

Part II - Parasite Remains Preserved in Various Materials and Techniques in Microscopy and Molecular Diagnosis

15. Paleoparasitological Diagnosis

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FUGASSA, M.H. Paleoparasitological Diagnosis. In: FERREIRA, L.F., REINHARD, K.J., and ARAÚJO, A., ed. *Foundations of Paleoparasitology* [online]. Rio de Janeiro: Editora FIOCRUZ, 2014, pp. 223-254. ISBN: 978-85-7541-598-6. Available from: doi: [10.7476/9788575415986.0017](https://doi.org/10.7476/9788575415986.0017). Also available in ePUB from: <http://books.scielo.org/id/zngnn/epub/ferreira-9788575415986.epub>.



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Paleoparasitological Diagnosis

Martín H. Fugassa

Paleoparasitology emerged in the early 20th century using light microscopy studies. Although numerous tools have been added since then, light microscopy is still the most widely used method for paleoparasitological examination and study of microfossils.

Paleoparasitology has made significant progress in nearly a hundred years, partly through the adoption and adaptation of methodologies developed in other fields such as clinical parasitology and biology molecular. However, the inherent difficulties of archaeological materials require on-going improvement of methods and the introduction of new ones.

In the coming years, the extent to which paleoparasitology contributes to paleoecological and anthropological studies will depend largely on increasing the number of samples for examination. It is thus necessary to multiply the range of evidence beyond conventional sources like coprolites and mummified tissues. This will also require expanding the availability of powerful methods and techniques for recovering parasitological information. The following is a description of various methodologies and applied techniques in paleoparasitology, some still in the experimental stage.

SAMPLING

Enteroparasites (helminths and protozoa, or parasites of the gastrointestinal tract) commonly release eggs or other forms for dispersal in the feces. Helminth parasites of the respiratory tract can also be dispersed by passage of eggs into the host's digestive system when they are transported with mucous, as in the case of *Eucoleus aerophilus* and *Metastrongylus* sp. (Miyazaki, 1991).

Coprolites and various archaeological sediments provide the clearest source of evidence in the paleoparasitological study of enteroparasites, while mummified tissues and bones are generally associated with paleopathological and tissue parasite studies.

Regardless of their nature, paleoparasitological samples should be extracted and handled under aseptic conditions, because molecular and optical studies of microfossils involve a certain risk of contamination from current and ancient sources. Thus, the sample design should consider not only the necessary statistical aspects, but also the aseptic conditions for procedures.

The following is a list of general recommendations for handling samples in fieldwork:

- Use sterile, hermetic, and resistant recipients.
- Use new sterile and preferably disposable instruments (spoons, spatulas, tweezers, scalpels, etc.).
- Depending on the sampling conditions, in the field or laboratory, and on the type of sample, it is necessary to use a cap and mask to protect the face and hair. During excavations, one should use gloves without talcum powder (to avoid contamination with starch, which can influence the diagnosis of the use of corn by the prehistoric group), at least during the removal of samples for paleoparasitological examination and especially when they involve food remains.
- Samples should be extracted as quickly as possible to avoid leaving the sampling area uncovered for prolonged periods. One should avoid extracting samples under adverse weather conditions such as heavy wind or rain.
- Both in the field and in museums or other institutions where archaeological collections are kept, sample collection equipment and the resulting samples must be kept in clean, hermetic recipients, specific for the purpose.
- It is best if at least one subsample in each case is kept refrigerated at 0°C for molecular studies (Alena Iñiguez, personal communication).

For statistical purposes, collecting samples from coprolites and sediments require a prior design. This design should be conducted carefully in order to reflect as closely as possible the properties of the sampled population from which one intends to obtain inferences. Extraction of samples from sediments or coprolites should consider the spatial distribution and density of the parasite remains. It is thus possible to establish some basic hypotheses: 1) coprolites contain more parasites than free sediments, with the exception of latrine deposits; 2) distribution of parasites in the coprolites may not be random; and 3) distribution of parasites within a particular sector of the stratigraphic unit is random, or steps can be taken for random distribution to occur. These assumptions mean establishing different sampling plans for free sediments and for coprolites and other concretions.

Extracting samples from coprolites

Before extracting samples for the study of microfossils, one should macroscopically examine and describe the coprolites. This procedure is useful for both identifying them and for the detection of hairs, feathers, bone fragments, or any other remain from the individual's diet and which could be associated with parasites.

Macroscopic observation also allows obtaining taphonomic data on the sample's state of preservation, e.g., the presence of fungi or perforations resulting from scatophagous insects.

In order to interpret the distribution of parasites in coprolites, one must investigate the phenomena that allowed or inhibited their preservation over time. Morphometric studies of wildlife coprolites allowed obtaining criteria for the zoological identification of coprolites (Chame, 1988), while various experimental studies have helped assess the effects of desiccation and rehydration on the dimensions of the parasite remains (Confalonieri, 1988b; Araújo, 1988).

In current samples, Martín & Beaver (1968) showed that particles entering the digestive tract mix uniformly with the feces at the upper colon or slightly before. Likewise, Ye et al. (1997) found a uniform distribution of *Trichuris*

trichiura and *Ascaris lumbricoides* eggs inside and on the surface of current human feces. One can thus expect that the eggs of parasites living in the upper portion of the intestine, as well as parasites found in animals ingested as food, will be distributed uniformly in the fecal mass. However, parasite species located distally in the digestive tract, like *Enterobius vermicularis* and the tapeworms that release proglottids containing eggs, may be located mostly on the coprolite's surface.

Therefore, in coprolites and concretions found in pelvic sediment – called enteroliths – it is useful to obtain a surface sample and another from the interior (Fugassa, 2006). Since parasite remains may be aggregated, it is convenient to sample small amounts from various sites, both on the surface and inside the coprolite.

Usually, paleoparasitological examination is only done on parts of the coprolite, which often means finding a small number of eggs (Confalonieri et al., 1988b). The preservation of parasite remains can vary greatly between archaeological sites and between taxonomic groups of parasites. As a result, coprolite examination can yield a limited total number of parasites or of ones that preserve taxonomically valuable structures for identification.

Coprolite size can vary greatly, thus requiring different sampling strategies. As mentioned, parasites can be found both inside and on the surface of sample, or they can concentrate in one of these parts. Both inside and on the surface, parasite remains can be distributed in an aggregated way as the result of differential taphonomic processes. One strategy might be to examine the entire coprolite, but this would impede future studies such as the application of molecular biology techniques and investigation of other microfossils in the same sample.

Therefore, the suggested strategy is to take a small sample from the surface and another from the interior, about 0.5-1.5 g each, consisting of numerous subsamples representing the entire coprolite. Exceptionally, in very large coprolites such as those of megafauna, one could use a design including the removal of more and larger samples (Fugassa, 2006).

Thus, as we will describe below for free sediments, coprolites also require controls for adequate interpretation of the parasitological information recovered from them. A subsample from the coprolite surface can function as a control for the internal sample, supposedly less disturbed. Ideally, the sediment from the coprolite's setting (in a radius of about 4 cm) would be highly useful as a control. The sediment removed from under the coprolite would serve to assess whether there was vertical migration of parasite remains, carried by water or other liquids (Dommelier-Espejo, 2001).

Sampling of sediments

To date, the most widely used objects of study in paleoparasitology have been coprolites and enteroliths (Reinhard et al., 1988). However, paleoparasitological analysis of sediments can help elucidate the use of deposits such as latrines, garbage heaps, silos etc. (Bouchet, 1995). Pike (1968) was the first to propose the use of parasitological tests in archaeology as indicators of the use of space.

Taylor was the first to conduct paleoparasitological studies in latrines and wells, in the mid-20th century (Fernandes et al., 2005). Numerous studies were done on other free sediments. Specifically, the antecedents for the study of archaeological sediments rely on records in latrines and other soils from human occupation (Bouchet, 1995; Bouchet et al., 1999, 2002; Fernandes et al., 2005; Fugassa & Barberena, 2006; Moore, 1981; Pike, 1968; Taek Han et al., 2003), middens (Bathurst, 2005), various jars and other recipients (Harter et al., 2003), and skeletons (Aspöck, Auer & Picher, 1996; Bouchet et al., 2001; Dittmar & Teejen, 2003; Fugassa, Araújo & Guichón, 2006).

Free sediments are generally assumed to involve greater dispersal and exposure to deterioration of parasites, with low probability of finding parasite specimens (Confalonieri et al., 1988b). For example, the sandy sediments of the Patagonian steppe display a greater degree of complication, such as the percolation and hydraulic and thermal stress to parasite remains (Fugassa, 2006). These taphonomic events affect the expected paleoparasitological yield and should be included in the sampling design for sediments, both from skeletons and those representing the archaeological soil.

Parasite vestiges inside the sediment sample (e.g., from the sacrum) can display random, uniform, or aggregated distribution. Defining the type of distribution of such remains allows establishing a sampling plan. Jones (1990) found concretions of fecal matter greater than 1 mm in diameter during excavations from 1983 to 1987 in York, England, might suggest contamination or aggregation in the sample. However, this depends on the size of the selected sample unit and the size of the concretions. For example, when extracting a 10 g sample, the sampling unit is many times greater than these concretions and will definitely behave as a random distribution. To ensure that the sample is random, one should first homogenize it.

