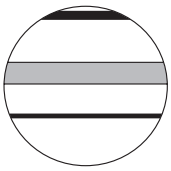

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The effects of dehydration and local soil on parasite recovery: A preliminary paleoparasitological evaluation on experimental coprolites

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Abstract

Experimental paleoparasitological approaches have been used in order to optimize the methodology previously to the application in archeological samples. In this study we evaluated the action of dehydration and local soil (Central Argentina) on the loss of parasite eggs in experimental coprolites, using two parasitological techniques: spontaneous sedimentation and sucrose-flotation. Experimental coprolites comprised fresh human feces, positive for *Hymenolepis nana*, *Ascaris* sp., and *Enterobius vermicularis*, submitted to controlled artificial dehydration. Experimental coprolites with soil addition were prepared by mixing archeological sediment with equal mass of fresh feces. Helminth eggs were counted and eggs per gram were estimated in each subsample. Statistical analyses were applied to compare subsamples before and after desiccation and with and without addition of soil sediment. The performance of parasitological methods statistically differed, the sucrose flotation technique being the less effective when fresh feces and experimental coprolites were analyzed. Partial deformation of eggs was observed via both techniques only in subsamples containing *H. nana* eggs. However, this was not seen in *Ascaris* sp. subsamples, possibly due to eggshell composition. We found that sample desiccation significantly decreased the number of eggs in the experimental coprolites. Mixing archeological sediment with the fecal material also resulted in significantly fewer eggs surviving, independent of desiccation. This shows that climate and soil in which archeological fecal samples are found can strongly influence the survival of parasite eggs from past populations. The small amount of parasite evidence often found in paleoparasitological analyses, including Central Argentina, could be attributed to the action of taphonomic processes rather than to the real absence of infection in these ancient populations. Importantly, the study highlights the role of local soil, confirmed for the first time by empirical data. The research provides valuable insights into the understanding of the paleoparasitological results of the region and of general paleoparasitology.

Keywords

coprolites, helminth eggs, parasite load, parasite preservation, sediment, taphonomy

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Introduction

Paleoparasitology aims to study parasites in archeological and paleontological material (Araújo et al., 2013). Paleoparasitological research allows making inferences about the diet and health conditions of past populations (Ferreira et al., 2014; Iñiguez, 2020). Possible samples to be analyzed mainly consist of coprolites, that is, desiccated and/or mineralized fecal matter, and sediment from the pelvic and abdominal cavity of skeletons, as well as from latrines, trash pits, and spaces of occupation at archeological sites (Borba et al., 2019; Fugassa et al., 2008; Guedes et al., 2020; Ramirez et al., 2021b; Reinhard et al., 2008; Vieira de Souza et al., 2018; Yeh et al., 2019). Less frequently, mummified tissues and regurgitation pellets of birds of prey have been processed (Beltrame et al., 2011; Fugassa, 2014; Gonçalves et al., 2003).

Depending on the sample, one can expect to find diverse parasite evidence: protozoan cysts, helminth eggs, or arthropod appendices. Taphonomic processes affect differently each one of these materials and, accordingly, the remains contained within them (Morrow et al., 2016). While some eggs are particularly

