PALEONTOLOGY

Paleoparasitological analysis of a coprolite assigned to a carnivoran mammal from the Upper Pleistocene Touro Passo Formation, Rio Grande do Sul, Brazil

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Abstract: A paleoparasitological analysis was carried out on a large coprolite assigned to a carnivoran mammal, recovered from the Municipality of Uruguaiana, in the western region of the State of Rio Grande do Sul, Brazil, where the Upper Pleistocene Touro Passo Formation crops out. For this, an individual sample was extracted from the specimen using an electric drill, dissociated with 10% hydrochloric acid solution, washed with distilled water, and sifted through a 500 mesh Tyler sieve. After laboratory processing, the sediment retained on the sieve was mixed with glycerin and examined by optical microscopy, which revealed the presence of 14 protozoan oocysts and three nematode eggs. The morphological characteristics of the oocysts (i.e., spherical shape, thick-walled, internal zygote apparently at the beginning of sporulation, as well as their size) and of the eggs (i.e., ovoidal shape, rounded ends, smooth surface, thin-shelled, embryo in their interior, along with their morphometry) suggest that these specimens belong respectively to the orders Eucoccidiorida and Strongylida (Family Ancylostomatidae) represented by several parasitic species of the alimentary tract of modern carnivore. This is the first record of paleoparasites discovered in a vertebrate host from the Touro Passo Formation.

Key words: Ancylostomatidae, Eucoccidiorida, Ichnology, Lujanian, Paleoparasitology.

INTRODUCTION

The Upper Pleistocene Touro Passo Formation occurs in the western region of the State of Rio Grande do Sul, Southern Brazil and has an expressive fossil record, originated probably from fluvial deposits and alluvial sedimentation occurred in the last 42,000 years (Lujanian) (Kerber & Oliveira 2008a). The main fossils, discovered since the early 1970s, include several vertebrates (Bombin 1976, Oliveira 1996, Martins & Oliveira 2003, Oliveira et al. 2003, Hsiou 2007, Scherer et al. 2007, Kerber & Oliveira 2008a, b, Gasparini et al. 2009, Kerber et al. 2010, 2014), mollusks (Bombin 1976, Oliveira & Milder 1990, Kotzian & Simões 2006) and phytoliths (Bombin 1976).

The term coprolite, denotes petrified feces, droppings or excrement and is used in paleontological and archaeological studies (Ferreira et al. 2008, Hunt et al. 2012). Coprolites provide interesting paleoecological evidences, such as the diet and physiology of the producer, the presence of organic inclusions, intestinal microbiota and endoparasitic fauna, which can not be accessed through body remains (De

Baets & Littlewood 2015, Bajdek et al. 2016, Vajda et al. 2016, Dentzien-Dias et al. 2018, De Baets et al. 2020). Additionally depending on the type of paleoparasite found in a coprolite, it is also possible to infer about their probable hosts and pathogenic effects on them, as well as food chains, paleoenvironmental and paleoclimatic conditions of the ecosystems in the geological moment which these organisms were fossilized, using the necessary requirements for survival and maintenance of the biological cycles of the species of similar modern parasites as proxies.

Despite advances in paleoparasitological analysis of human coprolites, including the current possibility of DNA extraction from parasites, especially by some South American parasitologists since the 20th century (Ferreira et al. 2011), there are few contributions on this topic regarding animal coprolites worldwide. Nevertheless these studies have increased in recent years and have already been able to reveal the presence of parasitic protozoans and helminths in coprolites of sharks (Dentzien-Dias et al. 2013), dinosaurs (Poinar & Boucot 2006), crocodyliforms (Cardia et al. 2018, 2019a, b, Dentzien-Dias et al. 2018), dinornithiform birds (Wood et al. 2013), non-mammalian therapsids (Hugot et al. 2014, Silva et al. 2014, Bajdek et al. 2016, Francischini et al. 2018), and different mammals, such as rodents (Sardella & Fugassa 2009a, b, 2011, Sardella et al. 2010, Beltrame et al. 2012, 2013, 2014, 2018, 2019, Souza et al. 2012, Mowlavi et al. 2014), ruminants (Fugassa et al. 2008, Sianto et al. 2012, Taglioretti et al. 2015, 2017, Beltrame et al. 2017a, b, Nunes et al. 2017) and carnivorans (Fugassa et al. 2006, 2009, 2013, 2018, Beltrame et al. 2010, 2016, Sianto et al. 2014, Mowlavi et al. 2015, Fugassa & Petrigh 2017, Perri et al. 2017, Petrigh et al. 2019, Tietze et al. 2019). The oldest confidently identified paleoparasitological record in a coprolite derives from the Permian and is dated in up to 259.8 million years (Dentzien-Dias et al. 2013), although possible even older Carboniferous remains have been reported (Zangerl & Case 1976), demonstrating an universe of possibilities still to be explored in the Veterinary Paleoparasitology and Paleoichnology areas, mainly in geological units with rich and abundant paleofaunas, such as the Touro Passo Formation.

