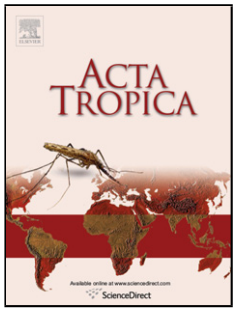


Title: Fox parasites in Pre-columbian times: Evidence from the past to understand the current helminth assemblages

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# Fox parasites in Pre-columbian times: Evidence from the past to understand the current helminth assemblages

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Graphical abstract

Highlights

- The study of the entero-parasitism of ancient carnivores provides data on the history of the biogeography of different parasite species.
- Coprolites were found in Campo Cerda 1 archaeological site (CCe1), Chubut Province, Argentina.
- A 246bp fragment of the mitochondrial control region was amplified from the coprolites and showed 99% of identity with culpeo fox, *Lycalopex culpaeus*.

- The presence of *Alaria* sp. and *Clonorchis* sp. in both coprolites represents the first Holocene record for pre-Columbian America.
- Alternating occupations between carnivores and humans suggest the potential for human exposure to zoonoses due to fecal contamination with helminth eggs.

**Abstract**

This work aims to increase the information on the entero-parasitism in Holocene carnivores, by examining coprolites found in Patagonia. Molecular analysis was conducted following the Authenticity Criteria to Determine Ancient DNA sequences. The nucleotide sequences showed 99% of identity with the Control Region sequences of *Lycalopex culpaeus* (culpeo fox). Coprolites were positive for gastrointestinal parasites. The presence of *Alaria* sp. and *Clonorchis* sp. represents the first record for pre-Columbian America. The parasitological findings suggest the importance of these carnivores for the dissemination of their own parasites and those to their prey in rockshelters, areas with high re-use of space.

*Keywords*: Coprolites, Helminthes, Patagonia, Piedra Parada archaeological locality.

## 1. Introduction

The knowledge about parasitism is built on the study of ecological relationships and environmental configurations that have changed over time. For instance, several studies in archaeological sites of Patagonia have shown entero-parasite assemblages between camelids different from those observed today, likely due to the introduction of novel parasite species associated with the introduction of domestic mammals during the European colonization (Amalfitano et al., 2017; Fugassa, 2015). Ancient parasites studies have provided evidence of the parasitic relationships under different ecological or cultural configurations. Specifically, parasites of Holocene mammals provide insights into the natural baseline of parasite assemblages prior to exotic invasions.

Carnivores represent keystone species in the structure and dynamics of ecosystems (Berger et al., 2001; Soulé et al., 2003). Also, carnivorous mammals play a critical role in the parasite supra-

community, because, like other mammals, they disperse their monoxenous entero-parasites through their feces. However, they disperse parasites of indirect cycle and monoxenous parasites in transit, both acquired by ingestion of parasitized prey. In this framework, the study of the entero-parasitism of carnivores that frequented rockshelters in the Holocene provides data on the history of the biogeography of different parasite species (both of their own and their prey), being critical to understand the processes that shaped current relationships.

A high diversity of entero-parasites was identified in rockshelters occupied by humans and other mammals in southern Patagonia during the Holocene. This would suggest that these environments functioned as transmission nodes, exchange of parasites and maintenance of parasitic cycles in the region (Fugassa and Petrigh, 2017; Fugassa et al., 2009), as happen for other parasites in other regions (Reinhard, 2008). This work aims to increase the information on the entero-parasitism in Holocene carnivores, by examining coprolites found in a rockshelter of the Piedra Parada archaeological locality (Chubut, Argentina), a new area of study.

## 2. Materials and Methods

Three concretions presumably of fecal origin (catalog number 770-1, 770-2 and 770-3, collection of the Lab. de Parasitología de Sitios Arqueológicos, Univ. Nac. de Mar del Plata, Argentina) were examined. The coprolites were found in level 5 (8) of the Campo Cerda 1 archaeological site (CCe1), located in the archaeological locality Valle Piedra Parada (42°37'S; 70°12'W). The site is located inside a rockshelter, with a front of 55 m wide and a room of 20 m deep (Bellelli et al., 2007). This rockshelter is at 300 m from the north coast of the Chubut River (Bellelli et al., 2007). Campo Cerda 1 site has six stratigraphic levels. Layer 5, which has a depth of *ca*. 100 cm, is composed of sandy sediment containing artifacts, zooarchaeological remains, and two hearths. It was dated to *ca*. 1,700 – 2,900 radiocarbonic years before present (BP) (Bellelli et al., 2007). In

