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Short Communication: First report of a morulated Ascaridoidea (Nematoda) egg in an avian coprolite from the Paleogene of the Paraíba Valley, State of São Paulo, Brazil

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Abstract

Ascaridoidea (Nematoda) is a widespread superfamily of nematodes that comprises gastrointestinal parasites from all major groups of vertebrates. Although this taxon probably has emerged in the Carboniferous, its Brazilian fossil record includes mostly eggs, found in ancient remains, collected in paleontological and archeological sites from the Mesozoic and Cenozoic Eras. The Tremembé Formation (Oligocene of the Taubaté Basin) has become an important source for paleoparasitological studies in avian coprolites during the third decade of the 21st century, with reports of eggs only at one single cell stage, of embryonic development. Here we present the first egg of Ascaridoidea preserved containing morula, from a bird coprolite recovered from the shales of the Tremembé Formation. Three coprolites, from the outcrop of Aligra Comércio de Argila S/A, Taubaté municipality (State of São Paulo), were rehydrated and subjected to spontaneous sedimentation. Based on morphological and morphometric features, diet and zoopaleontological context, the trace fossils were assigned to piscivorous birds. The egg found showed morphological characteristics typical of Ascaridoidea namely spherical form, ornamented and somewhat thick shell. Moreover, this superfamily includes several taxa that infect piscivorous birds and fish in heteroxenous life cycles, and produce eggs with similar features as the egg found in the present study. The paleoparasitological information associated with the paleofaunistic diversity of birds and fish from the Tremembé Formation, reveal that the ancient Brazilian paleoenvironments provided subsidies for the rise and success of nematodes infecting these animals during the Paleogene.

Keywords: Oligocene; Helminth; Nematoda; Parasite; Tremembé Formation; Taubaté Basin.

Introduction

Ascaridoidea (*sensu* Hodda, 2022) is a widespread superfamily of nematodes, which comprises gastrointestinal parasites infecting mammals, including humans, birds, reptiles, amphibians and fish (Hartwich, 2009). This taxon probably emerged in the Carboniferous parasitizing terrestrial tetrapods and, subsequently, moving to aquatic environments and infecting other hosts like bony and cartilaginous fishes (Li *et al*., 2018).

The Brazilian fossil record related to ascaridoids includes mostly eggs found in ancient remains collected in paleontological and archeological sites and, even though the egg morphology is rather uniform and unspecific, some species have been proposed based on these immature forms. For example *Ascarites rufferi*, was described from an egg found in a coprolite of Cynodontia, the taxonomic bridge between reptiles and mammals, which was recovered in the Santa Maria Formation, Paraná Basin, Brazil (Silva *et al*., 2014). *Bauruascaris cretacicus* and *B. adamantinensis* were proposed based on eggs found in coprolites of Crocodilomorpha from the Adamantina Formation (Bauru Group, Brazil) (Cardia *et al*., 2018; 2019). In addition, Poinar & Boucot (2006) described the species *A. priscus* and *A. gerus* from eggs in a single dinosaur coprolite. In addition, ascaridoid parasitic forms have been more frequently reported in Cenozoic ancient remains dated from the Holocene (Gonçalves *et al*., 2003; Leles *et al*., 2008; Camacho *et al*., 2013; Jaeger *et al*., 2013a, 2013b; Sianto *et al*., 2014; Guedes *et al*., 2020; Iñiguez *et al*., 2022).

The Tremembé Formation (Oligocene of the Taubaté Basin), aged between 33.9 and 23.03 million years ago, is an intercalation between shale and clay layers with lacustrine origin, and it has become an important source for paleoparasitological studies in avian coprolites, providing different well-preserved morphotypes of helminth eggs, protozoan cysts and non-sporulated oocysts (Carmo *et al*., 2023; Carmo *et al.*, 2024a). Currently, the helminth eggs from this geological unit have been reported only at one single cell stage of embryonic development (Carmo *et al*., 2023). Here we report the first egg of Ascaridoidea preserved containing morula, from a single bird coprolite recovered from the Paraíba Valley, as well as discuss some insights into the potential of its lower taxon of origin.