Before sampling, the sediment should be observed with the naked eye, since it can contain interesting remains such as rodent coprolites (Fugassa, Araújo & Guichón, 2006; Fugassa, 2006) or coprolite fragments from the individual himself.

As emphasized by Jones (1992), any comparative study requires using the same testing techniques on all the samples. This allows comparisons to assess the presence of parasites from distinct locations within the archaeological site using the chi-square (χ^2) test (Daniel, 2008). In this case, the test of independence is intended to test the hypothesis of dependence of the presence of parasites in relation to the samples' location.

Sampling of sediments in deposits associated with the archaeological site's occupation (such as dwelling or food-processing areas) requires more controls due to the diverse origins of the parasite remains. Such remains can come from different stratigraphic units or sectors from the same level, due to highly complex processes in the formation of the archaeological record.

SEDIMENTS ASSOCIATED WITH SKELETONS

Starting with the natural or artificial deposition of the corpse, taphonomic processes condition the fate of the bony remains and soft tissues. After the individual's death, the digestive system is generally deposited on the pelvic bones (Reinhard et al., 1992). Reinhard et al. (1992) and later Berg (2002) proved that remains of intestinal contents are usually deposited on the sacrum in skeletons, including those placed in the vertical position at the time of death (Shafer, Marek & Reinhard, 1989).

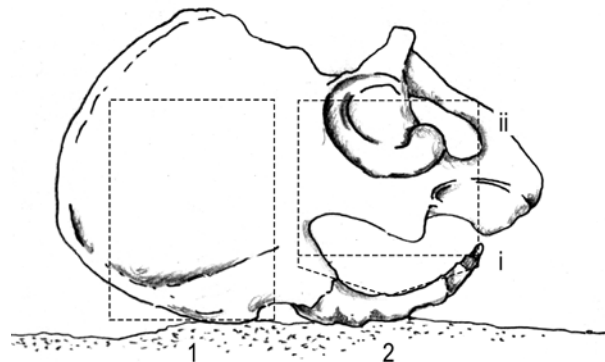
Studies on sediments from the pelvic cavity in human skeletons showed the presence of parasite eggs together with food remains. Examination of the pelvic cavity also revealed coprolites inside mummified bodies (Sianto et al., 2005). However, unlike the situation in mummified bodies, coprolites in skeletonized remains undergo greater dispersal due to various factors such as fragmentation and compression (Reinhard et al., 1992), although enteroliths can occasionally be found in skeletonized bodies (Shafer, Marek & Reinhard, 1989). It is thus recommended to extract the sediment without disaggregating the concretions that may be present.

Remains of parasites can be identified in various excavations and in sediments associated with pelvic bones (Bouchet et al., 2001; Dittmar & Teejen, 2003; Jones 1982b; Reinhard et al., 1992). Using palynological studies, macrobotanical remains, and fauna, Berg (2002) also demonstrated the importance of sediments from the sacrum, particularly from the sacral foramina, for studying digestive remains in skeletons.

Although sediments from skeletal remains usually display a lower density of parasite remains when compared to other deposits, they offer an opportunity to contribute results to the elaboration of epidemiological studies. In some regions there are more skeletons than coprolites available, and they can be used to establish sex and age by osteological examination, as well as information on location in time and space, allowing an ideal association between parasites and individuals. Thus, the analysis of a larger number of individuals allows interpreting this information from a population perspective.

The samples examined by Reinhard et al. (1992) were obtained from a column of sediment located between the sacrum and the pubic symphysis (Figure 1), in supine position. The sample close to the sacrum showed the largest amount of intestinal content.

Figure 1 – Obtaining sediment from the pelvic girdle of a skeleton



- (1) sediment at the level of the last lumbar vertebrae
 (2) column of sediment over the sacrum, separated into two fractions:
 i) sacral concavity and ii) over the sacrum.

Modified from Reinhard et al. (1992).

It is possible to add a column of sediment at the level of the 4th and 5th lumbar vertebrae and the hepatic region (Figure 2) to this sampling scheme, although these locations have the disadvantage of lacking a bony structure to surround the deposited remains. As for the individual's position, the sacrum can also retain material in bodies close to the anatomical position (Reinhard et al., 1992).

Figure 2 – Distribution of samples and controls in sediments associated with human skeletons



- | | |
|---------------------------|------------------------|
| (A) Control next to skull | (B) Sample from sacrum |
| (C) Sample from pelvis | (D) Control from femur |

Photograph: Martín Fugassa.

The use of controls facilitates the discussion on possible sources of contamination (Berg, 2002; Reinhard et al., 1992). Sediment samples are also exposed to changes resulting from infiltration of water and erosive processes. In free sediments, the water flow can carry parasites and other microfossils to lower strata by percolation (Bouchet et al., 2003; Dommelier-Espejo, 2001; Pike, 1968; Reinhard et al., 1988). This phenomenon is probably more intense in sandy soil from open-air sites than in caves or rock shelters.

It is important to conduct sampling at different depths, ideally using probes to minimize impact and contamination with sediments from other strata. This permits a comparative reading between different levels and interpretation of results (Bathurst, 2005; Dommelier-Espejo, 2001). In human remains, one should also examine sediments removed from below the skeleton due to the probable leaching of microfossils (Fugassa & Barberena, 2006).

In processing samples, in addition to sediments that are observable under the microscope, one should also examine the macroscopic remains. A stereoscopic microscope can also be used to examine remains retained in the screen or gauze immediately after the paleoparasitological processing (see below). However, before proceeding to

paleoparasitological techniques, one should examine the sediments to separate potentially interesting remains, such as small bones, seeds, fragments of insects, and others, which could be lost during the processing. This preliminary observation has been important for identifying rodent feces in pelvic sediments from skeletons (Fugassa et al., 2006b; Fugassa & Barberena, 2006), including the pupae of flies and other insects, indicative of the individual's funeral conditions (Fugassa et al., 2008a; Reinhard et al., 1992). Observation of sediments should be systematized, as suggested by Jouy-Avantin (2003) for coprolites.

FIRST STEPS FOR PROCESSING SAMPLES

Before processing and studying samples, one should consider the molecular and microscopic studies that can be altered by the presence of contaminants of different origins:

- contaminated materials introduced into the laboratory (clothing, hands, articles, shoes, instruments, etc.);
- inefficient cleaning of the study instruments.

Cleaning should thus be performed in microbiology laboratories, with the following most important precautions:

- reduce the exposure of samples outside their recipients;
- preferably use disposable material and instruments;
- wear mask, gloves, and cap;
- restrict the circulation of persons inside the laboratory;
- use a protocol for historical follow-up of samples with a daily laboratory log (Dommelier-Espejo, 2001).

Rehydration and separation of microfossils

Regardless of the origin of the samples (coprolites or sediments), they should all be properly stored to allow submitting them to some of the techniques for concentration and enrichment of parasite remains.

Organic sediments are usually aggregated. Disaggregation is the process of separation of carbonates and/or humic acids by chemical or mechanical means (Bouchet, 1995; Coil et al., 2003). The alternatives include a commercial detergent solution (Kruger apud Reinhard et al., 1988), but the group of Françoise Bouchet (France) uses a porcelain mortar after rehydration and later exposes the sample to ultrasound for a period of 20 seconds to a maximum of 3 minutes (Bouchet et al., 1999, 2002).

The nature of the soil in question is important for determining whether other techniques are necessary. For example, limestone soils may need treatment with hydrochloric acid to recover parasite remains from the carbonate matrix. Jones added the use of diluted hydrochloric acid to dissolve possible carbonate concretions and retain microfossils (Reinhard et al., 1992). Hydrochloric acid may also be necessary in mineralized coprolites (Ferreira, Araújo & Duarte, 1993).

According to Coil et al. (2003), glacial acetic acid can be a less aggressive alternative that avoids loss of mineral microfossils like calcium oxalate phytoliths. Although the focus here is parasite remains, the possible inclusion of other studies requires contemplating a multidisciplinary protocol design.

Coil et al. (2003) distinguish between disaggregation and deflocculation, defined as the separation (by electric repulsion) of mineral particles the size of clay (less than 4 μm) that can hinder observation. If disaggregation and deflocculation are ineffective, the subsequent filtrate may retain microfossils, making them absent to microscopic observation.

Rehydration has been attempted by various means, like distilled water, EDTA and sodium hydroxide, alcohol, and others (Harter et al., 2003, Hidalgo Arguello, 2006), although aqueous trisodium phosphate is still the most popular. Initially applied by Van Cleave & Ross (1947), as well as by Van Cleave & Delinger and Benninghoff in microfossils (Figuerero Torres, 1982), and soon thereafter by Callen & Cameron (1960), rehydration in aqueous sodium phosphate solutions also allows deflocculation of the sediments (Coil et al., 2003). Certain groups in paleoparasitology, like Françoise Bouchet, use rehydration in aqueous trisodium phosphate and add 5% glycerin (Le Bailly, 2005), a technique used successfully by other groups.

Rehydration time varies by author and the material's conditions. Typical rehydration time is usually three days to a week and can be extended if the material is very dry or hard (Le Bailly, 2005). During rehydration, the sample should be observed to avoid proliferation of fungi or bacteria. Any such growth can be stopped by adding 10% alcohol.

The choice of a fixing method should include the possibility of applying specific molecular, immunological, or staining tests. Therefore, researchers increasingly attempt to keep the samples refrigerated in order to prevent formalin (an inhibitor of the polymerase chain reaction, PCR) from interfering in the latter technique.