Therefore the present study describes the first record of paleoparasites in a unique coprolite found in the Touro Passo Formation, assigned to a carnivoran mammal.

MATERIALS AND METHODS

Coprolite

The coprolite analyzed in this study was preliminarily described by Kerber & Oliveira (2008a) and it comes from the Milton Almeida outcrop (29°40'20.57" S; 56°51'59.20" W), in the margins of the Touro Passo stream, located in the Municipality of Uruguaiana, in the western region of the State of Rio Grande do Sul, in Southern Brazil (Fig. 1). This fossil locality belongs to the Touro Passo Formation, which is Lujanian (Late Pleistocene) in age and lithologically composed by silty-sandy rocks of fluvial origin with abundant carbonate concretions layers (Kerber & Oliveira 2008a). The coprolite was originally housed in the Setor de Paleovertebrados of the

Figure 1. Coprolite (MCP-5176-PV) assigned to Carnivora mammal recovered from the Milton Almeida Outcrop, Touro Passo Formation (Upper Pleistocene), Municipality of Uruguaiana, Western region of State of Rio Grande do Sul, Southern Brazil.

Museu de Ciências Naturais of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS at Uruguaiana), under the collection number MCPU-PV-141. After the deactivation of this museum, the specimen was transferred for the collection of the Setor de Paleovertebrados of the Museu de Ciências e Tecnologia of the PUCRS located at the Porto Alegre campus, with a new collection number: MCP-5176-PV.

This coprolite was preserved through phosphatization and is apparently complete, with a size of 18.0 cm in length and of 4.0 cm in diameter. Additionally the coprolite had grayish white coloration and cylindrical with rounded ends, consolidated and homogeneous shape, divided into four blocks by constrictions, besides the presence of large amount of organic material, such as parts of undigested bones. This morphology resembles that proposed for big felids by Chame (2003), such as the genera *Panthera* and *Puma*, however the large size of the coprolite and the finding of bones may also suggest other extinct large Carnivora mammals, such as the genera *Smilodon, Protocyon, Theriodictis* and *Arctotherium*, which were able to hunt large prey like ground sloths or feed on their carcasses. Fossil remains of carnivorans are still unknown from the Touro Passo Formation, but other Late Pleistocene (Lujanian) records from the Rio Grande do Sul, include material assigned to canids (*Dusicyon* cf. *D. avus, Protocyon troglodytes,* and *Theriodictis* sp.), felids (*Smilodon populator*) and ursids (cf. *Arctotherium* and indeterminate remains) (Rodrigues et al. 2004, Ribeiro & Scherer 2009, Pereira et al. 2012, Lopes 2013 and references therein).

Laboratory processing

Two individual samples (≅ 1.0 g) were extracted from the coprolite surface and its internal portion using an electric drill, resulting in some macerated material (following the procedure made by Silva et al. 2014). The resulting product of each sample was stored individually in properly labeled two Falcon 15 mL polypropylene tubes. A 10% hydrochloric acid solution was added to the first tube, as proposed by Ferreira et al. (2011). Upon dissociation of the minerals, the reaction was stopped by adding a double volume of distilled water.

The resulting solution was washed several times with distilled water, and then sifted through a 500 mesh Tyler sieve, following Bouchet et al. (1999). The residual material from sample retained on the sieve was washed again in distilled water. A drop of this material was then placed on a microscope slide, three drops of glycerin were added, and the slide was covered with a coverslip. The material was analyzed by bright field microscopy at 100x and 400x magnifications using a Quimis® optical microscope and images of the paleoparasites observed were recorded with a Sony® digital camera coupled to it. Each paleoparasite was measured individually using a Bel Photonics® ocular micrometer. Measurements of the length and width of the paleoparasitic specimens were expressed in micrometers (μm), as mean ± standard deviation and range in parentheses.