Materials and methods

Three coprolites were recovered from sedimentary rocks of the Tremembé Formation, Taubaté Basin, in 2023 at the outcrop of Aligra Comércio de Argila S/A, Taubaté municipality (State of São Paulo). Samples were rehydrated in 10 mL of 0.5% trisodium phosphate (Na_3PO_4) solution for 72 h (Callen, 1967; Fugassa *et al*., 2006), and subjected to spontaneous sedimentation for 24 h (Hoffman *et al*., 1934). A drop of sediment was placed on a glass slide, together with a drop of glycerine, covered with a coverslip (Ferreira *et al*., 2014), and observed using a light microscope Nikon Eclipse Ei with image capture system PrimeCam Intervision 12. A total of 20 slides were mounted for each sample of coprolite. Parasitological identification was performed based on morphology and morphometry (Ferreira *et al*., 2014); since there is no general literature for taxonomic identification of nematode eggs the present identification was based on the expertise of the authors and comparisons with specific literature (see results and discussion for details). The avian origin of the coprolite was interpreted based on criteria

adopted by Castro *et al*. (1988), Souto (2017) and Carmo *et al*. (2023). Two females of *Contracaecum* Railliet & Henry, 1912 (Ascaridoidea, Anisakidae) were collected from a single bird of the species *Nannopterum brasilianum*, and their uteri were dissected in order to obtain eggs that were used for comparison with the fossilized material. The genus *Contracaecum* was identified based on the following criteria: three well-developed labia, excretory pore at the base of subventral labium, presence of ventriculus with posterior appendix and intestinal caecum (Hartwich, 2009; Gibbons, 2010; Supplementary Figure S1). We also provided an micrography of an eimeriid oocyst (Fig. 1B), found by our research group in a previously analyzed sample, from the same geological formation, for comparisons.

Results and discussion

Coprolites were ovoid, grayish-yellow in colour, 10.80 to 28 mm long and 6.93 to 16.30 mm wide, containing fragmented remains of fishes (Fig. 1A). Based on morphological and morphometrical features, diet remains and zoopaleontological context, the samples were assigned to piscivorous birds (Avialae, Neornithes). Some bird species may have benefited from periodic fish mortality due to seasonality (dry and wet seasons), which possibly supported a great success of this feeding habit in the Paraíba Valley, during the Oligocene (Olson & Alvarenga, 2002).

A single slide from a single coprolite was positive for the parasite form, which was represented as a spherical egg, measuring 34.21 x 32.44 μm, containing morula, translucent, with somewhat thick and ornamented shell (Fig. 1D). The identification of immature parasitic forms (*i.e.* eggs and larvae), preserved without adult worms or genetic material, is rather difficult and generalist, since these forms hold ancestral ontogenetic traces that are common within a higher taxa (*i.e.* family, order), show homogeneous morphology and lack specific traits (Carmo *et al.*, 2024b). Nevertheless, the present egg exhibited morphological features typical of the Ascaridoidea nematodes, i.e., the shell with ornamentations (resembling a mammillated layer) and the pronounced spherical form. These characteristics are especially common in eggs from representatives of Ascarididae, Anisakidae and Raphidascarididae, all belonging to Ascaridoidea (Koie & Fagerholm, 1995; Anderson, 2000; Carrera-Játiva *et al*., 2014; Carvalho, 2020).

The family Anisakidae was of particular interest in the present context, because it includes some parasites that use piscivorous birds as definitive hosts and fish as intermediate or paratenic hosts, as well as produce eggs with similar morphometry and morphology as that found in the present study (Køie & Fagerholm, 1995; Anderson, 2000; Carrera-Játiva *et al*., 2014; Carvalho, 2020). It should be mentioned that the present egg was quite similar to those reported by Carrera-Játiva *et al.* (2014), and mainly to the morulated stages reported by Huizinga (1967), all assigned to the genus *Contracaecum*.