layer 5, sub-levels 5(1) at 5(8) were defined according to their sedimentary characteristics and cultural remains. Coprolites were found in sub-level 5(8) where a fragment of an artifact made of vegetable fibres was dated in 2,913 calibrated years BP (3,061-2,784 calibrated years BP; probability 95.4%)[1]. The sandy sedimentary matrix did not allow a precise correlation between the dated artifact recovered and the coprolites from sub-level 5(8) due to their low degree of compaction. Although the coprolites were not dated, the upper layer (level 4) is distinguished by a strongly compacted consolidated sand that would have sealed the level, and thus guarantees that the coprolites correspond to the dates cited (Bellelli et al., 2007).

All coprolites were found in the same micro-sector (H15A), although their appearance suggested that they corresponded to different fecal event (Fig. 1). Therefore, samples of 3 ml of each coprolite were separately rehydrated in a trisodium phosphate aqueous solution at 4°C within chemical fixation, and then subjected to filtration by a 300-µm-opening mesh and spontaneous sedimentation (Fugassa et al., 2006; Reinhard et al., 1986). Transient slides of each sample were examined by light microscopy for parasite identification. Parasitic remains were identified taking into account specific bibliography (e.g., Benbrook and Sloss, 1965; Miyazaki, 1991; Soulsby, 1987).

Molecular analysis of coprolites was performed according to Petrigh and Fugassa (2017) to identify their zoological origin. To ancient DNA (aDNA) studies specific protocols must be applied to minimize contamination risk with modern or exogenous DNA following the authenticity criteria to determine ancient DNA sequences guidelines (Fulton, 2012; Hofreiter et al., 2001; Willerslev and Cooper, 2005). Therefore, coprolites fractionation, aDNA extraction, PCR amplification, electrophoresis, PCR products purification and sequencing were carried out in separate places using special reagents, sterile disposable supplies and exclusive equipment for each process. Room, laminar airflow workbench and surfaces were decontaminated with 10% bleach and 70% ethanol

[1] Calibrated in Oxcal v4.3.2. SHCal 13 atmopheric curve (Hogg et al., 2013). Radiocarbon age: 2850±50 years BP (UGAMS 7454; Bellelli, 2000-2002).

and irradiated with light UV prior their use. Ancient DNA extractions, PCR amplifications and sequencing were performed into horizontal laminar airflow workbenches located in separate areas to the other process.

Internal fragments of 50 µg of each coprolite was isolated by duplicated. A sterile 1.5 ml tube was separated as negative control for aDNA extraction process. DNA extraction was performed with the Geneclean for Ancient DNA™ Kit (MP Biomedicals) following manufacturer instructions with modifications reported in Petrigh and Fugassa (2017). PCR reactions and thermocycler steps were the same previously reported in Petrigh and Fugassa (2017). A negative control was included in all PCR experiments.

Canid Specific primer pairs: PEX2bF- PEX2bR and PEX3F-H3R (Nystrom et al., 2006) was used to amplify ~ 154 bp and ~ 141 bp, respectively, of mitochondrial control region that allow to construct a overlapped fragment. These primers were selected based on the presumption that the coprolites belonged to a canid according to morphological characters and internal contents (Chame, 2003). PCR products were purified by using the MinElute Gel Extraction Kit (Qiagen) in a room distant. Duplicates of the specific aDNA fragment were sequenced in forward and reverse sense by Sanger method in the Genomic Unit of National Institute of Agricultural Technology -INTA-, Argentina. This facility have a special area to work in ancient DNA. Chromatograms were analyzed using BioEdit v7.2.0 (copyright © 1997e2013, Tomm Hall, Ibis Biosciences). The consensus sequences obtained were compared with the GenBank sequences by using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Center for Biotechnology Information (NCBI) to determine sequence identity.