The macerated material of the second tube was rehydrated by immersion in a 0.5% solution of trisodium phosphate and submitted to spontaneous sedimentation following to Iñiguez et al. 2006. Upon 72 h, aliquots of the resulting sediment were used for the extraction and detection of the *Toxoplasma gondii* DNA. For this, these aliquots were grinded by Bio-vortex homogenizer (Biospec Products Inc, USA). DNA extraction was carried out by using the IllustraTM Tissue & Cells Genomic Prep Mini Spin kit (GE Healthcare, USA) and quantification in a spectrophotometer (Epoch-Biotek, USA). PCR reactions were performed by employing

the primers described by Homan et al. (2000) to amplify a 529bp fragment. Primers TOX4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and TOX5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') were used. The reactions (25 µL final volume) were run with the following reagents: 10 mM Tris HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP, 10 pmol of each primer, 0.2 units of Taq DNA polymerase, and 10 ng of DNA template. Amplification was performed in a Veriti (Life Technologies®, Carlsbad, USA). Initial denaturation for 7 minutes at 94°C was followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C, and final extension for 10 minutes at 72°C. The sequence was analyzed by electrophoresis in 1.5% agarose added with 0.1 µL/mL of SYBR® safe DNA gel stained (Invitrogen, USA), recorded using the transilluminator (Syngene, USA), and the image was captured by the digital documentation system.

RESULTS

The parasitological analysis revealed 14 oocysts of spherical shape, with a thick wall and internal zygote apparently at the beginning of sporulation, measuring 11.9 ± 1.1 µm (10.0 µm – 12.5 µm) long and 11.5 ± 1.2 µm (10.0 µm – 12.5 µm) wide, compatible with Eucoccidiorida (Apicomplexa: Coccidia) protozoans (Fig. 2a, b, c). In addition, three eggs of ovoidal shape, with bluntly rounded ends and a thin and smooth hyaline shell, apparently embryonated, measuring 60.8 ± 1.4 µm long (60.0 µm – 62.5 µm) and 30.8 ± 1.4 µm (30 µm – 32.5 µm) wide, similar to Strongylida (Nematoda: Rhabditia) nematodes, probably from the Family Ancylostomatidae, were recovered (Fig. 3a, b, c). Other organic inclusions were observed under optical microscopy in this ichnofossil, such as
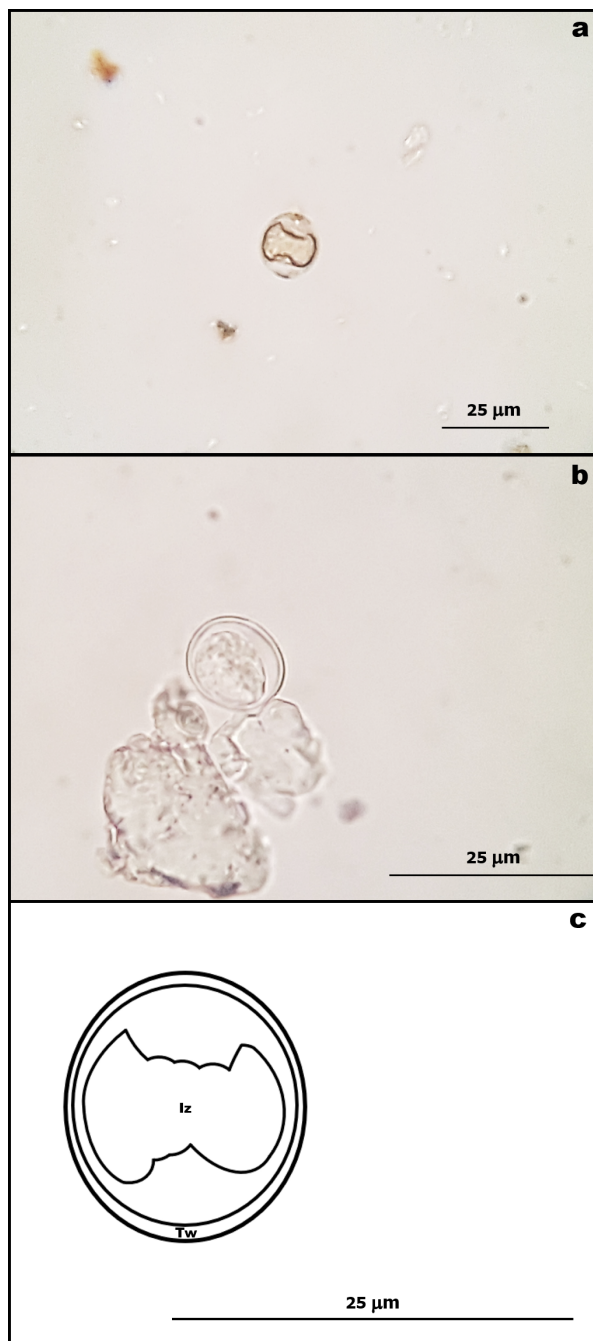