Rehydration and preservation with refrigeration, without using fixing solutions, has allowed recognizing the natural smell of rehydrated coprolites, especially from canids and felines dated to 6,500 BP (Fugassa, 2006; Fugassa et al., 2006). It is thus recommended that both the processing and observation be done without fixing solution and with refrigeration (from 0 to 4°C). However, this cold storage should not be maintained for too many days, because microorganisms can proliferate despite it in some samples. It is recommended to add ethanol 70% to the sediments, since it is the preserving agent that causes the fewest problems for molecular techniques. In addition, the design of immunological tests should include refrigeration of rehydrated sediments rather than preservation with formalin solutions, due to possible interferences in antigens and antibodies (Le Bailly, 2005).

To separate particles larger than 300 μm from those that may contain parasite remains, the sample is sifted with screens soon after rehydration. Importantly, the sediment should be completely rehydrated and disaggregated, or the microfossils could be trapped or adhered to other particles and become lost during filtration.

Bouchet et al. (1999) use a series of metal screens (315 μm , 160 μm , 50 μm , and 25 μm) through which the rehydrated sediment is sifted to recover remains retained in the last screens, with 50 μm and 25 μm meshes, respectively. The particles retained in the larger screens (from 315 to 160 μm) serve for studies on diet and sedimentology (Dommelier-Espejo, 2001), but they can retain eggs of trichostrongylidae such as *Nematodirus* sp., recorded in coprolites from Patagonia (Fugassa, 2006).

After sifting the rehydrated sediment, according to Coil et al. (2003) the elimination of clay avoids its interference in flotation solutions that can be applied, besides facilitating viewing under the microscope. In experiments with coprolites from Patagonia, visibility was hampered by fecal compounds that darkened the solution (Fugassa, 2006). For both sediments and coprolites, the sediment should be washed several times, eliminating these substances, plus the clay and possibly grains of sand (Coil et al., 2003). Thus, several rounds of washing should be performed in the rehydrated sediment and filtrate to improve the visibility, always taking care not to lose any microfossils that may be present.

Enrichment techniques used in paleoparasitology

Coproparasitological studies target the presence of parasites (larvae, eggs, and cysts) in fecal remains. The most commonly recommended techniques are flotation and sedimentation, which allow concentrating the parasite elements in a small fraction of the fecal matter.

For clinical analyses, Thienpont, Rochette & Vanparijs (1979) recommend using concentration techniques when direct techniques yield negative results (direct techniques are defined as those that analyze a minimal portion of the sample without concentrating the target elements before observation). The advantages of direct techniques are speed, ease, and minimal disturbance of delicate parasite remains, such as trophozoites of *Giardia* sp. or *Entamoeba* sp. (although the latter are relatively uncommon in archaeological samples).

Direct techniques are recommended for tests in recent reptile and bird droppings, but normally not for paleoparasitological tests, although they are used in some cases such as Hidalgo Arguello (2006).

Among the enrichment techniques, sedimentation consists of concentrating parasite remains by deposition in an aqueous solution, less dense than the eggs or other parasite forms. The most important sedimentation techniques in clinical parasitology are spontaneous sedimentation (Lutz, 1919) and the Telemann formalin-ether technique (Thienpont, Rochette & Vanparijs, 1979). Spontaneous sedimentation involves a prolonged reading time due to the abundance of debris, but it is more sensitive, quick, and economical than other available techniques.

The formalin-ether techniques (Ritchie technique) and its modifications (formalin-acetate [Telemann technique] and formalin-tween) consist of disaggregation of an amount of fecal matter in a solution of an organic substance (ether, tween) in a polar medium (formalin or acetate). These substances should be shaken vigorously with the fecal matter, separating the suspension into two phases (Kaminsky, 2003; Thienpont, Rochette & Vanparijs, 1979).

The upper, less dense phase is the organic phase, while the lower phase is the polar compound. Parasite eggs and cysts settle on the bottom of the tube, and various debris concentrate in both phases. Horne & Tuck (1996) used the formalin-ether technique to examine sediments from historical latrines in North America, recovering eggs of *Ascaris* sp., *Trichuris* sp., *Dicrocoelium dendriticum*, and *Taenia* sp. However, Reinhard, Ambler & McGuffie (1985) had tested this sedimentation technique, observing that simple or spontaneous sedimentation was more efficient. There is limited paleoparasitological experience with this technique, widely used in clinical parasitology, essentially due to efficient separation of parasite remains from debris and fat.

Enrichment techniques using flotation are based on the relative density of the parasite remains. For the remains to float, the flotation solution must be denser than they are. Various saturated solutions are used, in which one introduces the fecal matter previously sifted in a screen or gauze and rehydrated, in the case of paleoparasitological studies.

It is common to place the sample in the test tube and add a little flotation solution, then mix and fill the tube to the brim with solution, avoiding bubbles that could hamper visualization. The slide cover is placed on the surface such that it makes contact with the solution. After some time, varying from 15 minutes to two hours, the parasite remains float to the surface of the solution. Mild centrifugation can be used to shorten the time for the parasite remains to float (Kaminsky, 2003; Thienpont, Rochette & Vanparijs, 1979). The slide cover is then removed, and if there are any parasite remains, they are adhered to the lower surface of the slide cover, which is then placed on the slide for observation under the microscope.

Different groups of parasites have different densities and thus behave differently in flotation solutions, with trematode eggs requiring denser solutions (Thienpont, Rochette & Vanparijs, 1979). In addition, the techniques' effectiveness varies according to the conditions of the sediment being examined. Bouchet et al. (1999) used solutions with specific gravity ranging from 1.1 to 2.0, since taphonomic factors typically make the density of ancient eggs vary greatly from modern ones. Sucrose solutions at various concentrations, sodium chloride, sodium nitrate in sucrose solution and others have been used in archaeological samples in France (Dommel-ier-Espejo, 2001). Importantly, the eggs tend to warp, fade, and lose the operculum (in the case of operculated eggs) when very dense solutions are used,

or for a very long time (Thienpont, Rochette & Vanparijs, 1979). In ancient samples, the use of zinc sulfate causes deformations, breakage, or peeling in eggs of *Hymenolepis* sp. (Reinhard et al., 1988).

Although the flotation technique in sodium chloride solution (Willis technique) is more common, Marder et al. (2000) found no differences in relation to the Sheather technique (sucrose solution) and Faust (sodium sulfate solution) for the detection of modern *Toxoplasma* cysts and nematode eggs. However, using the Willis technique, Binda, Moriena & Alvarez (2003) obtained more negative results and fewer *Giardia* sp. cysts than using the zinc phosphate technique. Other solutions have been used in paleoparasitology, such as zinc chloride and zinc phosphate (Reinhard et al. 1988), with variations in their osmotic potential and density.

Various authors, like Navone et al. (2005), recommend the simultaneous use of sedimentation and flotation techniques for stool tests in current human samples. In archaeological contexts, the use of two techniques means greater consumption of sediment, which is not recommendable and can only be suggested in specific situations, for example with negative results and an abundant amount of sediment. As an alternative, Bouchet et al. (2001) suggest taking samples from the flotation solution surface and simultaneously from the sediment in the bottom of the tube. Thus, in archaeological sediments, the suggested method is the sequential application of the modified Stoll quantitative technique (Fugassa et al., 2006) and the flotation technique on the same rehydrated sediment (see later in this chapter).

As mentioned, spontaneous sedimentation has the most advantages. However, the final decision depends on various factors like the samples' characteristics, the context from which they come, and the availability of equipment for examination. The selected technique should cause minimum destruction or alteration in the microfossils, especially in parasites.

As for the number of preparations performed for each sample, usually 10 to 20 slides are made (Gonçalves, Araújo & Ferreira, 2003; Holliday, Guillen & Richardson, 2003; Taek-Han et al., 2003). More preparations do not appear to increase the number of species recorded (Harter, 2002) and consume more laboratory time. However, since ancient samples suffer various taphonomic processes, parasites are often found in small numbers, and expanding the number of slides increases the likelihood of positive parasite finds.

The number of slide preparations also depends on the study's objectives and availability of samples. For example, when hundreds of coprolites are available, one can plan to examine only a few slides per coprolite. However, when considering the material's limitations and study objectives, one should not overlook the inherent conditions of sampling. If only a few slides are examined they will probably not be very representative. The study design should address these limitations and prioritize the examination of fewer specimens and more slide preparations.

More preparations are needed in free sediments than in coprolites, because (as discussed previously) the dispersal and deterioration of parasite elements are greater. While some 20 slides are prepared for coprolites, in sediments this figure reaches 50 slides per sample location. In sediments from latrines, Fernandes et al. (2005) examined 20 slides per sample, while Taek Han et al. (2003) made 10 slides. Latrine sediments require fewer slides because they usually have a higher concentration of parasites compared to skeletons or soil samples from archaeological sites.

Quantification in paleoparasitology

Paleoparasitological studies should use a quantitative methodology to obtain comparable results. Quantification in paleoparasitology helps assess the comparative density between locations in the same site and thus discuss possible sources of contamination or the origin of the parasites.