The *Contracaecum* eggs (54.36 - 61.27 x 43.82 - 50.04 μm; Fig. 1C) recovered from the dissected females were found in an initial stage of development, before morulation, similar to those reported by Huizinga (1967). Although the stages of development from these eggs were different, they showed morphological similarities such as an oval to spherical shape, and an ornamented and relatively thinned shell that is typical from nematodes that have aquatic life cycles. Moreover, differences observed

in the egg size between the fresh and the fossilized materials, may be accounted by intrinsic features of females from each species. According to Herreras *et al.* (2007), spatial constrictions imposed by uterine size, may influence the number and size of eggs produced by females of anisakid nematodes.

Although the parasitic form reported here may have a size similar to that of some oocysts from Eimeriidae, previously found in the Tremembé Formation (Carmo *et al.*, 2024a; Fig. 1B), it lacks common traits such as micropyle and micropyle cap. Moreover, we believe that the internal embryonic structure represents morula rather than developing sporocysts. In fact, more than ten oocyst morphotypes were described in the Tremembé Formation, and none of them had preserved sporocysts or sporozoites (Carmo *et al.*, 2024a). In this sense, sporocysts or sporozoites most likely degrade before feces fossilization in this paleoenvironment, or the abiotic conditions essential of sporulation (e.g. heat, humidity and oxygenation) were absent (Fayer, 1980; Berto *et al.*, 2014).

Fossilization is a singular phenomenon that takes place under specific conditions, which partially explains the lack of parasitological studies on material found in paleontological sites (Dentzien-Dias *et al.*, 2013; Carmo *et al.*, 2024b). Although we found only one egg in the present coprolites, the record of a morula reveals good potential for preservation of parasitic forms in the Tremembé Formation. In addition, the identification of the Ascaridoidea egg provides insights on the biogeographic distribution of this taxon, in the Brazilian territory, during the Oligocene.

The paleoparasitological information associated with the paleofaunistic diversity of birds and fishes from the Tremembé Formation (Carmo *et al.*, 2024c) indicate that the ancient Brazilian paleoenvironments provided conditions for the rising and success of nematodes infecting such hosts during the Paleogene.

Previous paleoparasitological studies from the Tremembé Formation were performed in coprolites exclusively from the site Fazenda Santa Fé (Carmo *et al*., 2023). Therefore, this is the first parasitological analysis in trace fossils from the outcrop of Aligra Comércio de Argila S/A, providing new perspectives related to the paleoparasitological studies in this lithostratigraphic unit, and expanding the knowledge on helminth infections in birds that inhabited the Paraiba Valley millions of years ago.

Declarations

- *Ethical Approval*

Not applicable.

- *Additional headings with statements on consent to participate and consent to publish.*

Not applicable.

- *Conflict of interest*

The authors declare none.

- *Authors' contributions*

Gustavo Macêdo do Carmo: conceptualization, methodology, investigation, writing-original draft, writing-review & editing, funding acquisition. Ana Hadassa da Silva Guilherme Luiz: methodology, investigation, writing-original draft. Jotapê Fernandes Passos: writing-original draft. Sueli de Souza Lima: validation, resources, supervision, funding acquisition. Hermínio Ismael de Araújo-Júnior: validation, resources, supervision. Felipe Bisaggio Pereira: validation, resources, supervision, funding acquisition.

- *Funding*

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- *Availability of data and materials*

All data and materials used are available in this manuscript.

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Captions:

Fig. 1 Avian coprolite with fish bone fragments (A), eimeriid oocyst (B), *Contracaecum* egg (C), and fossilized egg of Ascaridoidea (C) from the Tremembé Formation, Taubaté Basin, State of São Paulo, Brazil.

Belo Horizonte, July 2024.

Dear Editor-in-Chief and Associate Editors of Journal of Helminthology,

First, thank you for the feedback on our manuscript. We also would like to thank the reviewers for taking time for review the work and for all their comments and suggestions. Their attention, detailed and insightful comments, as well as expertise, were crucial on improving the manuscript revised version. Most of the suggested changes were adopted accordingly. Please, find as follows the responses for the most critical comments.

Comment: "The manuscript 'First report of a morulated Ascaridoidea (Nematoda) egg in an avian coprolite from the Paleogene of the Paraíba Valley, State of São Paulo, Brazil', reports the first egg of Ascaridoidea preserved containing morula, from a single bird coprolite recovered from the Paraíba Valley. I consider the work to be well written. The methodology is well planned and developed. However, the finding is very poor and does not present consistency. It is a unique and very dubious find. It is not possible to identify its origin, it looks like an insect egg. I consider that this finding does not merit publication in the journal. It would be interesting to do interdisciplinary work with the samples, which are very interesting, and to publish more robust results."