**3. Results and Discussion**

The two mitochondrial DNA fragments of 154 bp and 141 bp of the control region were amplified in two replicates of coprolite 770-1. No DNA amplification was achieved in coprolites 770-2 and

770-3. In addition, no amplification were achieved in any of three samples when carnivore-specific (including human) *cytochrome b* gene set of primers (Farrell et al., 2000) were tested (data not shown). The nucleotide sequences of each fragment were overlapped and a 246-bp sequence was obtained. BLASTN analysis showed 99% of identity with the control region sequences of culpeo fox, *Lycalopex culpaeus* (e.g. Accession Number: JX890323.1), 96-97% of identity with gray fox, *L. griseus*, and 93-94% identity with pampas fox, *L. gymnocercus*. The sequence obtained in this work was submitted to GenBank (Accession Number: MG570076).

Coprolites 770-1 and 770-3 were positive for gastrointestinal parasites. They contained eggs of 11 helminth species: one cestode, two trematodes and eight nematodes.

Coprolite 770-1 exhibited 330 eggs of eight helminth species, four of which were not found in coprolite 770-3. In addition, coprolite 770-1 contained six *Physaloptera* sp. eggs (60.5[55-66.25; n=2] x 37[33.75-42.5; n=5] μm). The latter were thick- and smooth-walled eggs, translucent, and contained a developed larva that occupied all the inside of the egg (Fig. 2a). Coprolite 770-1 also exhibited only one broken egg of *Heteroxynema* sp. (123.75 x 75 μm). This egg was elliptical and with an operculum composed of irregular plates; the eggshell was brown in color, single, and with a rough surface. Coprolite 770-1 also had 21 eggs of Capillariidae gen. sp. (64.54[62.5-67.5; n=11] x 38.62[37.5-41.25; n=10] μm). These eggs were bi-operculated, had an ornamented surface and a thick shell similar to that of *Calodium hepaticum*, although with the polar plugs extruded (Fig. 2b). Finally, coprolite 770-1 contained 30 *Eucoleus* sp. asymmetrical eggs (61.5[53.75-65; n=18] x 33.1[30-37.5; n=18] μm), with a wall thinner than that of Capillariidae gen. sp., ornamented with small holes and the polar plugs extruded.

Coprolite 770-3 contained 245 eggs of seven helminth species, three of which were not found in coprolite 770-1. Twenty-four semi-spherical eggs were assigned to *Toxocara* sp. (87.25[83.75-91.25; n=10] x 74.63[65-85; n=10] μm). In these eggs, the brown and reticulated eggshell is similar to that of the Subfamily Toxocarinae, specifically to that of *T. canis* (Fig. 2c). Coprolite 770-3

exhibited only one *Uncinaria* sp. egg (77.5 x 55 μm). This egg was elliptical, with dissimilar poles and a thin, smooth and translucent eggshell, and contained an embryonic mass. Finally, one poorly preserved cestode egg was assigned to Anoplocephalinae gen. sp. (57.5 x 55μm). This egg showed a triangular shape and contained an embryo and a pyriform apparatus.

Four helminths were found in both coprolites: *Toxascaris* sp., *Trichuris* sp., *Clonorchis* sp., and *Alaria* sp. *Toxascaris* sp. eggs (81.7[77.5-87.5; n = 6] x 66.7[65-67.5; n= 6] μm) were found in low density. These eggs were elliptical, had a wall composed of a thin external brown membrane, a thick and translucent middle membrane, and a laminar inner membrane (Fig. 2d), and contained a developed larva. *Trichuris* sp. eggs were found in both coprolites (68.44[63.75-73.75; n=8] x 37.92[28.75-42.5; n=9] μm). Small eggs (33.54[30-36.25; n=6] x 20[18.75-22.5; n=6] μm) with a rough eggshell, and operculated eggs with thickening at the level of the opercular suture were assigned to *Clonorchis* sp.; these eggs also showed a thickening in the ad-opercular region (Fig. 2e). *Alaria* sp. eggs (133.78[121.5-152.5; n= 29] x 75.87[67.5-87-5; n= 28] μm) were yellowish brown, elliptical and had a characteristic thickening in the ad-opercular region (Fig. 2f).