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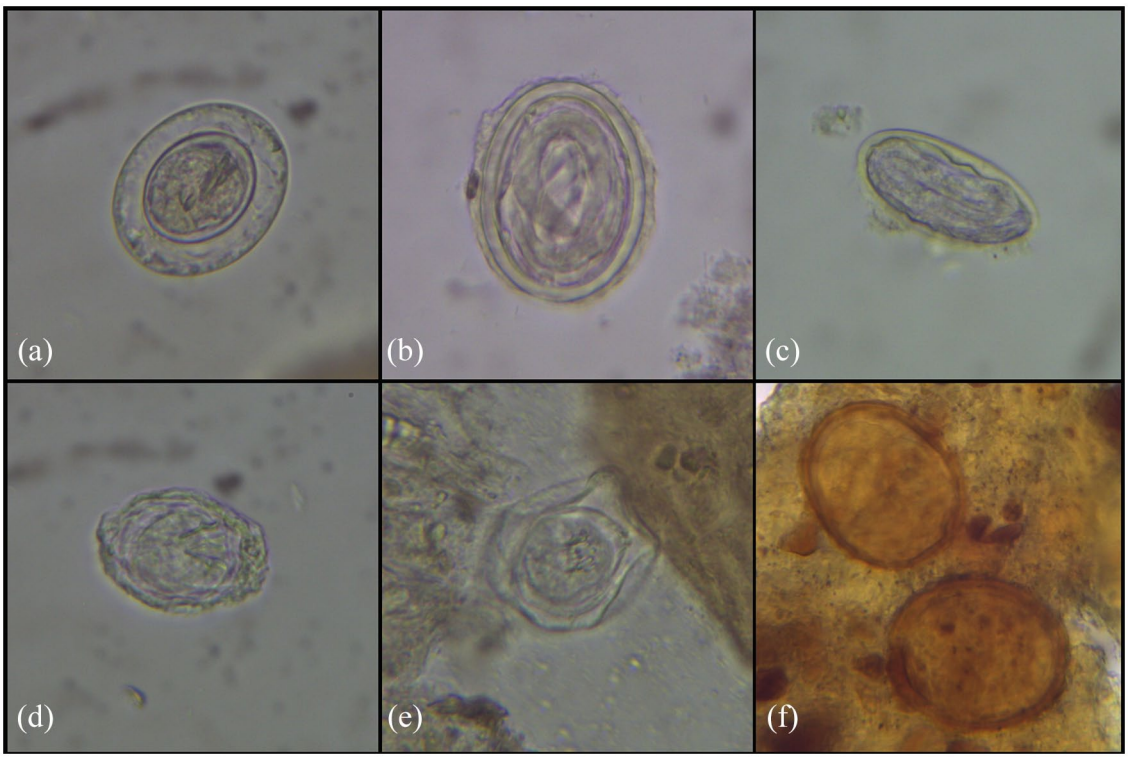

Figure 1. Helminth eggs detected in the samples of the present study. Eggs found in fresh feces subsamples: (a) *Hymenolepis nana*, (b) *Ascaris* sp., and (c) *Enterobius vermicularis*; Eggs found in experimental coprolites subsamples: (d and e) *H. nana* and (f) *Ascaris* sp. (d) and (e): Note the deformation in the walls of *H. nana* eggs.

resistant due to their multiple layers, cysts are very fragile and, thus, are not commonly found in ancient material (Gonçalves et al., 2003). In addition, some environments are not suitable for the preservation of parasite remains (Camacho et al., 2016; Jaeger et al., 2013a, 2013b; Morrow et al., 2016; Rácz et al., 2015). For example, humid-warm climate environments allow a high microorganism activity, damaging cysts, eggs, and arthropod remains. Hence, paleoparasitological examinations can be challenging when working with naturally degraded or poorly conserved samples.

Paleoparasitological studies had an important development in Argentina over the last 15 years, through the examination of samples mainly from the Patagonia region, leading to the finding of both helminth eggs and protozoa cysts (Beltrame et al., 2010, 2018, 2020; Fugassa et al., 2008, 2010, 2018). Most were well preserved, sometimes allowing identification at a species level. On the other hand, in the central region of the country, the application of paleoparasitological techniques (Ramirez et al., 2021a, 2021b) showed scarce results, probably attributed to the taphonomic processes acting negatively on the preservation of remains.

In this sense, this work aimed at evaluating the effects of dehydration/desiccation and local soil in the recovery of parasite remains on experimental coprolites, that is, artificially desiccated modern feces (Iñiguez, 1998) by conventional paleoparasitological techniques and a quantitative approach.

Materials and methods

Recent human feces ($n=8$), positive for parasite infection by *Hymenolepis nana* (Figure 1a), *Ascaris* sp. (Figure 1b) and *Enterobius vermicularis* (Figure 1c), were donated for this study. Fecal samples were separated in five different subsamples: (1) fresh feces; (2) experimental coprolites; (3) experimental coprolites with the addition of soil sediment; (4) control coprolites; and (5) control coprolites with the addition of soil sediment.

First, fresh feces subsamples were processed by spontaneous sedimentation technique (Lutz, 1919) for 1 h, to concentrate parasite remains. Aliquots of 200 µg were recovered from each sample. In addition, 1 mL was separated for sucrose flotation technique (Sheather, 1928), performing a 30-min flotation in a sucrose-saturated solution ($\delta=1290$).