Response: We acknowledge the reviewer's feedback. However, we believe that the finding is not very poor, since this is a material aged between 33.9 and 23.03 million years ago, making it very difficult to analyse regarding the possible parasite forms, fossilized in it. Moreover, as the reviewer commented, the methodology is adequate, which means that we took all precautions to avoid any source of contamination. The samples used for analysis were taken from the inside of the coprolite and, in this sense, it is unlikely that an insect egg would be fossilized there. In addiotion, based on the suggestions by reviewer 2, we improved much the evidences towards the our hypotesis that this is a morulated egg from Ascaridoidea, which exhibits a morphology totally different from that of the broad definition of "insect". Based on all these arguments we believe that the result is worth publishing as it provides interesting biological insights on the parasites from a far far way time.

Comment: "I think their identification would be much stronger by showing comparative pictures of relevant taxa. For instance, Thapa et al. (2017) Figure 1 G is comparable. And

Dziekońska-Rynko, et al. (2007) has a good picture of *Contracaecum* eggs at different stages. I believe it would be worth trying to get permission to reproduce one or more of these, or something similar, to bolster support for their identification."

Response: We would like to thank the reviewer for the pertinent suggestion. However, we have already cited a study in which it is possible to see *Contracaecum* eggs similar to the one found in our study. Moreover, we added the study by Huizinga (1967) that evaluated in details the egg development of *Contracaecum multipapillatum*, in which figures 1C, D (see at the end of this file) of it show morulated eggs very similar to the one fossilized, found in our work. In addition, we believe that it is more important to use original images, since the photos of the eggs from the indicated references can be restricted by copyrights. In this sense, two females of *Contracaecum* from a single bird of the species *Nannopterum brasilianum* were dissected in order to recover eggs that were used for comparison. Finally, we also added a picture of an oocist from our personal archive, which was found in a coprolite from Tremembé, in order to make it clear its differences for nematode eggs.

Comment: "I note that in Carmo et al. (2024) references are made to a number of papers reporting bird presence in the palaeoenvironment at Tremembé. It would be interesting to the parasitology reader to know what bird taxa could potentially be the producers of the guano?"

Response: We would like to thank the reviewer for another important suggestion. However, it is impossible to make a more precise identification of the zoological origin of these coprolites. The samples were all found unassociated to their producing organisms. In addition, the Tremembé Formation has more than one bird taxon that fed upon fish, which could be the probable zoological tracemaker. Both we and other paleontologists have discussed this issue in previously published papers (mentioned in this manuscript) and the specific origin of these coprolites remains uncertain.

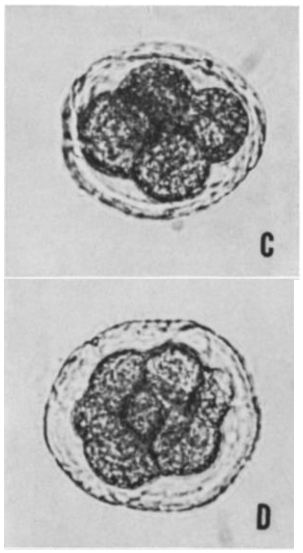
Thank you for your time and I look forward to hearing from you.

Sincerely,

MSc Gustavo Macêdo do Carmo (on behalf of all authors)