The molecular results complemented with the morphological features confirmed the zoological origin of coprolite 770-1. Culpeo fox has a wide geographic distribution in Patagonia and Los Andes mountain range (Novaro, 1997). On the other hand, negative result of aDNA analysis was consistent with parasitological analysis which showed that coprolite 770-2 was negative to parasites. These results, and the atypical morphology observed allow to suggest that this sample was not an intestinal concretion. Although coprolite 770-3 failed to amplify aDNA fragments, its parasitological profile suggests a canid origin because *T. canis* eggs were found. The largest diameter of feces from carnivores is another useful diagnostic tool (Cornejo Farfán and Jiménez Milón, 2001); in this case, the largest diameter of coprolites 770-1 and 770-3 was 26.3 mm and 22.4 mm, respectively, thus being compatible with feces from culpeo fox. The helminths in transit and

those of indirect cycle found in both coprolites indicate that they belonged to canids with a similar ecological niche but were different individuals because they differ in seven species of helminths. Species-specific identification of the origin of coprolites in parasitology is rare (e. g., Boast et al., 2018; Iñiguez et al., 2003; Petrigh and Fugassa, 2017). The confirmation of the zoological origin of the coprolites by molecular tools allow to formulate more precise hypotheses about the parasite assemblage, and specifically, in fox found in ancient rockshelters. The parasitic richness of the two coprolites analyzed in the present study was high (seven and eight species, respectively) and can be associated with the trophic position of the host as an opportunistic predator (Novaro, 1997). Numerous helminth species have also been previously found in other Patagonian canid coprolites (e. g., Fugassa et al., 2006).

The presence of *Physaloptera* sp. suggests the consumption of arthropods or paratenic hosts such as reptiles and amphibians (Soulsby, 1987). On the other hand, the presence of *Heteroxynema* sp., a rodent parasite which has been previously reported for Patagonia (e. g., Beltrame et al., 2012), represents a pseudo-parasitism due to the ingestion of rodents. Parasites of indirect cycle whose definitive hosts are carnivores (*Clonorchis* sp. and *Alaria* sp.) suggest the consumption of amphibians and fish or paratenic hosts, indicating a relationship with the aquatic environment. In the layer studied, several taxa that could be hosts of these parasites were found: rodents (Sigmodontinae, Octodontidae and Caviidae), reptiles (Family Tropiduridae) and fishes (Siluriforme and *Percichthys* sp.), and at least part of them was accumulated by carnivores (Fernández, 2010). Carnivores chew marks and evidence of digestion on *Lagidium viscacia* (a rodent) and fish bone surfaces were also identified. In addition, two vertebrae and an articular bone of fish had attached hairs and evidence of digestion, suggesting that these remains were deposited into the site through feces. One of those vertebrae exhibited a chewing marks (puncture), compatible with the action of foxes (Fernández, 2010).

The present findings broaden the knowledge of the biogeographic history of gastrointestinal helminths. The finding of *Physaloptera* sp., *Toxascaris* sp., *Heteroxynema* sp., *Eucoleus* sp. and *Uncinaria* sp. have a background in meridional Patagonia and extend the reports, specifically, to the Late Holocene of Central Patagonia. *Toxocara canis* was found for the first time in coprolites from Argentina and Capillaridae gen. sp. has features not previously identified among other capillariids identified in Patagonia (Fugassa et al., 2006; Fugassa et al., 2008; Taglioretti et al., 2014). The presence of *Alaria* sp. and *Clonorchis* sp. in both coprolites represents the first Holocene record for pre-Columbian America. In this fluke, the surface, profile, and structure of the egg allowed discerning it among other genera with similar eggs, such as *Opistorchis*, *Metagonimus* and *Heterophyes* (Miyazaki, 1991).

From the ecological perspective, the entero-parasites identified contaminated a domestic hunter-gatherer area. The high latitudes of Patagonia induce a rigorous climate for the region and this could indirectly determine the entero-parasitic profile of these populations, by forcing the use of rockshelters by both humans and other mammals (Fugassa and Petrigh, 2017; Fugassa et al., 2009). The alternation in the use of CCe1 is supported by the low frequency of archaeological materials deposited during the period covered in layer 5 (Bellelli et al., 2007) as well as by the large quantity of rodent bone remains accumulated mostly by the decomposition of raptor pellets (Fernández, 2010). Alternating occupations between carnivores and humans indicate exposure to zoonoses due to fecal contamination with eggs of monoxenous parasites: *Eucoleus* sp., *Toxocara* sp., Capillariidae gen. sp., *Trichuris* sp., *Uncinaria* sp. and *Toxascaris* sp. (Taylor et al., 2001). Likewise, the evidence of heteroxenous parasites for which foxes are definitive hosts (*Physaloptera* sp., *Alaria* sp., *Clonorchis* sp., and Anoplocephalidae gen. sp.) indicates their presence outside the rockshelter. The parasitological findings suggest the importance of these carnivores for the dissemination of their own parasites and those to their prey in rockshelters, areas with high re-use of space.