Figure 2. Eucoccidiorida subspherical oocysts recovered in coprolite assigned to Carnivora mammal from the Milton Almeida Outcrop, Touro Passo Formation (Upper Pleistocene), Municipality of Uruguaiana, Western region of State of Rio Grande do Sul, Southern Brazil (400 X magnification). a-b. Photomicrographs; c. Representative drawing. Abbreviations: Tw = thick wall; Iz = internal zygote apparently at the beginning of sporulation.

fungal microconidias. The sample was negative by PCR with specific primers for *T. gondii*.

DISCUSSION

Thick-walled subspherical sporulated oocysts and thin-shelled ovoidal embryonated eggs are respectively the typical evolutive stages of most modern species of Eucoccidiorida protozoans and Strongylida nematodes (including Ancylostomatidae) that parasitize vertebrates which can be found in the feces of their final hosts during their sexual reproduction phases (University of Illinois 1933, Anderson 2000, Berto et al. 2014).

Eucoccidiorids are intracellular obligatory parasites that usually injure intestinal tissues (Monteiro 2017). Oocysts of this group of protozoans contain the zygotes and, generally, are unsporulated when liberated in the feces by their hosts. Extant genera with parasitological significance for carnivoran mammals include *Besnoitia, Cryptosporidium, Cystisospora, Eimeria, Hammondia, Hepatozoon, Neospora, Sarcocystis,* and *Toxoplasma,* being some of these acquired by the predation of other vertebrates (Taylor et al. 2015). This latter, represented by the species *Toxoplasma gondii*, is from special interest because of its medical and veterinary importance (Rey 2010). The oocysts described here have the size and shape expected for the genera *Toxoplasma, Hammondia and Neospora*, but the lack of well-defined sporocysts, sporozoites and other secondary structures within the oocysts precludes a more assertive classification of the materials. The final hosts *Toxoplasma* and *Neospora* are respectively felids and canids, while *Hammondia* can inhabit the small intestine of both groups. However, these three genera can parasitize other mammals as intermediate hosts, besides some species of

Figure 3. Strongylida ovoidal eggs (probably Ancylostomatidae) recovered in coprolite assigned to Carnivora mammal from the Milton Almeida Outcrop, Touro Passo Formation (Upper Pleistocene), Municipality of Uruguaiana, Western region of State of Rio Grande do Sul, Southern Brazil (400 X magnification). a-b. Photomicrographs; c. Representative drawing. Abbreviations: Ths = thin hyaline shell; E = embryo.

birds as in the case of *Toxoplasma* and *Neospora* (Dubey 2010, Taylor et al. 2015, Dubey et al. 2017).

Most species of strongylids in their adult form inhabit the alimentary tract of the major lineages of modern vertebrates, where they generally consume the blood, tissues and food ingested by their final hosts (Monteiro 2017). The Family Ancylostomatidae contains the main genera of strongylids, such as *Ancyclostoma, Uncinaria* and *Necator,* which can parasitize domestic and wild carnivoran mammals (Anderson et al. 2009, Taylor et al. 2015). These hematophagous intestinal strongylids, also known as hookworms, normally have monoxenous cycles, though some species also require, during their development, other vetebrates as paratenic hosts. The life cycle of these nematodes is usually completed from ingestion or skin penetration of infective larvae that previously developed in the environment from embryonated eggs eliminated in the feces of other final hosts. Additionally, some species can also be transmitted by via lactogenic or ingestion of paratenic hosts, especially rodents (Anderson 2000). The eggs described in this paper have a similar shape and size to the genera *Ancyclostoma, Uncinaria* and *Necator,* which also does not rule out the possibility of these specimens belonging to an extinct genus of hookworm from Pleistocene, whose life cycle would be unknown. The hypothesis that the oocysts described here belong to an extinct lineage of Eucoccidiorida can also not be dismissed. Because the eggs of these ancilostomids are practically indistinguishable, normally their differentiation is made through the morphological and morphometric analysis of their infective larvae cultivated in fresh fecal cultures or adult forms recovered from the small intestine during necropsy of the final host, however this laboratory procedures were