PhD student in Parasitology

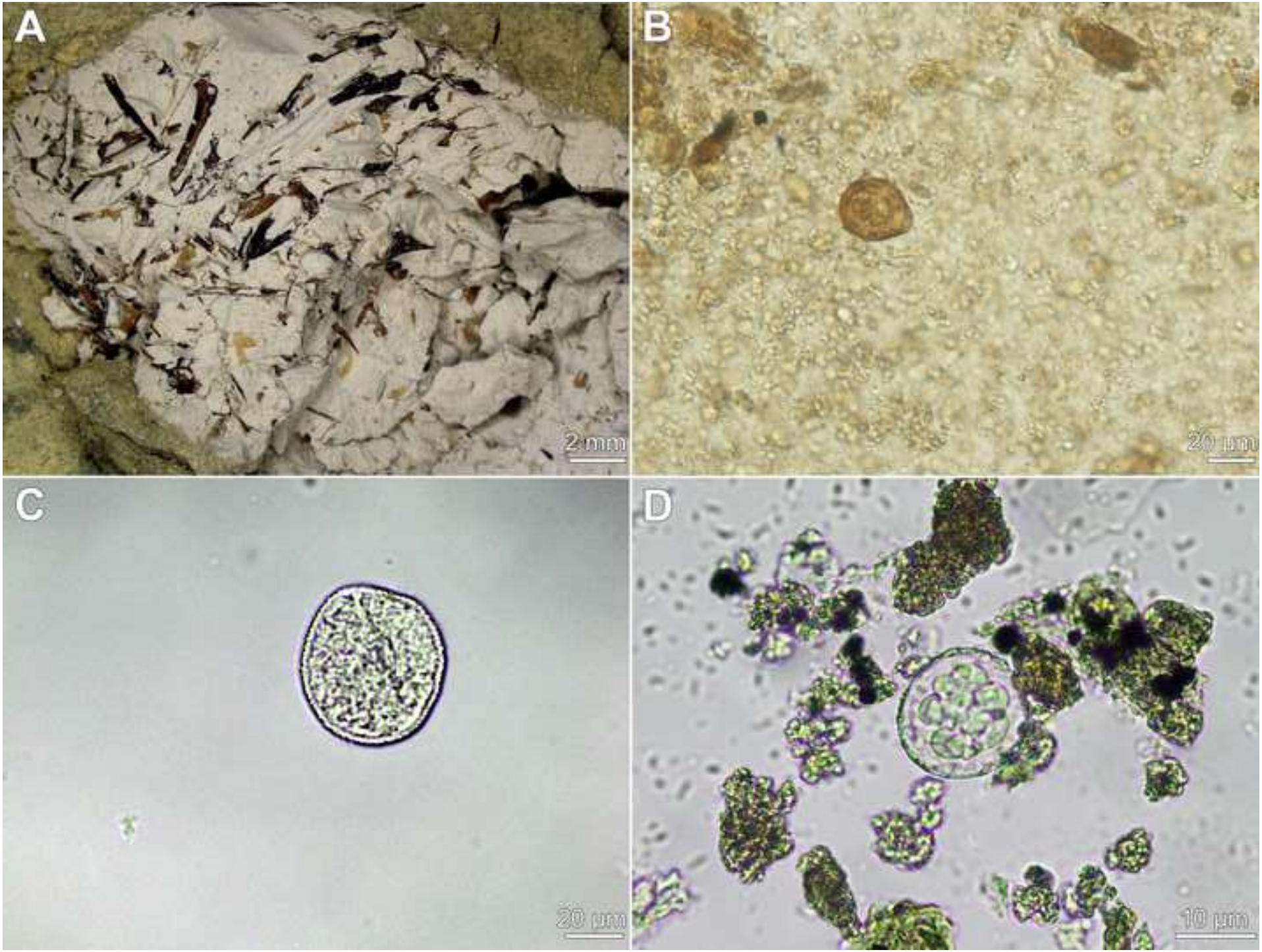
Institute of Biological Sciences / Federal University of Minas Gerais



Images of morulated eggs from *C. multipapillatum* by Huizinga (1967)

Figure

Click here to access/download;Figure;Figure 1.tif



Journal of Helminthology

Short Communication: First report of a morulated Ascaridoidea (Nematoda) egg in an avian coprolite from the Paleogene of the Paraíba Valley, State of São Paulo, Brazil

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Supplementary Figure S1. Female of *Contracaecum* sp. parasite of *Nannopterum brasilianum*. A: anterior end, lateral view. B: cephalic end, lateral view. C: oesophageal region, lateral view. D: oesophago-intestinal junction, ventral view. Abbreviations: ep, excretory pore; ic, intestinal caecum; v, ventriculus; va, ventricular appendix.