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**Legends to Figures**

Figure 1. Coprolites 770-1(A), 770-2(B) and 770-3(C). Scale Bar = 20mm.

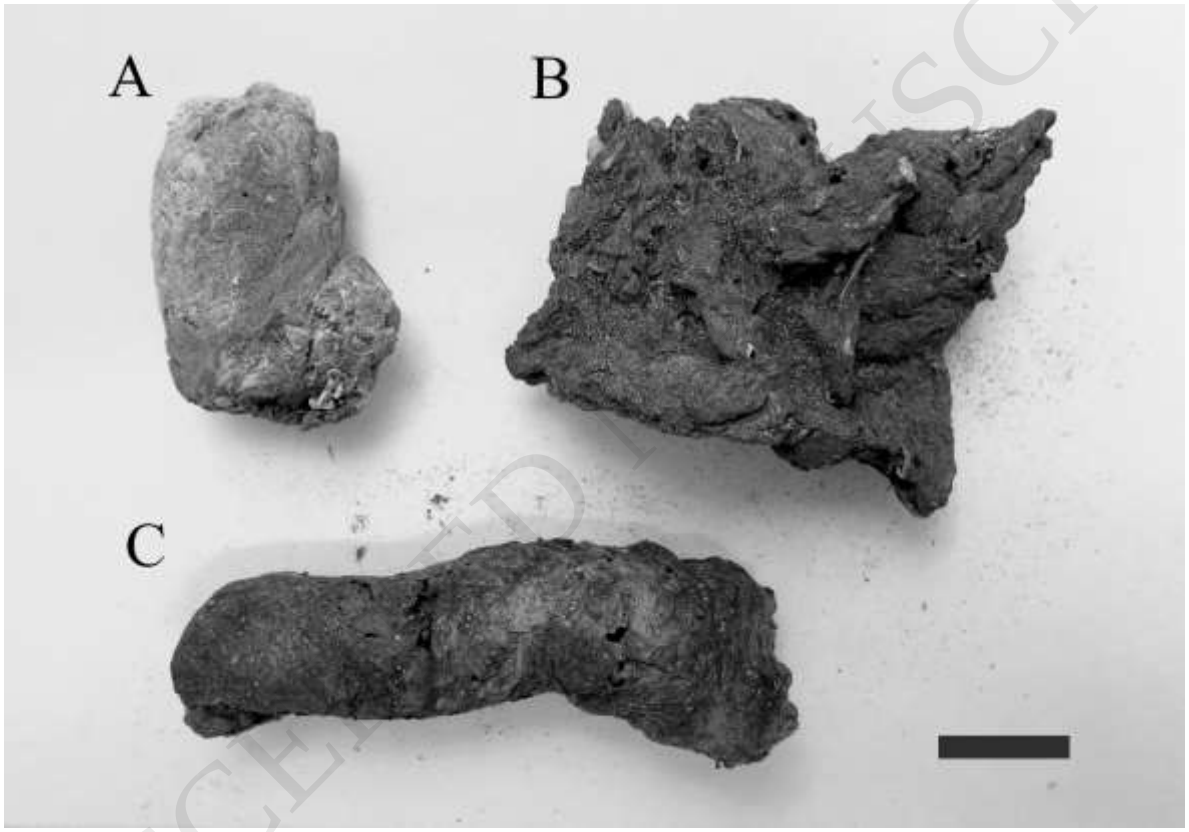

Figure 2. Eggs found in coprolite 770-1. (a) *Physaloptera* sp. in coprolite 770-1 (b) Capillaridae gen. sp. in coprolite 770-1. (c) *Toxocara* sp. in coprolite 770-3. (d) *Toxascaris* sp. in coprolite 770-1. (e) *Clonorchis* sp. in coprolite 770/3. (f) *Alaria* sp. in coprolite 770/3. Scale bar= 40μm.