not possible, precluding also a more assertive classification of these specimens.

Although normally the type of paleoparasite found facilitate the identification of the probable producer of a coprolite (Ferreira et al. 2008, 2011), it was not possible by means of this device to distinguish if the examined fossil material belonged to particular group of carnivorans, since both groups of parasites observed can currently be found in these mammals (Borka-Vitális et al. 2017, Oudni-M'rad et al. 2017, Solórzano-García et al. 2017), with species of very similar morphology and dimensions, distinct from each other only by their larval and adult forms in the case of Ancylostomatidae or when their oocysts are totally sporulated in the case of Eucoccidiorida, besides the fact that some species can be shared between different Carnivora families. Still with the intention of filling this gap, biomolecular analyzes by polymerase chain reaction (PCR) were performed (following Homan et al. 2000, Iñiguez et al. 2006), aiming to detect the specific DNA of *T. gondii* in the studied coprolite, since the oocysts of this Eucoccidiorida can be found only in the feces of their definitive hosts, represented by practically all modern felid species (Dubey 2010). However, it was not possible to detect the DNA of this parasite in MCP-5176-PV, probably due the lack of *T. gondii* oocysts on the analyzed sample or due the destruction of the nucleic acids during the fossilization process.

A considerable number of paleoparasitological investigations with animal coprolites, including extinct species, that lived during the Pleistocene, were carried out in different regions worldwide (Table I). The first of these studies, conducted by Ringuelet (1957), revealed eggs of an unidentified nematode in the coprolite of a giant sloth of the species *Mylodon darwini* (formerly *M. listai*) from the Última Esperanza, in Chile. Ferreira et al. (1991) found

Table I. Paleoparasitological records from Pleistocene coprolites published until now.

		Taxon	Material	Unit / Cave / Site	Locality	Reference
Metazoa	Nematoda	Gen. et sp. indet.	Eggs	Cueva del Milodón	Última Esperanza, Chile	Ringuelet 1957
		Trichuris sp., Enoplida	Eggs	Pedra Furada Archeological Site	Piauí, Brazil	Ferreira et al. 1991
		Ascarididae, Ascaridida	Eggs	Rampart Cave	Arizona, USA	Schmidt et al. 1992
		Agamofilaria oxyura, Oxyurida	Larvae			
		Strongyloides shastensis, Rhabditida	Larvae			
		Gen. et sp. indet.	Larvae	Fontana Acetosa, Costa San Giacomo, Grotta Guattari and Grotta del Fosselone	Lazio, Italy	Ferreira et al. 1993
		Toxocara sp., Ascaridida	Egg	Haro River Quarry	Punjab, Pakistan	Perri et al. 2017
		Gen. et sp. indet.	Egg			
		Toxascaris leonina, Ascaridida	Eggs	Peñas de las Trampas	Catamarca, Argentina	Petrigh et al. 2019
		Toxocara cati, Ascaridida	Eggs	Cueva Huenul 1	Neuquén, Argentina	Tietze et al. 2019
		Dioctophymatidae, Enoplida	Eggs			
		Oxyurida	Eggs			
		Trichostrongylidae, Strongylida	Eggs			
		Ancylostomatidae, Strongylida	Eggs	Touro Passo Formation	Rio Grande do Sul, Brazil	**This paper**
	Plathyhelminthes	Schistosomatidae, Trematoda	Eggs	Rampart Cave	Arizona, USA	Schmidt et al. 1992
		Dicrocoeliidae, Trematoda	Eggs	Caune de l'Aragó	Occitanie, France	Jouy-Avantin et al. 1999
		Trematoda or Pseudophyllidea, Cestoda	Eggs	Sopas Formation	Artigas, Uruguay	Duarte et al. 1999
		Viscachataenia quadrata, Anoplocephalidae, Cestoda	Eggs	Cueva Huenul 1	Neuquén, Argentina	Beltrame et al. 2012
		Trematoda	Eggs	Cueva Huenul 1	Neuquén, Argentina	Tietze et al. 2019
Protozoa	Apicomplexa	*Archaeococcidea antiquus*, Eucoccidiorida	Oocysts	Rampart Cave	Arizona, USA	Schmidt et al. 1992
		Archaeococcidea nothrotheriopsae, Eucoccidiorida	Oocysts			
		Cystoisospora felis, Eucoccidiorida	Oocyst	Cueva Huenul 1	Neuquén, Argentina	Tietze et al. 2019
		Eucoccidiorida	Oocysts	Touro Passo Formation	Rio Grande do Sul, Brazil	**This paper**