Both Reinhard et al. (1988) and Araújo et al. (1998) claim that quantification of parasite remains can contribute to a paleoepidemiological reconstruction, since it allows comparing egg density between skeletons from burials in the same site, even though parasite density is not highly related to an individual's parasite burden in life. Likewise, Moore (1981) described findings in a medieval latrine and observed a certain stable quotient between the number of *Ascaris* sp. and *Trichuris* sp. eggs. This could suggest comparisons of rates in the number of parasite eggs found, or the use of such an index as a parameter in studying ancient human remains in a given region.

Importantly, quantification does not serve to discuss the intensity of infections, because taphonomic processes extensively modify the initial conditions. Even in current samples, fecal parasite burden is not a good indicator of intensity of infection, since the elimination of eggs, larvae, and cysts in feces depends on numerous parasite, host, and environmental factors, e.g., the number of parasites in the individual, the host immune status, time of year, etc. (Thienpont, Rochette & Vanparijs, 1979). However, parasite burden may be a useful indicator in some circumstances.

Quantitative techniques were first used by Taylor and soon after by Pike (Reinhard et al., 1988), who estimated the number of eggs per gram of sediment by directly counting the parasite eggs in a gram of sample.

In the search for appropriate methods, Warnock & Reinhard (1992), Dittmar & Teejen (2003), and Sianto et al. (2005) used a quantitative palynological technique that consists of adding a known amount of a tracer – *Lycopodium* spores – to a known amount of sediment. The number of eggs per gram of sediment can be estimated by the formula

$$\text{HPG} = h \times \text{ELM} / \text{ELC} \times \text{PM}$$

where h is the number of eggs counted on the slide, ELM is the number of *Lycopodium* spores in the sample, ELC is the number of spores counted on the slide, and PM is the weight of the sample.

In addition to serving as a quantitative tool, the state of preservation of the *Lycopodium* spores measures the possible aggressiveness of the paleoparasitological processing conditions on the microfossils, especially parasites. A possible disadvantage to this modified sedimentation technique is that it assumes homogeneous distribution of *Lycopodium* spores in the column of rehydrated sediment. As with eggs and cysts, spores deposit differentially, and their abundance can vary according to the depth at which the sediment is obtained.

Quantification of sediments can be performed with the use or modification of clinical techniques such as Stoll (Thienpont, Rochette & Vanparijs, 1979). Jones (1984) first used this procedure to analyze archaeological samples in England. He used 150 μl aliquots of a mixture of 3 g sediment in 42 ml of water, assuming a sediment density of 1 g/ml. The number of eggs per gram (HPG) is estimated by the formula

$$\text{HPG} = \text{no. of eggs counted} \times 100$$

As stated previously, the modified Stoll technique (Jones, 1982a) assumes that the sediment density is equal to 1. However, this assumption has not proven true for sediments of various origins analyzed in Patagonia. This demonstrates the need to measure the sediment density before calculating the number of eggs per gram of sediment and to make the necessary corrections in the HPG formula.

Recently, Fugassa, Araújo & Guichón (2006) successfully modified the Stoll technique with the following changes: 1) using 5 g of sediment in 10 ml of solution, which increases the density of sediment in the solution and thus the likelihood of recovering parasites, 2) reducing the size of the aliquot examined per slide, making the technique more operational, and 3) using aqueous trisodium phosphate as the liquid medium instead of water.

The modified Stoll quantitative technique (Fugassa, Araújo & Guichón, 2006) showed acceptable sensitivity compared to the technique applied by Jones. Bouchet, Harter & Le Bailly (2003) suggest that the latter is especially useful in samples with parasite densities greater than 400 eggs per gram.

The quantitative technique also allows quantification of other remains, such as soil mites and pollen grains (Fugassa, Sardella & Denegri, 2007). The modified technique led to an operational advantage: mean observation time for a 24 x 36 mm slide cover was only 13 minutes, due to the uniform density of the material. Equally important is the ease in slide preparation due to the use of an automatic micropipette and absence of grains of sand in preparations of sandy soil samples.

Taek Han et al. (2003) used another modification of Stoll. Samples with 10 g of sediment were previously rehydrated in 50 ml of trisodium phosphate (TSP), which was then replaced with 20 ml to eliminate the turbidity. The authors used 20 μ l aliquots for the preparations. Washing the sediments can be a useful modification in soils with large amounts of organic matter, although unnecessary in sandy soils.

The modified Stoll technique has proven to be a useful quantitative tool. However, it should be accompanied by a highly sensitive qualitative technique such as the Sheather flotation technique. If debris is recovered and some parasite remains are deformed, the risk of false negatives decreases. Thus, the combination of a quantitative technique with a qualitative one is highly useful both to expand the results and to prove the former's sensitivity.

In the pelvic sediment of a skeleton from the Alero Mazquiaráns site in Chubut, Argentina, with large amounts of organic matter (Fugassa, 2006), the modified Stoll technique failed to yield positive results, while the Sheather technique proved efficient for recovering helminth eggs. In the latter case, the large amount of organic matter had retained the scarce parasite remains, and the flotation technique thus produced more efficient results.

Ideally, various flotation techniques should be tested, as suggested by Bouchet et al. (2001), although the time spent and the greater consumption of the sample are significant disadvantages.

Observation and diagnosis

Once the archaeological sample has been properly processed, one should use a small aliquot for microscopic examination. Next, drops of sediment are mounted on slides covered with slide covers, available in various sizes according to the planned use. Small slide covers (18 or 20 mm) dehydrate less after sealed and are resistant and form a thin layer of solution, especially by adding a small drop of glycerin (Fugassa, 2006). Larger slide covers, 24 to 36 mm, allow the drop of sediment to dehydrate more easily, although they form a layer of preparation that is thinner and easier to examine. Larger slide covers are recommended for quantitative techniques using larger volumes, such as 50 to 150 ml (Fugassa, Araújo & Guichón, 2006; Jones, 1984).

Slides should be examined exhaustively under magnification 10 in the ocular lens and 10 in the objective lens. Correct measurement of the parasite elements is essential for diagnosis and should be performed under a magnification of 400. Deformed or broken parasite remains should not be measured. In species with operculated eggs as in genera *Trichuris* and *Calodium*, the eggs should be measured without considering the polar opercula, since they can vary widely; even more importantly, they are often absent from archaeological material (although many authors consider the measurements of these species with the plugs in opercula, so the measurements should be taken with and without them). Additional measurements such as the width of the operculum or egg wall may be useful in some cases.

During parasite identification, the use of photographs is indispensable for guaranteeing the results and for consultation with other specialists. Kliks (1990) criticized numerous paleoparasitological findings, sparking an interesting debate (Ferreira & Araújo, 1996; Hawdon & Johnston, 1996). His observation emphasized the lack of images to back the diagnoses. Findings should thus be properly recorded, especially when they contain species with numerically low representation in the samples.

Some authors seal the slides with transparent commercial nail polish (Bathurst, 2005; Dittmar & Teejen, 2003). The paleoparasitology laboratory at the Oswaldo Cruz Foundation in Rio de Janeiro uses a hot mixture of equal parts of beeswax and resin (Araújo, personal communication), which is very useful. However, nail polish is quick and can be used in unexpected situations when the dehydration needs to be stopped. Care should be taken to avoid contamination of the brush.

In samples containing interesting remains, a gelatin-glycerin solution can be used (Ruzin, 1999), which is good for preservation. The mixture should be heated to approximately 50°C in water bath, and once fluidity is obtained, one drop of the preparation and one drop of gelatin-glycerin solution are mixed on a slide. The slide cover is then placed on top, and the preparation can be preserved for a long time. Preliminary results indicate that ancient eggs of *Ascaris lumbricoides* and *Trichuris trichiura*, as well as current *Hymenolepis nana* eggs and *Giardia* sp. cysts are well-preserved when immersed in gelatin-glycerin.

Eggs and cysts of given taxa have typical characteristics that facilitate their identification. For example, it is relatively easy to diagnose genera *Calodium* sp. and *Trichuris* sp. eggs by the opercula on the two extremities, their shape, and the ornamentation on the wall. However, species determination is less simple and should be measured in probabilistic terms (with the eggs' length, width, and morphology as parameters) and comparative ones. The addition of other circumstantial evidence (e.g., diet items, host, parasite biogeography) can lead to a more robust probabilistic diagnosis.

As suggested by Noronha et al. (1994), identification should also consider the coprolite's morphological characteristics and distribution of possible hosts in the region, besides the other above-mentioned circumstantial evidence. Concerning possible hosts, the distribution of both current and past wildlife should be observed.

Eggs from some different parasite species can be confusing. Certain ascarids such as *Lamanema* and *Nematodirus*, or *Ancylostoma* and *Trichostrongylus*, have similar eggs that require careful measurements of numerous specimens to enable estimating their mean morphometry and if necessary apply an appropriate statistical technique. Other genera can also be confusing if there is no microscopic method to help distinguish between them. For example, *Necator* and *Ancylostoma* cannot be distinguished by the appearance of their eggs, nor can species of genus *Taenia* from other tapeworms such as *Echinococcus*.

Distinctions within a genus are even more difficult. For example, the egg morphometry of *Ascaris suum* and *A. lumbricoides* or *Trichuris suis* and *T. trichiura* does not serve to diagnose the eggs' species. Importantly, the paleoparasitological study of coprolites from wild animals can prove even more complex, since there are fewer parasitological studies that serve as the basis or reference for the paleoparasitological findings. One should also consider the possibility of changes in the parasites' life cycle and morphological alterations in the eggs, larvae, and cysts, which can hinder the correct diagnosis (Dommelier-Espejo, 2001).