Experimental coprolites with sediment were prepared by mixing archeological sediment with equal mass in grams of fresh feces. In order to obtain dehydrated samples emulating original coprolites, experimental coprolites with and without soil sediment were then desiccated at 37°C and daily dehydration was controlled by weighing, until no further reduction in weight occurred, which took place after 2 weeks. Then, they were kept at 37°C for the same time they were initially dehydrated (Iñiguez, 1998), to ensure total dehydration. However, no weight variation was observed during this additional period, counting a total dehydration time of 4 weeks. On the other hand, coprolite controls, with and without sediment, were kept at −20°C throughout the process to avoid the influence of ambient temperature on the samples, resulting in decomposition, for example, and mainly, as a parallel comparison with samples subjected to the effects of desiccation. Soil sediments were taken from the cranium of an individual from El Diquecito site (2562 ± 47 BP AA93742), located in the coast of Mar Chiquita Lagoon, province of Córdoba. This individual was negative to paleoparasitological examination (Ramirez et al., 2021b). Soil texture was silt loam with a basic pH (8), and showed a violent reaction when immersed in hydrochloric acid, suggesting the presence of large amounts of calcium carbonate (Tavarone et al., 2016). When humid, the sample was yellowish brown (10YR5/4, according to the Munsell Soil Color Chart); when dried, it was very pale brown (10YR7/3) Tavarone, 2014).

Conventional paleoparasitological techniques were applied to experimental and control coprolites. First, they were rehydrated in a 0.5% water solution of trisodium phosphate for 72 h (Callen and Cameron, 1960) at 4°C. Spontaneous sedimentation was used for 24 h and aliquots of 200 µg of each subsample were taken for the analyses. Flotation technique was applied as described.

The observations were made with a light microscope (Labklass XSZ 107 CCD) using 100× magnifications. All the eggs

Table 1. Samples, helminth species, and egg quantification in fresh feces, experimental, and control coprolites.

Sample ID/parasite	Fresh feces					Experimental coprolites					Control coprolites			
	NoHo		EPG			NoHo		EPG			NoHo		EPG	
	SS	FL	SS	FL	ID	SS	FL	SS	FL	ID	SS	FL	SS	FL
ID01 *Ascaris* sp.	1272	353	3180	882.5	EC	33	22	82.5	110	CC	159	237	397.5	592.5
					ECsed	6.5	2.5	65	25	CCsed	131	32	1310	320
ID03 *H. nana*	190	25	475	62.5	EC	111	–	277.5	–	CC	233	12	582.5	30
					ECsed	–	–	–	–	CCsed	38.5	1	–	10
ID04 *Ascaris* sp.	43	3	107.5	7.5	EC	18	–	45	–	CC	–	–	–	–
					ECsed	1.5	–	15	–	CCsed	–	–	335	25
ID05 *H. nana*	499	142	1247.5	355	EC	35	5	87.5	25	CC	57	7	142.5	17.5
					ECsed	–	–	–	–	CCsed	33.5	2.5	–	–
ID06 *E. vermicularis*	2	4	5	10	EC	–	–	–	–	CC	–	–	–	–
					ECsed	–	–	–	–	CCsed	–	–	–	–
ID07 *H. nana*	275	33	687.5	82.5	EC	89	2	222.5	10	CC	64	17	160	42.5
					ECsed	2	–	20	–	CCsed	0.5	–	–	–
ID08 *H. nana*	117	3	292.5	7.5	EC	20	5	50	25	CC	127	15	317.5	37.5
					ECsed	0.5	–	5	–	CCsed	8.5	1.5	85	15

NoHo: number of helminth eggs; EPG: eggs per gram of feces; EC: experimental coprolite without addition of soil sediment; ECsed: experimental coprolite with sediment addition; CC: control coprolite without sediment addition; CCsed: control coprolite with sediment addition; SS: parasitological technique of spontaneous sedimentation; FL: parasitological technique of flotation in sucrose-saturated solution.

present were counted and eggs per gram (EPG) were estimated using the formula $EPG = NEO/0.02$, where NEO means Number of Eggs Observed. The divisor 0.02 refers to the 200 µg aliquot that was totally analyzed from each sample. In subsamples without addition of soil sediment, including fresh samples, the formula was adjusted to $EPG = (NEO/0.02)/2$. The integrity of the retrieved eggs was also evaluated by morphological analysis considering subsamples and both techniques. A total of 40 subsamples and approximately of 400 slides, 10 per subsample, were examined.

Non-parametric of Friedman and Wilcoxon signed-rank tests were performed in a Microsoft Excel® program through Data Analysis Toolpak for Excel, with the null hypothesis of no difference in parasite recovery ($\alpha = 0.05$), using the EPG calculated in each experimental procedure. The analyses were corroborated with R statistical software version 4.0.2 (R Core Team, 2020).

Results

An important loss of parasite eggs was observed in all the samples when comparing fresh feces subsamples with experimental and control coprolites, both with and without sediment soils added (Table 1). When donated, one sample positive to *E. vermicularis* (ID02) showed no eggs, even in the fresh feces subsample, thus it was not used for evaluation. Another sample positive to pinworm infection (ID06) only showed parasite eggs in the fresh feces subsample.

Statistical analyses demonstrated significant differences among subsamples when both, spontaneous sedimentation Lutz ($p = 0.01$) and Sheather flotation techniques ($p < 0.01$), were applied. The detection of eggs was statistically significant using spontaneous sedimentation when compared to the performance of flotation technique regarding fresh feces ($p = 0.03$) and experimental coprolites ($p = 0.03$). Considering all subsamples ($n = 40$) using the two techniques, the difference among EPG values was highly significant ($p < 0.01$; Supplemental Appendix 1).

Statistical analyses revealed a particularly significant difference concerning dehydration effects between the initial EPG values in the fresh feces and those obtained from experimental coprolites ($p < 0.01$; Figure 2a). Regarding the effects of sediment soil, a marked difference was noted between the initial EPG values in the fresh feces and those recovered in experimental coprolites with sediment ($p < 0.01$; Figure 2b). A significant difference was observed when comparing EPG from fresh subsamples with those of control coprolites without soil ($p = 0.02$) and highly significant with control coprolites with soil sediment ($p = 0.01$). EPG significantly differed from control coprolites with sediment subsamples in relation to their corresponding experimental coprolites with sediment ($p = 0.02$). When experimental coprolites without soil sediment were compared with their respective control subsamples, a near to α value was observed, showing no significant difference ($p = 0.06$; Figure 2a and b). Importantly, there was a highly significant difference in egg loss between experimental coprolites with and without sediment addition ($p < 0.01$; Figure 2c; Supplemental Appendix 1).

Partial deformation of egg walls and internal content was observed only in subsamples containing *H. nana* eggs, using both techniques. However, these morphological modifications of eggs were evidenced in a larger number of experimental coprolites with sediment (ID07 = 75, 0% of deformed eggs by Lutz, Sheather, respectively) and without sediment (ID03 = 62, X%; ID05 = 45, 60%; ID07 = 54, 50%; ID08 = 50, 0%; 0 = No eggs deformed; X = No eggs retrieved; Figure 1d and e) than in fresh feces (ID03 = 24, 32%; ID05 = 23, 28%; ID07 = 4, 0%; ID08 = 0, 0%) that were predominantly intact (Figure 1a). The same tendency was also observed in control subsamples containing *H. nana* eggs, with a percentage of modified eggs in subsamples with sediment: ID03 = 11, 0%; ID05 = 45, 100%; ID07 = 100, 03%; ID08 = 0, 33%, and without sediment: ID03 = 13, 0%; ID05 = 45, 43%; ID07 = 30, 29%; ID08 = 4, 13%. *Ascaris* sp. eggs showed no evidence of deformation in the subsamples (Figure 1b and f).

Discussion and conclusions

The influence exerted by environmental and climatic conditions and the different elements present in the soils on the preservation of the micro remains is a major issue when working with ancient parasites recovery. Thus, paleoparasitologists often find helminth eggs, with their diagnostic elements, altered in different ways, from slightly damaged to total absence, preventing parasite identification.

In the present work, we evaluated the influence of dehydration and local soil adding to modern feces on loss of parasite eggs. We

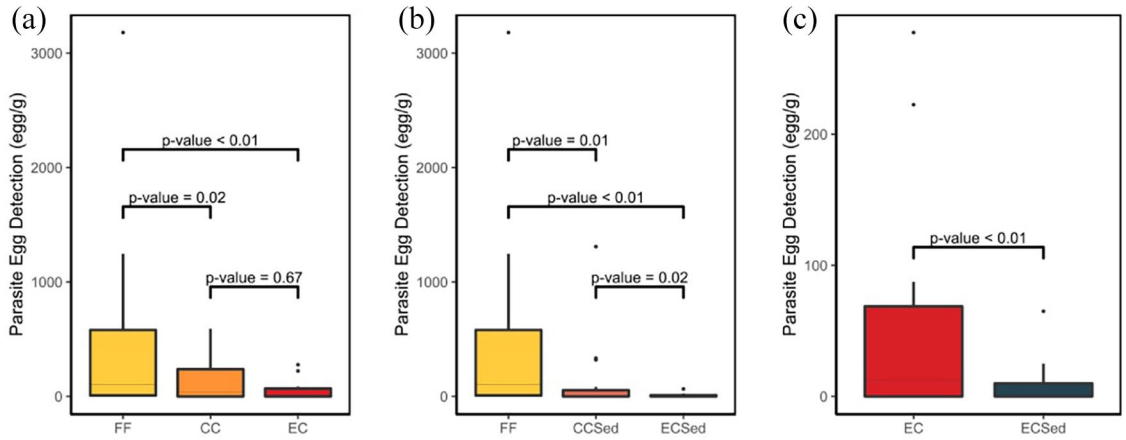

Figure 2. Boxplots displaying EPG values for each subsample evaluated. Eggs loss due to (a) desiccation effect, (b) sediment addition effect, and (c) desiccation effect versus desiccation plus sediment addition effect.
FF: fresh feces; CC: control coprolites; EC: experimental coprolites; CCsed: control coprolites with sediment addition; ECsed: experimental coprolites with sediment addition.

analyzed 40 final subsamples including fresh feces, experimental coprolites with and without sediment from local soil, and control coprolites with and without sediment.

We collected data that allow us to infer about post-depositional influence on parasite recovery. A highly significant loss of eggs of *H. nana*, *E. vermicularis*, and *Ascaris* sp. was observed between the fresh feces subsamples and the experimental coprolites or experimental coprolites with sediment (Figure 2), evidencing the importance of both desiccation and local soil in parasite degradation.

Previous research employing experimental coprolites showed that, although suffering gross morphological alterations after desiccation, *Trichuris trichiura* eggs presented no statistically significant morphometric changes. Therefore, whipworm could be identified at a species level (Confalonieri et al., 1985). As noted by Araújo (1988), modification of ancylostomid eggs after experimental desiccation was not significant either. In the present study, in most fresh subsamples with *H. nana*, the egg structure, including walls and filaments, was found in ideal conditions, allowing precise identification (Figure 1a). In contrast, in their experimental coprolite subsamples, a high number of eggs with deformed walls was detected (Figure 1d). If this phenomenon was observed in authentic coprolites, deformation could hamper identification at a species level. Although *H. nana* and *H. diminuta* eggs are not close in size to each other (Atías, 1998), when considerable shrinkage in their walls occurs, the distinction between these common species by morphometric means could be hindered. On the contrary, *Ascaris* sp. eggs showed no morphological modifications when desiccated (Figure 1f). This is possibly due to eggshell composition, which includes multiple thick albuminoid and lipid layers (Atías, 1998). Hymenolepid eggs were more prone to deformation and degradation, both due to soil composition and desiccation. However, the deformation of some eggs in fresh feces shows how fragile the eggs of this species are and how easily they tend to decay. In addition, *E. vermicularis* eggs were found in one fresh feces subsample (Figure 1c), but none in the rest of the subsamples. This case might also result from the structure of the parasite, since pinworm eggs have thin layers that decompose easier than those of other helminth eggs. This highlights the importance of performing more sensitive assays like those of Molecular Paleoparasitology which have allowed detecting *E. vermicularis* infection by paleogenetic means in individuals without eggs found by microscopy (Iñiguez et al., 2003, 2006; Jaeger and Iñiguez, 2014).

In this work, the effect of soil sediment had a high statistically significance ($p < 0.01$; Figure 2b and c), which could be attributed to both organic and inorganic soil matter.

Among abiotic and inorganic compounds, there are metals, salts, and other chemicals that can have an aggressive effect on the structure and composition of parasite remains, and alter their morphology and morphometry. Partial deformation in *H. nana* was observed in experimental coprolites with sediment samples, which could result from the presence of some compound in the soil (Figure 1e). Nevertheless, in the control coprolites from other subsamples, egg deformation of the same species was also seen, thus indicating that there should be another factor besides soil composition that modifies parasite structures. Interestingly, we observed that morphological alterations caused by cold storage did not show the same pattern in the three helminth species analyzed. Roundworm eggs did not seem to suffer morphological distortion caused by desiccation, addition of soil, or freezing; instead, well preserved *Ascaris* sp. eggs were identified.

In this sense, since control coprolites were kept at −20°C, freezing could be a factor that prevents a totally good preservation of remains, causing instead some kind of damage, as indicated by Sianto et al. (2013), and significant egg loss, as demonstrated in this study (Figure 2a and b), when compared to the initial EPG. Morono et al. (2015) showed that freezing and storage at −20°C of environmental samples decreased microbial cell count to 10.7%, and lowered their viability compared to that of fresh samples. Cryopreservation has been traditionally regarded as the best method for overcoming decomposition and putrefaction in the preservation of biological samples, despite damage caused to cellular structures by ice crystal formation (Morono et al., 2015). These contrasting properties could have been involved in the destruction of helminth eggs observed in this work, but this might have been caused also by the microorganisms contained in the soil. Notably, when EPG from experimental coprolites and control subsamples without soil were compared, close to α, no significant difference was observed between them, showing similar influence of temperature and cold storage, in sharp contrast with the significant decrease of EPG values in subsamples of experimental coprolites (desiccation) with soil added (vs freezing).

The pH of soil sediment could be a further factor leading to the deformation of *H. nana* eggs. Previous research showed notably well-preserved eggs and larvae found in Korean mummies recovered from an extremely alkaline environment, in tombs with addition of lime-soil mixture (Shin et al., 2009). Samples from a mummified bog body from acidic pH environments also showed particularly good preservation; yet, no fragile parts, that is, those parts of the eggs made up of soft structures, were observed (Searcey et al., 2013). Rácz et al. (2015) recovered parasite eggs from coprolites of skeletonized human remains from three different alkaline

environments; they observed that embryos and plugs were not properly preserved, suggesting that a basic pH would not allow good preservation for the most fragile egg parts. In the present work, the alkaline pH of the sediment used for the elaboration of the experimental coprolites could also be another aggressive element for the preservation of some parts of the eggs, particularly in those of *H. nana*. However, the influence of pH on parasite preservation has been controversial (Reinhard et al., 1986) and, as shown, no consensus on this regard has yet been reached. Therefore, further research will allow gathering more empirical evidence for this issue.

As mentioned above, for biotic and organic elements present in soil, there is wide diversity of free-living nematodes, fungi (including nematophagous and coprophagous species), insects, and bacteria. Many of these organisms have the potential to destroy parasite remains, thus hindering paleoparasitological findings in certain sediment contexts, such as Brazilian *sambaquis* (Camacho et al., 2013), urban post-Contact archeological sites in Brazil (Jaeger et al., 2013a, 2013b), and latrines in USA (Reinhard et al., 1986). Furthermore, Camacho et al. (2016) suggested that the loss of *Ascaris lumbricoides* eggs in an experimental approach emulating stratigraphic levels was most probably due to biodegradation.

In the present study, we proposed that both the chemical composition and pH of the soil, as well as the presence of living organisms, seem to magnify the damage done to parasite eggs, since their loss in experimental coprolites in the presence of the soil was dramatic.

Yet, we are aware of the limitations of this study. More experimental coprolites need to be elaborated to increase observation of the effect brought about by desiccation and local soil on preservation. We also observed that the parameters evaluated affected parasite species differently, thus a larger number of samples need to be studied, including other parasites not examined here. Other types of soils with chemical and granulometry data also need to be tested. Future research will require testing experimental coprolites with other soils, from different locations and with other characteristics. Additionally, a formula for estimating the EPG values which includes species gravity and sample density should be designed in future experimental approaches.

In sum, we observed a significant decrease of parasite eggs when desiccation and soil effects were evaluated separately. The destructive effect of both parameters was amplified when considered together. The decrease of original parasite load suggests that paleoparasitological results are underestimated and that they depend not only on the climate of the archeological site region, but also on the type of soil and on the characteristics of structures of parasites. Paleoparasitological findings, often consisting in scarce or only one egg, do not reflect the real parasite infection rate that the individual had at the moment of death.

Although preliminary, this study provides new insights into the interpretation of paleoparasitological results, and consequently, into the understanding, at a regional level, of the health status of past human groups, in addition to contributing to Paleoparasitology in general.

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Supplemental material

Supplemental material for this article is available online.

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