Enoplida nematode eggs, identified as belonging to the genus *Trichuris,* in rodent coprolites of the species *Kerodon rupestres* from State of Piauí, in Brazil. Schmidt et al. (1992) recovered Schistosomatidae trematode and Ascaridida nematode eggs in coprolites of a Shasta ground sloth (*Nothrotheriops shastensis*), from State of Arizona, in USA. In addition, these authors also described two new species of nematodes based on their first stage larvae, *Agamofilaria oxyura* (Order Oxyurida) and *Strongyloides shastensis* (Order Rhabditida), and two new species of Eucoccidiorida protozoans based on their oocysts, *Archeococcidea antiquus* and *Archeococcidea nothrotheriopsae*. Ferreira et al. (1993) recorded larvae of an unidentified nematode in Hyaenidae mammal coprolites collected from central Italy. Jouy-Avantin et al. (1999) registered Dicrocoeliidae trematode eggs in a coprolite of an unidentified carnivorous mammal from Region of Occitanie, in France. Duarte et al. (1999) discovered eggs of a flatworm, probably trematode or Pseudophyllidea cestode, in coprolites of an unidentified Carnivora mammal from Department of Artigas, in Uruguay (Sopas Formation). Beltrame et al. (2012) observed Anoplocephalidae cestode eggs assigned to species *Viscachataenia quadrata* in coprolites of rodents *Lagidium viscacia* from Northern Patagonia, Province of Neuquén, in Argentina. Perri et al. (2017) found Ascaridida nematode eggs, characterized as belonging to the genus *Toxocara,* in the extinct hyena (*Pachycrocuta brevirostris*) coprolite from Attock District, Punjab Province, in Northwestern Pakistan. Additionally, the thin sections analysis of this same coprolite showed an egg of an unidentified nematode. According to these authours, it may represent another parasite known to infect hyenas, such as *Ancylostoma* sp. Petrigh et al. (2019) using morphological and molecular analysis, identified eggs of the Ascaridida nematode species, *Toxascaris leonina*, in coprolite of puma (*Puma concolor*) from Southern Puna, Province Catamarca, in Argentina. Tietze et al. (2019) registered different nematode eggs, such as *Toxocara cati,* Dioctophymatidae (Enoplida), Oxyurida and Trichostrongylidae (Strongylida), besides trematode eggs and Eucoccidiorida protozoans oocysts assigned to species *Cystoisospora felis* in feline coprolites also from Northern Patagonia, Province of Neuquén, in Argentina.

It is noteworthy that most of the Pleistocene parasitic record comes from coprolites collected in caves (Ringuelet 1957, Schmidt et al. 1992, Ferreira et al. 1993, Jouy-Avantin et al. 1999, Beltrame et al. 2012, Tietze et al. 2019) or archeological sites (Ferreira et al. 1991, Perri et al. 2017, Petrigh et al. 2019). Recording paleoparasites from buried coprolites (i.e., excavated from rock outcrops) is rare and must be related to taphonomic biases that act destroying the organic remains. These biases are still understudied, but the Upper Pleistocene Sopas and Touro Passo formations are good examples of preservation of paleoparasites in this context.

Thus the present paper reveals the first vertebrate paleoparasites from the Late Pleistocene of the Rio Grande do Sul, as well as it brings clues about the composition of the endoparasitic fauna of carnivoran mammals, including possibly extinct species of these hosts, which inhabited South America during the Lujanian.

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