Although less common, other difficulties in paleoparasitological diagnosis relate to some parasite remains that can resemble artifacts and other structures like pollen grains, air bubbles, fungal spores, and plant fragments (Thienpont, Rochette & Vanparijs, 1979).

Although the dehydration process may not alter the eggs' morphometry (Araújo, 1988; Confalonieri, 1988b; Confalonieri et al., 1988a), taxonomic diagnosis assumes that morphometry is a stable characteristic in each species. Still, some studies have proven that egg size can vary in some helminth species, due to the host among other reasons. For example, *Fasciola hepatica* eggs differ significantly in size, depending on the host's body mass (Valero et al., 2002). In pseudophyllid cestodes, egg size varies according to the host species and intensity of infection (Andersen & Halvorsen, 1978). Although this variability cannot be generalized to all helminth taxa, one should recall the morphological plasticity that some species can display.

Eggs containing first-stage larvae can often be found. This is common in such parasites as hookworms, ascarids, and pinworms and is a useful criterion for differentiating them from artifacts and other helminth eggs that are only found embryonated. Third-stage larvae have been found in coprolites and sediments (Ferreira, Araújo & Confalonieri, 1980; Reinhard et al., 1988), some as old as 1.5 million years (Ferreira, Araújo & Duarte, 1993), thus demonstrating their potential for preservation, although the diagnosis is difficult.

On this point, Reinhard, Hevly & Anderson (1987) emphasize the need to differentiate between parasite larvae and free-living nematodes. Kliks (1990) contends that some paleoparasitological diagnoses of parasite larvae may actually correspond to free-living nematodes. However, several characteristics can be used to determine whether they are free-living larvae or parasites (Fiel, Steffan & Ferreyra, 1998), or even to distinguish them from plant parasites (Chaves, Echeverria & Torres, 1995).

During microscopic observation, it is common to find various mites that can provide important information on ectoparasitism, such as the finding of *Demodex* sp. in a regurgitation pellet from a bird of prey (Fugassa, Sardella & Denegri, 2007), or on intermediate hosts, such as oribatid mites, intermediate hosts of Anoplocephalidae tapeworms (Fugassa et al., 2006). The presence of mites in coprolites of diverse zoological origins has provided relevant paleoecological information (Guerra et al., 2003).

The preceding paragraphs highlight some difficulties that can arise during diagnosis and that relate basically to confusion between parasite remains and artifacts, pollen grains, or fungi and errors in species diagnosis. Taxonomic determination of eggs, larvae, and cysts is often difficult in both ancient settings and in modern clinical diagnostic laboratories. For example, in a recent quality control exercise in Spain, human stool samples containing eggs of a helminth species and cysts from two species of protozoa were sent to 200 laboratories. Only 6.9 % identified all three parasites, and in 27.2% of the samples different parasites were identified from those known to the reference laboratory (SEIMC, 1998).

Observation and identification of parasite remains require great attention and caution at the moment of taxonomic determination. It is important and highly useful to consult several colleagues and discuss the identity of ambiguous findings. A detailed description and illustration should precede a rigorous and solidly based discussion on the possible diagnosis. The erroneous communication of a finding for a given region and time can alter the correct understanding of the biogeographical history of certain parasites and their hosts.

Staining and electron microscopy

In paleoparasitology, microscopic observation allows diagnosing helminth eggs and only occasionally protozoan cysts (Faulkner, 1991; Ferreira et al., 1992; Fugassa et al., 2008c; Leguía, Casas & Jane, 1995). The latter is due to the fact that most cyst-producing species are not only differentially preserved, but also difficult to recognize due to their small size or morphological similarity to other microfossils. Pollen grains, fungal spores, crystals, mite eggs,

free-living nematodes, and phytoliths are present in the sediments, so microscopic analysis involves some degree of uncertainty in identifying the observed structures.

The use of stains that react with given components of microfossils facilitates diagnosis. However, experience with staining in paleoparasitology is very limited and related to a few studies that used Lugol's solution (Moore, 1969; Taylor, 1955), merthiolate-iodine-formalin solution (MIF) (Harter, 2002), or iron hematoxylin (Horne & Tuck, 1996).

Although trichrome staining has not been recorded in paleoparasitology, it is one of the most widely used stains in modern material. Some studies recommend it for detecting various intestinal parasites in fecal matter (Kellogg & Elder, 1999), thus suggesting its potential use in ancient samples. Protozoa of the phylum Apicomplexa, like *Cryptosporidium* sp., *Cyclospora* sp. and *Isospora belli* may require special staining techniques, for example with modified acid-fast solutions (Kaminsky, 2003).

The lack of standardized protocols for differential staining in paleoparasitology further increases the complexity of its use in sample testing. Despite limited attempts in paleoparasitology, differential staining deserves inclusion in routine paleoparasitological examination.

Based on the design by Horne & Tuck (1996), who used iron hematoxylin in three of 15 preparations, this staining can be recommended in 50% of the slides for each sample, examining under 400 x magnification. Examination of protozoa in current samples uses 20 slide cover fields with magnification of 1,000 (Del Coco et al., 2006).

In archaeological samples, it is recommended to examine a large area due to the expected low density of cysts. The proposal for each stained slide is to examine 20 more fields under magnification of 1000, i.e., a total of 40 fields.

In parasite genera with morphometrically similar eggs, microstructural characteristics can help distinguish between them (Bouchet et al., 1999), as exemplified by scanning electron microscopy (SEM) in ancient *Diphyllobothrium* tapeworms (Le Bailly et al., 2005) and in current *Toxocara* sp. eggs (Ubelaker & Allison, 1975). The authors emphasize that taphonomic factors can alter the surface structure characteristics. SEM can also be used satisfactorily to observe third-stage hookworm larvae in coprolites (Araújo et al., 1988b).

Immunology has contributed other techniques to paleoparasitological research. Direct immunofluorescence uses antibodies marked with fluorescent substances and allows the specific detection of surface antigens in parasite forms (Atías, 1998). In paleoparasitology, it has been used to diagnose *Giardia duodenalis* (Faulkner, Sharon & Johnson, 1989), *G. duodenalis* and *Cryptosporidium parvum* (Allison, Bergman & Gerszten, 1999; Le Bailly et al., 2008; Ortega & Bonavia, 2003), and later *Cryptosporidium/Giardia* in coprolites from France (Le Bailly, 2005).

INTERPRETATION OF PALEOPARASITOLOGICAL RESULTS

When one reaches a degree of diagnostic certainty or probability with parasite remains, it is not the end of the job, but only the beginning. The identified remains represent forms of dispersal of a life cycle in a parasite species.

Eggs, larvae, or cysts found in samples can be the result of patent infections or (in the case of eggs and cysts) the ingestion of prey infected with the parasites in question. Thus, the presence of parasites in sediments from archaeological sites involves more possible origins, so their analysis requires both ecological and cultural knowledge. Contamination from unknown sources can introduce a bias into the results. For example, finding rodent coprolites in sediment from the pelvis of human skeletons can alert the researcher to contamination of material with their parasites (Fugassa & Barberena, 2006; Fugassa, 2006). To reiterate, painstaking planning of sampling and macroscopic observation of the sediment can help predict such situations.

Even under ideal conditions with highly sensitive and specific methods, false-negative results may occur. For example, in addition to the taphonomic phenomena specific to ancient samples, a mild infection, oviposition behavior as in *Enterobius vermicularis*, the reduced number of eggs in trichostrongylids of herbivores, intermittent oviposition, elimination of eggs in gravid proglottids, parasites in pre-patent periods, and other situations can lead to underestimation of ancient parasitic infections.

Adequate interpretation of results definitely requires in-depth knowledge of parasite diversity and biogeography, the natural history of the species under study, and the regional archaeology. The latter requires effective communication among paleoparasitologists and archaeologists studying the region.

STATISTICAL ANALYSIS APPLIED TO PARASITE REMAINS

As discussed previously, paleoparasitological diagnosis poses various challenges, from determination of the coprolite's zoological origin to that of the respective parasite species. We have described various methodological tools to overcome such problems. We will now present some available statistical techniques for analyzing the observed parasite remains.

Egg morphometry is commonly employed for diagnosing intestinal parasites (Thienpont, Rochette & Vanparijs, 1979). Statistical analysis of egg and cyst measurements can be highly useful for diagnosis in contexts where both human and other animal coprolites are present (Confalonieri et al., 1988b).

To compare eggs measurements between two situations, for example, eggs found in a body and in the surrounding sediment – the chi-square test can be used, with the median as the reference (Araújo, 1988), mounting a contingency table with the eggs' length and width.

When only a single egg is available (a frequent situation in paleoparasitology), Confalonieri et al. (1988b) used the Student t-test separately for length and width to determine whether the egg belonged to a given species. If a frequency table is available for a large population of eggs with length and width measurements, this statistical test can be applied to the species to which the egg supposedly belongs. The authors show that this method is equally useful for the identification of single coprolites.

Mounting these tables requires comparative samples of reliably identified parasite eggs and coprolites.

In another study on the taxonomical determination of a set of parasite remains, Joyner (apud Confalonieri et al., 1988b) used the linear regression coefficient of the measurements obtained from a set of *Eimeria* sp. oocysts to determine whether they belonged to a given species for which the oocyst morphometry was known. This method can complement other statistical tests.

Case study: discriminant analysis applied to capillarid eggs

In some cases, the eggs' qualitative characteristics are as important as their morphometric ones. In capillarids, the egg's ornamentation is highly useful for diagnosis (Moravec, 2001). Capillarid species have cycles that include a host from a lower trophic level and a carnivore as disseminator. They are thus commonly identified as parasites in transit in predator feces. Coprological examination in carnivores or omnivores can often show eggs from different capillarid species, some produced by parasites in transit, or false parasitism, and others as true parasitism, causing infection in the host.

Paleoparasitological records of eggs belonging to capillarid species are rare. In the Americas, Confalonieri described eggs from this genus in animal coprolites in Brazil (Confalonieri, 1988a; Araújo, Confalonieri & Ferreira, 1998). Bouchet

(1997) later found eggs from this genus in 21 coprolites from France. Still later, Dittmar & Teejen (2003), Fernandes et al. (2005), and Rocha et al. (2006) also recorded *Capillaria* sp. in archaeological sediments.

With the exception of an endemic of *Pseudocapillaria philippinensis* in the Philippines, reports of capillariasis in humans are now rare (Atías, 1998; Benenson, 1992), although risk of infection was probably greater in the past due to different ecological conditions.

Gonçalves, Araújo & Ferreira (2003) contend that the occurrence of capillariasis in humans is due mainly to the ingestion of raw meat, especially liver, where the eggs of *Calodium hepaticum* are located (Miyazaki 1991). However, numerous species of capillarids located in the gut are recorded in other mammals, for example *Capillaria puttori* and *Pearsonema plica* (Soulsby, 1987). Various stool tests with *Capillaria* sp. eggs have been recorded in Amerindian groups (Coimbra & Mello, 1981; Coimbra et al., 1985).

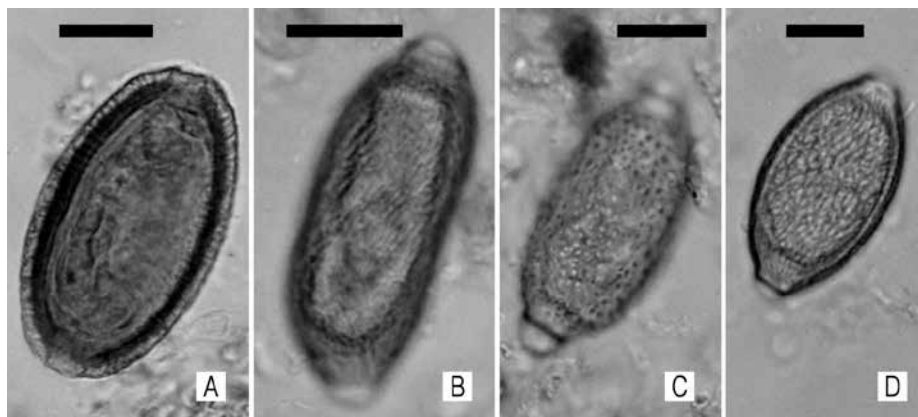
From the trophic point of view, the situation would be similar in canids, although more species of parasites have been reported in these hosts. *Capillaria puttori* and *Eucoleus aerophilus* have been recorded in canids (Thienpont, Rochette & Vanparijs, 1979), and *Calodium hepaticum* was found in necropsies of wild dogs in Brazil (Ruas et al., 2003).

As mentioned, the presence of capillarid eggs in archaeological remains can be interpreted as either true parasitism or the product of parasites in transit. The current impossibility of elucidating the origin of such eggs precludes an interpretation of their cultural and/or ecological meaning. A better diagnosis would allow discussing the presence or absence of true parasitism, thus clarifying the meaning of such findings in the host population.

Fugassa et al. (2006) analyzed morphometric and morphological data from a total of 171 capillarid eggs found in a single coprolite from the province of Santa Cruz, Argentina, attributable to a canid.

The eggs varied greatly in size, diversity of shell ornamentation, and shape of the operculum (Figure 3). Some had a surface with long longitudinal stripes and prominent opercula (type B), others had opercula, but short and with the surface covered with irregular stripes (type D), others with nearly flat opercula and the surface ornamented with small radially arranged dots (type A), and still other eggs with larger circular orifices on the surface (type C). There were also eggs in which the appearance of the shell could not be determined, and they were excluded from the analysis. Table 1 summarizes each group's dimensions.

Figure 3 – Morphotypes of capillarid eggs viewed in M8



Letters indicate the morphotype. Black bar = 20 μ m.

Source: Fugassa et al. (2008b).

Legendre & Legendre (1998) analyzed whether morphometric differences explain the morphological groups observed under light microscopy, testing whether the differences between groups were significant in multivariate space. They examined the width and length by linear analysis of the original variables that best discriminated the groups, using discriminant analysis.

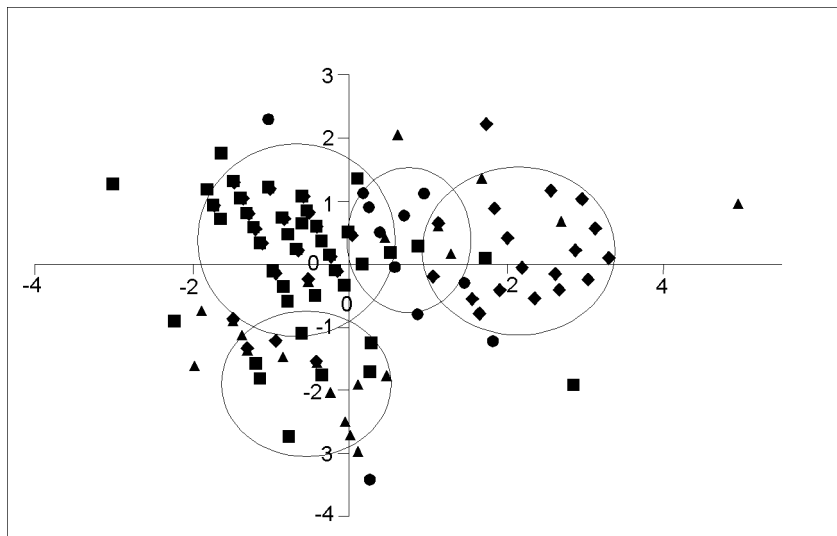
Table 1 – Morphometry of the different morphological groups

Morphology	n	%	Length (μm)		Width (μm)	
			Mean	Range	Mean	Range
A	20	13.4	63.7	57.5-75.0	38.1	33.8-41.3
B	85	57.0	59.8	42.5-67.5	28.8	22.5-37.5
C	19	12.8	62.1	40.0-71.3	32.7	27.5-37.5
D	25	16.8	54.2	42.5-72.5	29.3	22.5-47.5

Source: Fugassa et al. (2008b).

The results of discriminant analysis suggest that discrimination between groups was significant (Wilks' lambda = 0.419, $p < 0.05$) (Figure 4). Subsequent calculation of the square of the Mahalanobis distances between groups (Table 2) indicated that the separation between groups A and B was not significant, such that the morphometric variables would not explain the morphological differences between groups A and B (Figure 5).

Figure 4 – Morphotypes of capillarid eggs A (◆), B (■), C (○) e D (△)

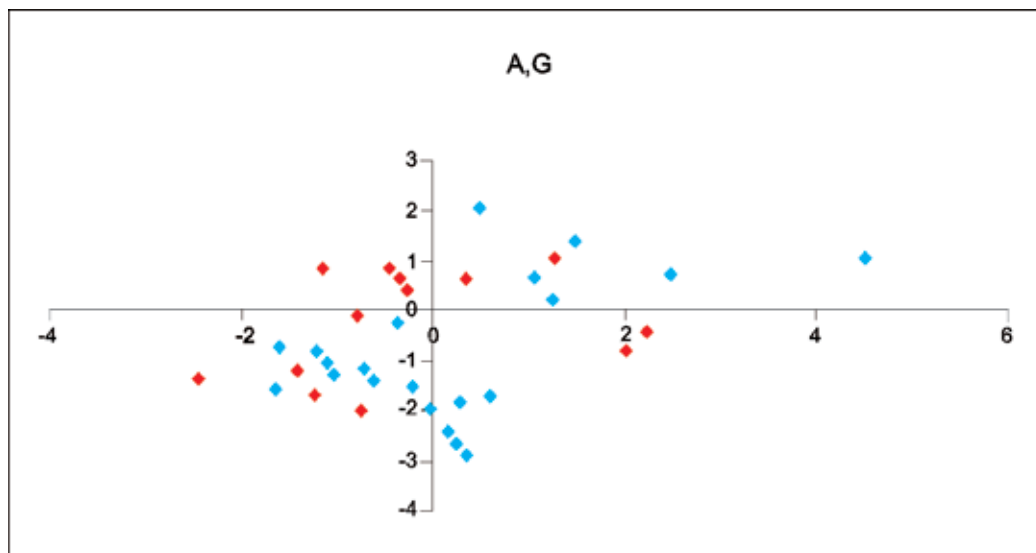


Source: Fugassa et al. (2008b).

Table 2 – Probability associated with Mahalanobis distance

	P	R	A	G	Q
P	0				
R	0.000103533	0			
A	0.005114172	0.02267275	0		
G	0.000173964	5.8133E-07	0.13483232	0	
Q	6.1475E-06	1.9853E-20	3.5023E-12	4.39407E-12	

Figure 5 – Discriminant function 1 and 2 for groups A and B



Correlation analysis suggests that the variable making the greatest contribution to discrimination between groups is width (Pearson=0.950).

The results suggest the existence of three species of capillarids in this coprolite.

The study of the shape, relative abundance, morphology of the polar opercula (when present), and dimensions of the eggs allowed discussing whether the morphological differences correlated with the morphometric differences.

The capillarid species located in the digestive tract in modern canids are *Calodium hepaticum* and *Capillaria putorii*. *Eucoleus aerophilus* locates in the respiratory tract, but the eggs can reach the digestive tract by swallowing. Analysis of the proportions among the four proposed species is: 14.7% (A), 50% (B), 11.17% (C), and 11.76% (D).

For morphotype A, the shape and metric mean fall within the range proposed in the literature for *Calodium hepaticum*. This parasite locates in the liver. The eggs are also located in the liver and generally do not pass into the intestinal tract, nor are they eliminated with the feces. Thus, the morphology, low relative abundance, and measurements of morphotype D suggest that it is the product of ingesting a truly infected prey.

Morphotype B could correspond to *Capillaria putorii* based on its measurements, ornamentation, and relative abundance.

Morphotype C is within the categories of measurements of *Eucoleus aerophilus*. The low proportion of eggs can be explained by their location in the airways and their release into the environment through the digestive and respiratory tracts.

Morphotype D cannot be assigned to a known species and probably represents immature eggs from some of the species present, most likely morphotype A.

Discriminant analysis can be useful in the diagnosis of eggs belonging to the *Capillaria* genus that cannot be classified simply by their morphology, due to taphonomic processes or other causes. Although capillarids are one of the most complex groups of parasites (Moravec, 2001), experience has shown that the combination of morphological and morphometric analysis of capillarid eggs allows improving paleoparasitological diagnosis.

NEW SOURCES OF EVIDENCE

As shown, coprolites have been the main source of evidence in paleoparasitology, although sediments of various origins have also been used sporadically. Paleoparasitological studies have also used mummified tissues (Araújo et al., 1988a; Araújo, Reinhard & Ferreira, 2000; Aufderheide et al., 2004; Bastos et al., 1996; Cockburn et al., 1975), intestinal mucosa (Alisson, Pezia & Hasegawa, 1974), and even taxidermied animals from museum collections (Cantarino, 1998; Persing et al., 1990).

Together with the increase in the number of samples and available techniques, new archaeological materials should be explored for their usefulness as sources of paleoparasitological evidence. The next section presents new sources of evidence for paleoparasitological research based on two case studies.

Case study: sampling in sacral foramina from skeletons deposited in bone collections

Skeletons deposited in institutions have usually been cleaned, and thus paleoparasitological studies (at least for enteroparasites) cannot be performed. By examining various sacra from collections in Patagonia, the hypothesis has been tested that parasite eggs can be found in the small amounts of sediment remaining on the thighbones and sacrum after the skeletons' excavation and cleaning (Fugassa, 2006; Fugassa, Sardella & Denegri, 2007).

It is important to study sediments from bones in the pelvic region because paleoparasitological studies can be expanded to more individuals with sufficient bioanthropological and contextual information to increase the sample size needed in paleoepidemiological studies. Fugassa, Sardella & Denegri (2007) also call attention to the need for preservation strategies that include the recovery of this type of information.

The sediments were recovered in the laboratory, mainly from sacral foramina of skeletons from southern Patagonia (Figure 6, Table 3). The bones had been totally cleaned, and a small sample was taken from the sacral orifices and bony processes. The sediment was weighed and rehydrated in double volume aqueous trisodium phosphate for seven days. Next, the modified Stoll technique was applied, as described previously (Fugassa, Araújo & Guichón, 2006).

Figure 6 – Localization of the samples used in the examination of sacral foramina. Skeleton recovered from the Misión La Candelaria cemetery (Río Grande, Tierra del Fuego, Argentina)



Bar = 50 mm.

Photograph: Martín Fugassa.

Table 3 – Close-up of samples used in parasitological analyses of dry bones

No.	Site	Dating	Weight (g)
1	Nombre de Jesús-IV (NJ-IV)	Historical	8.99
6	Nombre de Jesús-I (NJ-I)	515 +/- 45 years	5.02
7	Caleta Falsa	850 years ¹	0.405
8	Las Mandíbulas	Historical	1.78
10	Nombre de Jesús-II (NJ-II)	Historical	5.84
12	Nombre de Jesús-III (NJ-III)	Historical	7.15

Dating performed in clamshell substrate (Chapman & Hester, 1973).

Source: Modified from Fugassa et al. (2008a).

In NJ-1, bodies similar to capillarid eggs were recovered. Scarce eggs consistent with *Ascaris lumbricoides* were also found. No parasites were observed in either NJ-2 or NJ-3. Large amounts of fungi were observed in the latter two cases. At the Las Mandíbulas site, capillarid eggs were found with ornamentation similar to that of *Calodium hepaticum*. In this case, quantitative analysis showed 120-360 (208 ± 95.5 ; $n=5$) HPG (eggs per gram of sediment). Charcoal and charred fragments were also found. The Caleta Falsa site yielded numerous charcoal particles and capillarid eggs with a density of 0-111.11 (22.22 ± 49.69 ; $n=5$) HPG.

Some *Ascaris lumbricoides* eggs, although mostly with eroded mamelons, keep their characteristic shell and an opercular region, as described by Ubelaker & Allison (1975). This finding also provided evidence on the individual's identity, initially described as belonging to the indigenous population; however, the presence of this nematode strongly suggests European origin due to the higher expected prevalence in European populations. Ancient DNA tests later confirmed this hypothesis (Guichón, personal communication). In addition, the Laboratory for Molecular Genetics of Microorganisms at the Oswaldo Cruz Foundation in Rio de Janeiro later reconfirmed the presence of *Ascaris* sp. using ancient DNA studies for the parasite (Iñiguez, personal communication).

The results corroborated the hypothesis that parasite eggs can be preserved in clean pelvic bones. The observations also suggest that it is possible to simultaneously recover some evidence on eating behavior, such as hair fragments, starch, pollen, charcoal, and bone and plant tissues. As highlighted by Jones (1982b), cleaning archaeological remains destroys valuable information, as also observed during this study. It is thus important to reconcile preservation of archaeological material with the recovery of relevant paleobiological information.

Case study: analysis of regurgitated pellets

Concretions found in the Cerro Casa de Piedra archaeological site 5, located in the Perito Moreno National Park in Santa Cruz province, Argentina, displayed a smooth texture coated with hairs. Closer examination showed that they were regurgitated pellets, typical of birds of prey that first swallow their prey and then regurgitate the undigested remains through their beaks, including hairs, feathers, scales, and/or bones (Marti, 1987). These particular specimens belonged to a deposit dated to $6,540 \pm 110$ BP (Aschero, 1996).

The sample was described, measured, and weighed just as coprolites (Jouy-Avantin, 2003). Two samples of approximately 0.5 g each were extracted from it, corresponding to the surface and interior of the concretion. The samples were then rehydrated in aqueous trisodium phosphate according to Callen & Cameron (1960). The sediment was later concentrated by spontaneous sedimentation (Lutz, 1919). The macroscopic remains were separated and dried at room temperature. Ten slides each were made from the surface and inner samples.

Very little sediment was obtained by sedimentation, but the sample from inside the pellet showed capillarid eggs. A *Demodex* sp. mite was also identified, measuring $112.5 \times 32.5 \mu\text{m}$. Fragments of rodent hairs predominated on all the slides from both places in the concretion.

Paleoparasitological examination was performed with little hope of finding parasites, since the sample had been identified immediately as a regurgitation pellet. However, examination yielded both enteroparasites and ectoparasites, common in rodents.

The limited amount of sediment and the large amount of hairs and bones are consistent with the diagnosis of a regurgitation pellet (Figuerero Torres, 1981). The size indicates that the zoological source was a large bird of prey. The state of fragmentation of the preys' bones also serves as an indicator of the bird species (Figuerero Torres, 1981). Rock shelters are usually occupied by different owls such as *Tyto alba* and *Bubo virginianus* (Narosky & Yzurieta, 1987). The site is currently occupied by barn owls (*T. alba*) (Civalero, personal communication).

Capillarid eggs found in the sample are consistent with *Calodium hepaticum* (Thienpont, Rochette & Vanparijs, 1979), common parasites of rodents. Numerous species parasitize birds, although dispersal of the eggs generally involves their elimination with the feces and subsequent ingestion by earthworms, as invertebrate hosts (Soulsby, 1987).

The presence of a mite from genus *Demodex* sp. was recorded for the first time in an ancient sample. Its species identification is usually based on its size and the host in which it is found (Soulsby, 1987). Perez Tort & Sigal

Escalada (2006) reported finding eggs of these mites in the fecal matter of infected dogs, so their presence should be investigated in future paleoparasitological studies of mammalian coprolites, since they can be ingested accidentally by these animals when they lick themselves.

Although regurgitated pellets do not reflect the bird's own parasite fauna, they do provide a parasitological record of their prey. Indirectly, it is possible to learn data on parasitism in mammals that inhabited the region around the archaeological site. The capillarid eggs regurgitated by the bird were also probably in the infective stage, thus representing a potential source of infection for humans inhabiting the caves in the past.

TAPHONOMY AND PRESERVATION

Ancient organic material is preserved when microbial activity is at least partially inhibited (Heizer & Napton, 1969). Interruption of the process of fecal decomposition must happen quickly in order for the coprolites to form and for their microfossil content to be preserved (Bouchet et al., 2003). Mountainous areas and dry and cold ecosystems provide the best conditions for preservation of fecal matter (Bang & Dahlstrom, apud Chame, 2003; Reinhard, 1992). However, some events prevent preservation of the material (despite favorable climatic conditions), such as certain changes in the consistency of feces that can be explained by seasonal variations in the availability of various food sources (Jouy-Avantin et al., 1999).

Various types of archaeological sediments, including coprolites, represent microenvironments with a differential impact on the preservation of parasite remains (Bouchet et al., 2003). As occurs with the preservation of a coprolite, anaerobic wet environments such as latrines or cold and dry environments show the best preservation of parasite remains (Gonçalves, Araújo & Ferreira, 2003).

On the other hand, open-air sites lead to exposure to more intense changes in temperature and humidity, besides the risk of percolation of parasite elements (Reinhard et al., 1988). Percolation from a recent stratum to another deeper and older one can also occur in latrines, due to the water flow inside them (Bouchet, Harter & Le Bailly, 2003). Differences have also been recorded in the preservation of eggs from different parasites in the same archaeological site. For example, where fungi were present in latrines, *Trichuris trichiura* eggs showed alterations and cracks that were absent in *Ascaris lumbricoides* eggs (Reinhard et al., 1988). This same article reports the recovery of rare *Taenia* sp. eggs in this type of environment.

Coprolites may yield fewer parasites than sediment under certain conditions, possibly due to the percolation of parasite remains (Bouchet, 1995). However, in some nematode species the larvae leave the eggs and migrate through the soil; thus, as commented previously, samples should be taken from the sediment around the coprolite.

Among the most frequently recovered parasite groups, ascarids, capillarids, and trichurids are preserved best, while the eggs of *Strongyloides* sp., *Enterobius vermicularis*, and *Trichostrongylus* sp. are more fragile (Bouchet et al., 2003). In Patagonia, eggs of capillarid species have been found with the greatest frequency. They are commonly found in felines and camelids and have also been recorded in palynological preparations from Patagonian coprolites (Burry, personal communication). Their wide distribution and abundance probably relate more to taphonomic aspects than to a higher prevalence compared to other helminths. These observations may reflect the diversity of factors influencing parasite taphonomy. Several combinations of factors probably affect the preservation of eggs, cysts, and larvae.

Although obvious, it is important to emphasize that paleoparasitology has access to the remains that have survived over time. Likewise, coprolite findings can represent a sample, the product of taphonomic phenomena related to the

climate and favorable to the preservation of parasites in the environment. As an extreme example, the only species represented in the material may be those with eggs and cysts that resist over time, subject to the coldest and driest episodes in the region's history.

After coprolites are extracted by archaeologists, they are exposed to new sources of deterioration such as disintegration by mechanical pressure during transportation, lack of asepsis in handling, and variable humidity and temperature during storage. Preservation of coprolites should follow similar procedures to those of other dehydrated materials. For example, one should avoid handling dry samples in humid environments, and it is advisable to have a storage facility with controlled temperature and humidity.

Storing flasks with the samples inside hermetically sealed boxes allows controlling the humidity and placing silica gel inside the boxes. The samples should also not be exposed to lower humidity than in their original environment. Thus, samples should be stored under constant conditions, since changes can cause alterations in organic remains (Doro & Corvalan, 2006).

Paleoparasitological studies result in new materials such as microscope slides, macroscopic remains for studying the diet, and remains from rehydrated sediment, preferably stored in Eppendorf tubes. In all cases, immediately after the paleoparasitological analysis, the sediments from each location that have been exposed to given processes can be preserved, washed, and dried for subsequent storage.

Preservation should include not only maintenance of the sample's initial characteristics, but also protection of the information obtained from the sample and its availability for future studies.

Preservation requires planning an efficient strategy for concentrating and managing the information produced on each sample. Jouy-Avantin (2003) proposes such a strategy, recommending a standardized series of observations on each coprolite. The author proposes a standard descriptive form for future use in order to build a global database that can help determine the zoological origin of new samples. Assembling and maintaining an image bank is also essential.

In summary, the preservation of archaeological materials and their parasitological contents can be designed according to the following recommendations:

1. Use a minimum fraction of the sample in each study.
2. Coordinate the paleoscatological studies (phytoliths, pollen, charcoal, plant tissue, hairs, bones, parasites etc.) so as to allow simultaneous use in the same coprolite fraction to be processed.
3. Keep a subsample in cold storage for molecular studies.
4. Maintain the original sample under stable physical conditions, similar to the original ones.
5. Coprolites and organic remains should be kept in proper areas for avoiding fungal and bacterial proliferation (Dommelier-Espejo, 2001).
6. Store the processed remains from each sample (macroscopic remains, rehydrated sediments, images, hairs, slides, and other preparations).
7. Create an information system connected to each sample (inventories of rehydrated sediment, preparations, unprocessed sediment, macroscopic remains, comparative samples, images, and lab notes).
8. Share the general recommendations for obtaining and preserving samples with archaeologists and anthropologists.

The above list summarizes the highlights in classical paleoparasitological methodology. Studies should also consider various specific situations that are beyond the scope of this chapter.

As general criteria, before beginning a sampling or examination, it is important to recall that obtaining samples is a unique moment, generally incapable of being repeated. The use of specific methods on a sample will probably prevent the application of other subsequent techniques with that same sample.

METHODOLOGICAL PERSPECTIVES IN PALEOPARASITOLOGY

In a second paleoparasitological review, Araújo et al. (1998) highlighted that progress in the field depends on the creation of new techniques and their application to new materials. The field first emerged with findings in mummified human tissues (Ruffer, 1910), but most of the later work was done in coprolites.

Various studies have been conducted in mummified tissues. Ferreira, Araújo & Confalonieri (1983) were the first to employ a rectosigmoidoscope to obtain tissues and sediments from the interior of a mummy in order to avoid damaging the body. It was possible to view tissue helminths such as *Taenia solium*, *Trichinella spiralis*, and *Echinococcus* sp. by detailed histological examination in mummies, and Bruschi et al. (2006) recently described cases of cysticercosis.

Hair and pottery have also been the focus of intense paleoparasitological study in recent years (Harter, 2002; Harter et al., 2003). In addition, the search for ectoparasites was extended to textiles, and *Pediculus humanus humanus* was recorded in archaeological tissues from Israel (Mumcuoglu et al., 2003). Historical documents can provide further information that proves the results of parasitological tests (Araújo et al., 1988a).

As discussed above, paleoparasitological examination of atypical archaeological materials (regurgitated pellets and sacral foramina) has yielded positive results. Various sediments such as *sambaquis* (shell middens) and food-processing areas, although still rarely used, have shown important results that reinforce their usefulness for archaeological studies.

Some protozoan parasites have been described on rare occasions in paleoparasitological studies using light microscopy. Such rare findings feature *Eimeria* sp. in deer coprolites dated 9,000 BP (Ferreira et al., 1992), *E. macusaniensis* in camelid coprolites (Leguía, 1999; Fugassa et al., 2008c), and *Giardia* sp. in coprolites from Tennessee (Faulkner, 1991). The scarcity of such evidence is being overcome with immunological techniques for the detection of protozoa in coprolites using specific antibodies or molecular biology techniques such as polymerase chain reaction (PCR).

The inclusion of molecular techniques is a fundamental stage in the set of studies involved in paleoparasitological knowledge at the population level.

Paleogenetics has contributed new and powerful analytical techniques. The finding of DNA from *Ascaris* sp. in archaeological deposits from 14th-century latrines (Loreille et al., 2001) gave great impetus to the development of molecular techniques in paleoparasitology. Later, Iñiguez et al. (2002) demonstrated the usefulness of PCR for the detection of *Enterobius vermicularis*. Molecular techniques are also useful for the paleoparasitological diagnosis of microparasites such as the etiological agents of Chagas disease and leishmaniasis (Ferreira et al., 2000; Aufderheide et al., 2004; Cantarino, 1998). The presence of *Trypanosoma cruzi* was recently recorded in *Pediculus humanus* found in human hairs and coprolites in Chile (Aufderheide et al., 2005), and DNA from *Borrelia burgdorferi* was detected in fleas found on rodents belonging to museum collections (Persing et al., 1990), which means a new stage in paleogenetics, consistent with the search for new sources of molecular evidence.

Genomic sequencing studies in parasites and the detection of parasite DNA also launched the tracing of phylogenetic studies that include changes in virulence in relation to pathoecological studies (Gonçalves et al., 2002). The molecular studies launched in paleoparasitology are obviously expanding the scope of studies with traditional microscopy.

Independently, other disciplines are producing ecological and cultural information through the microscopic examination of sediments. Studies of microfossils (pollen, diatomaceae, charcoal, fungi, parasites, phytoliths, starch, spherulites, plant tissues, hairs etc.) have limited interpretative power but are enhanced by the combination of various sources of evidence (Coil et al., 2003).

Palynology is perhaps the field that has developed the most, providing evidence on the consumption of plant species, their seasonality, and other issues such as their possible pharmacological use in the past (Chaves & