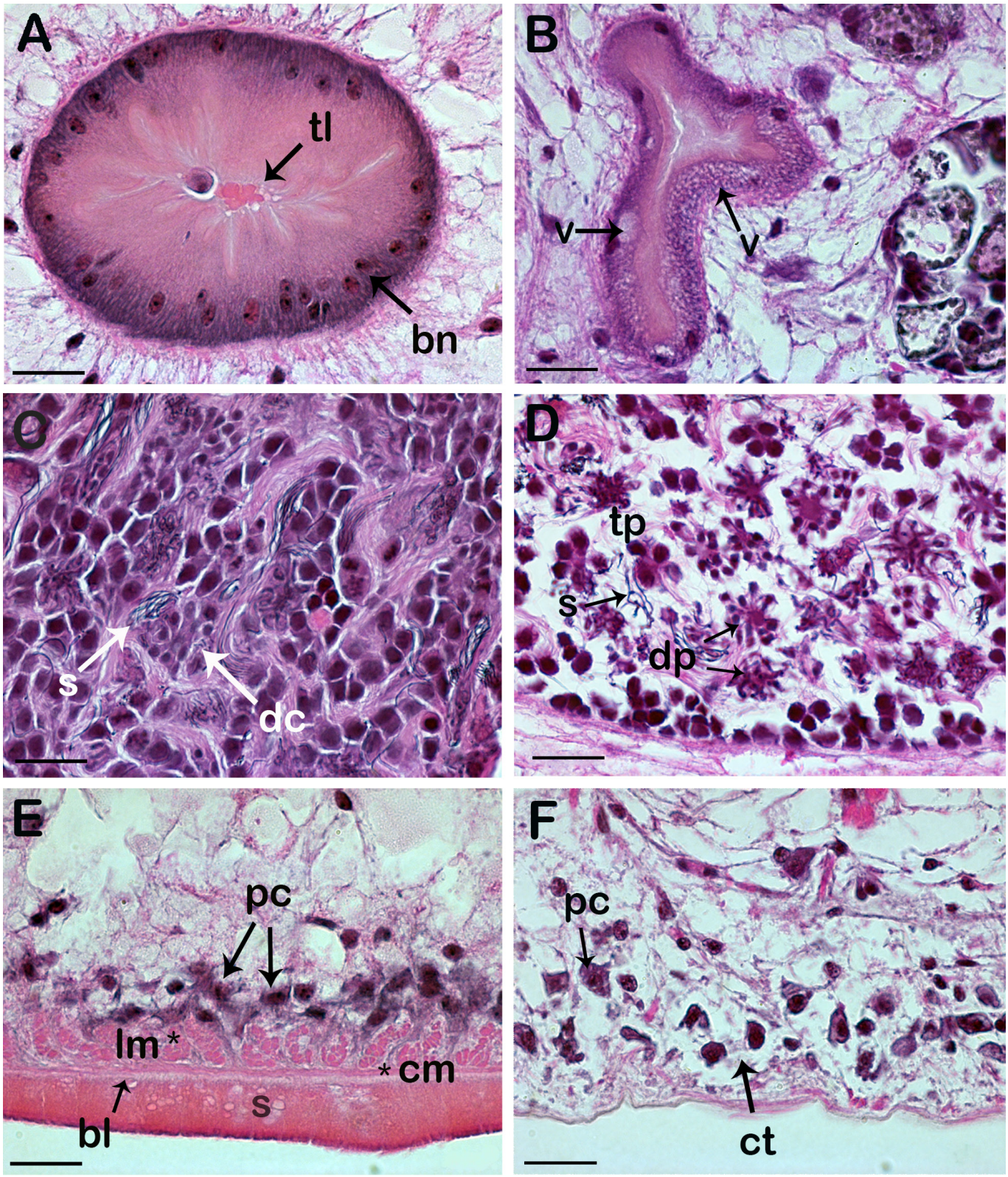

Figure 2. Histological sections of 14-old-day *Echinostoma paraensei* HE-stained. (A) Control group: Cecum with thin lumen (tl) and cylindrical cells with basal nucleus (bn) with one or two nucleoli; (B) Group exposed for two hours to 900 mg/L of Roundup®: Cecum cells with presence of vacuoles (v) (C) Control group: Testis with normal appearance of cell division (dc) with sperm bundle (s); (D) Group exposed for two hours to 900 mg/L of Roundup®: Disruption of the testicular parenchyma (tp) evidencing the differentiation process (dp), with presence of sperm (s); (E) Control group: Preserved tegument with syncytium (s), basal lamina (bl), circular (*cm) and longitudinal muscle layer (*lm), and loose connective tissue with parenchyma cells (pc); (F) Group exposed for two hours to 900 mg/L of Roundup®: Changed tegument (ct) with disordered parenchymal cells (pc). Bar: 50 µm.

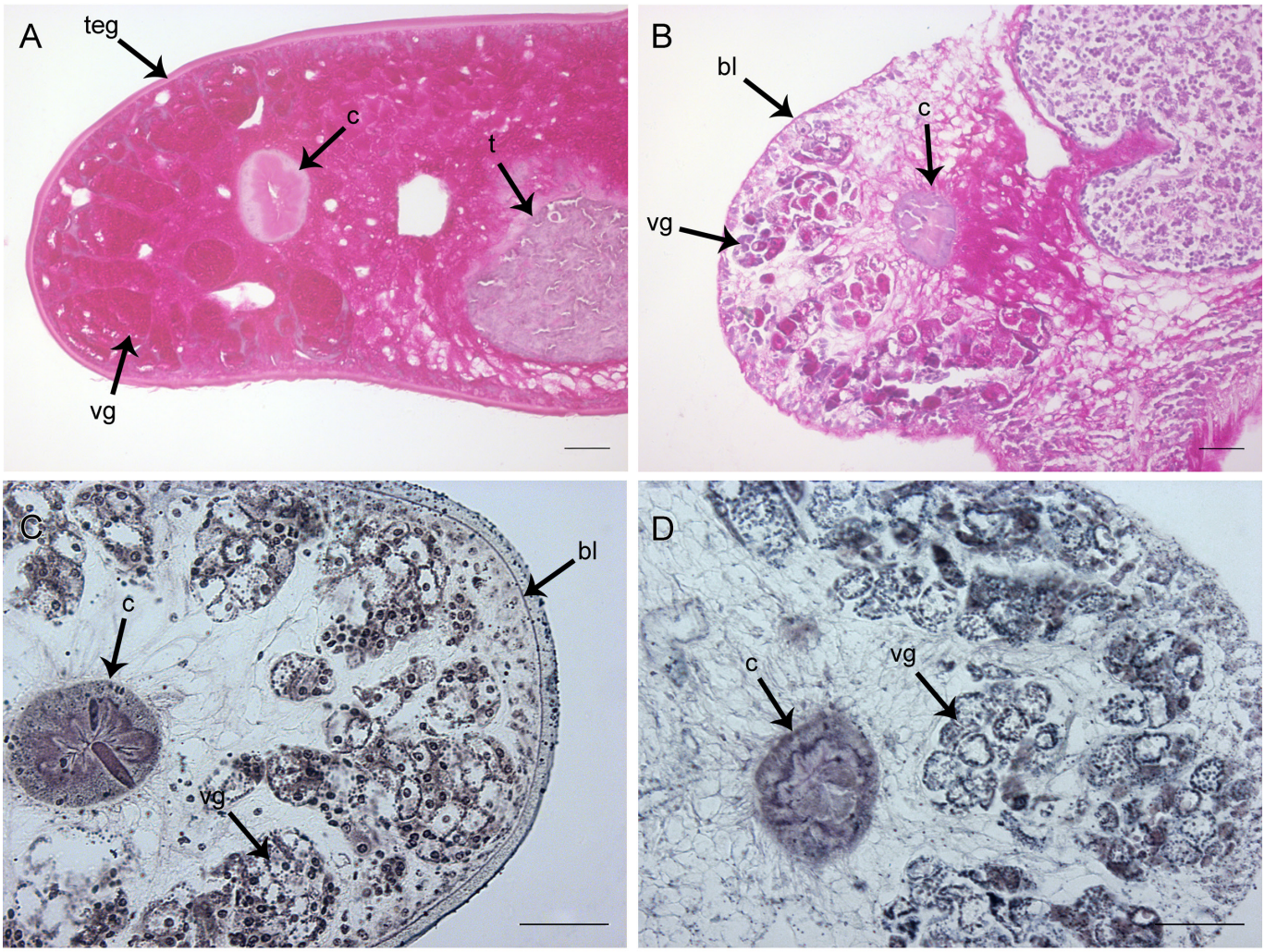

Figure 3. Histological sections of 14-old-day *Echinostoma paraensei* stained in PSA and Gomori's Reticulin. PSA: (A) Control group: Presence of intense glycidic content in the parenchyma and acinus vitelline glands (vg). Cecum (c), testis (t) and tegument (teg) preserved; (B) Group exposed for two hours to 900 mg/L of Roundup®: decreased glycidic content in the parenchyma and vitelline glands (vg) with basal lamina (bl) evident by loss of tegument. Cecum (c). Gomori's Reticulin: (C) Control group: Cecum (c) and vitelline glands (vg) with preserved morphology. Evident basal lamina (bl); (D) Group exposed for two hours to 900 mg/L of Roundup®: degeneration of the cecum (c) and vitelline cells (vg) showing no nuclei. Bar: 50 µm.

tegument loss with deteriorated basal lamina and degenerated cecum and vitelline cells with no nuclei (Figures 3C, D). No changes were observed in the parenchymal reticular fibers of the control and experimental groups.

Confocal scanning laser microscopy (CSLM)

CSLM revealed changes primarily to the vitellic glands and cecum. The control group revealed preserved acinus vitelline glands with vitellic cells presenting normal nuclei as well as normal body parenchyma (Figure 4A) and the cecum was preserved with a thin lumen (Figure 4B). After exposure the acinus vitelline glands were disorganized with cells revealing nuclei loss (Figure 4C), and the cecum was altered with epithelial damage to the cecum, evidencing an apparent dilated lumen (Figure 4D).

Discussion

Subsequent to the expanded use of herbicide in agricultural practices, studies have verified that herbicidal effects are not restricted to the target species for which they were designed (LANGIANO & MARTINEZ, 2008; LANCTOT et al., 2014). Since then accumulating evidence has shown the effects of herbicide on the diverse trophic levels, including parasitic helminths. However, there is little information on these effects and their mechanisms of action.

Recent evidence has show the effects of *in vitro* exposure to the herbicide, Roundup®, on the trematode parasite, *E. paraensei*, whose biological cycle requires fresh water. Exposure to low concentrations of the herbicide can lead to larval stages death in the cercariae and miracidia and impair larval embryonic development inside the eggs (MONTE et al., 2016). These findings were attributed

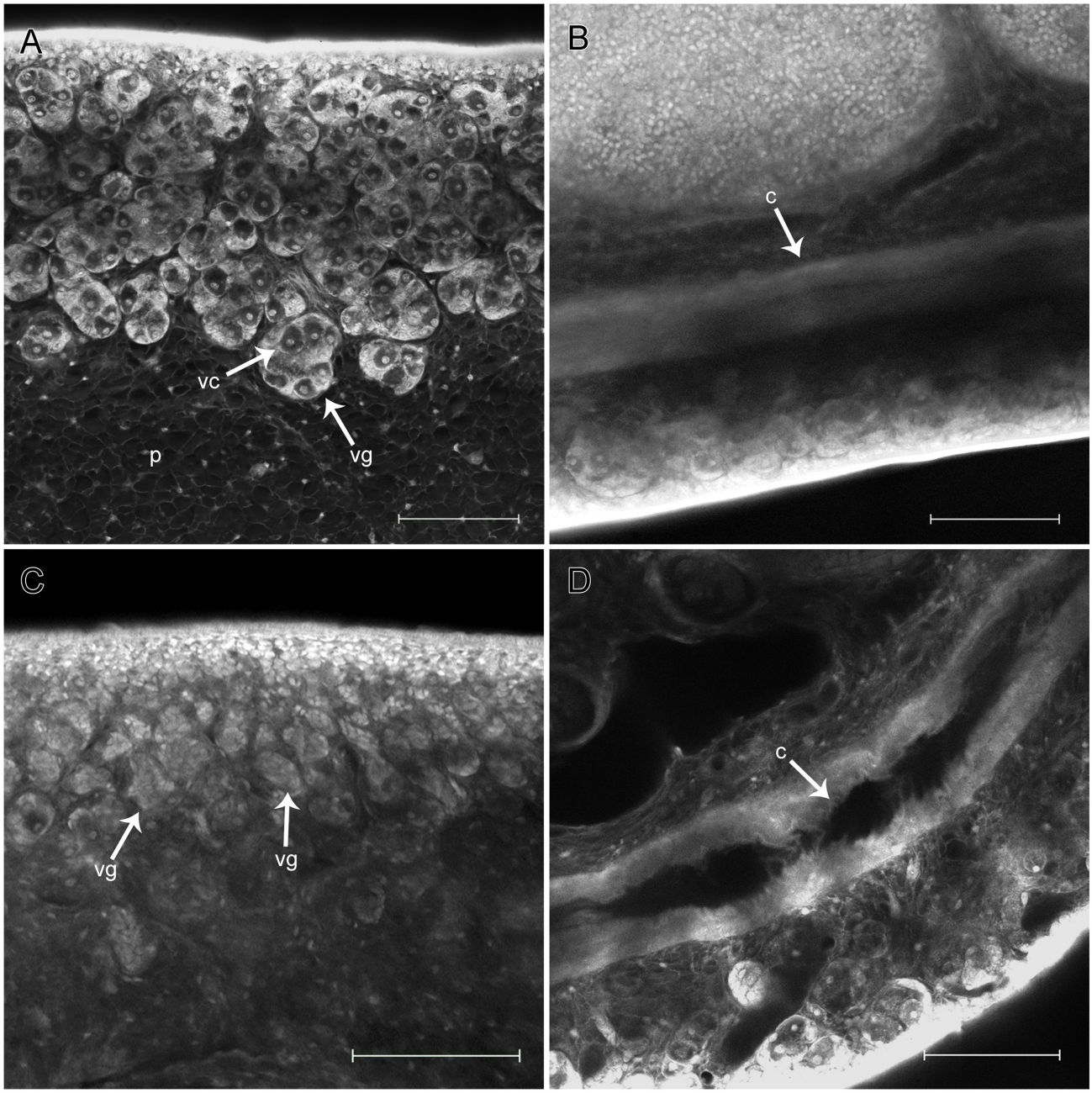

Figure 4. Confocal scanning laser microscopy images of whole-mounts preparations of 14-old-day *Echinostoma paraensei* stained with hydrochloric carmine: (A) Control group: Preserved acinus vitelline glands (vg) with vitellic cells (vc) with normal aspect as well as the body parenchyma (p); (B) Control group: Cecum with preserved form with thin lumen (c); (C) Group exposed for two hours to 900 mg/L of Roundup®: Acinus vitelline glands with cells with lost nuclei (vg); (D) Group exposed for two hours to 900 mg/L of Roundup®: altered cecum with apparent damage to the cecal epithelium (c). Bar 100 μm.

primarily to the presence of the surfactant in the commercial formulation, polyethoxylated tallow amine (POEA), an emulsifier that facilitates glyphosate entry and diffusion through plant cells walls to increase its effectiveness (WILLIAMS et al., 2000). In this study, morphological and histopathological alterations in this trematode after exposure to Roundup® were observed. However, few morphological studies are available on this herbicide that concern parasitic helminths (MONTE & MALDONADO, 2017).

Our results demonstrated that in the control group, all specimens showed general morphology similar to that previously described by Maldonado et al. (2001), such as an elongated and slender body with lateral borders curved ventrally on the longitudinal axes, enlarging from the ventral sucker onwards. The lowest concentration tested could promote tegumental surface damage. Similar alterations were observed after exposure to the antihelmintic, praziquantel, on the surface of trematodes, including swollen and pronounced changes

of the peristomic region and peeling and formation of vesicles (FERRAZ et al., 2012; GONÇALVES et al., 2013), suggesting that the anthelminthic acts by disrupting calcium homeostasis (GREENBERG, 2005).

Likewise, *in vitro* studies on *Echinostoma caproni* Richard, 1964 and a trematode of the genus, *Schistosoma*, also noted tegumental damage and curling, body shrinkage and vesiculation along the tegumental surface after treatment with different anthelmintics related to changes in muscular activity (praziquantel, tribendimidine, albendazole and quinine) (XIAO et al., 2009; PANIC et al., 2013). Changes in motor activity with intense body contraction and loss of the muscle layer underlying the tegument were also seen in *E. paraensei.*

Tansatit et al. (2012), compared the *in vitro* effects of triclabendazole and a derivate of artemisinin (artesunate) on *Fasciola gigantica* Cobbold, 1855 and observed similar morphological changes from both drugs, primarily consisting of swollen and disrupted tegument with blebbing and erosion. These findings were also observed by Souza et al. (2017) using *E. paraensei* newly excysted metacercariae treated with artesunate. Although the chemical species tested in these studies have different formulations, similar alterations were observed in our study, indicating that these formulations initially act on the same targets on the tegumentary surface, altering the membrane stability. The commercial composition of the pesticide tested in this study includes the surfactant POEA, which can altered tegumental integrity. This can be related to an imbalance in osmosis, resulting in a disturbed ion flux across the membrane, as already observed in other substances (MEHLHORN et al., 1983; SCHMAHL & MEHLHORN, 1985; SOBHON et al., 1986).

Histological changes caused by *in vitro* exposure to the herbicide, norflurazon, in planarian *Polycelis felina* Dalyell, 1814, revealed damage to the outer mucosal layer, epidermis and parenchymal cells (HORVAT et al., 2005). Degradation of parenchymal tissue with cell injury was also seen in this study, along with impaired tegument. These studies suggest potential cytotoxic effects from these herbicides on platyhelminths with consequent death based on the concentration to which they were exposed.

In a recent study, the earthworm, *Eisenia fetida* Savigny,1826, was compared histopathologically using five pesticides (insecticides and fungicides), and the results verified damage to the muscular layers, potentially resulting in nervous systems disorders (RICO et al., 2016). Although these studies involved other pesticide classes, we found that Roundup® Original led to disrupted and disorganized muscle layers, spastic movements and formation of a node in the post-acetabular region after two hours of exposure, suggesting nervous system involvement. More studies are needed to clarify these results.

Some studies have shown that glyphosate-based herbicide exposure decreases glycogen levels in different organisms (DORNELLES & OLIVEIRA, 2014; SINHORIN et al., 2014). This polysaccharide is essential for energy balance because it provides an internal energy reserve, and its depletion is associated with stress from environmental pollutants exposure, since the increased energy demand helps the metabolism involved in detoxifying xenobiotics (MOYES & SCHULTE, 2010). The present study evaluated acute exposure to the herbicide and observed a loss of glycidic content in *E. paraensei*, which may include glycogen, glycoproteins and glycolipids in the cells and cell membranes (VUTUKURU, 2005). This may be from the parasite's attempt to eliminate the toxic pollutant as previously described.

Few studies report the effects caused by glyphosate-based herbicides on the connective tissue, specifically on the reticular fibers that are primarily formed by type III collagen fibers. Histological observations of rodent liver cells exposed to the highest concentration of glyphosate-based herbicide, found increased connective tissue and reticulin fiber deposition, suggesting modified substance diffusion and impaired hepatic function (BENEDETTI et al., 2004). Our data did not show reticular fiber changes in the helminths exposed to Roundup®. This may be due to the short exposure period, with insufficient time for the increased deposition of type III collagen fibers.

Although different studies have evaluated the effects of the Roundup® on other model organisms, most of these studies focused on the biological and physiological impact of this herbicide (ADAM et al., 1997; LANGIANO & MARTINEZ, 2008; LANCTOT et al., 2014), and few studies exist regarding the ultrastructural effects. In conclusion, our data indicate that in addition to the biological effects on *E. paraensei*, morphological changes also occurred after exposure to Roundup® under experimental conditions. As *E. paraensei* is an intestinal parasite of the semi-aquatic wild rodent, *N. squamipes*, it is thus predisposed to agricultural pesticide exposure. Therefore, we emphasize the need to evaluate the herbicide's impact on parasitic helminths, since it is pivotal in regulating the hosts population.

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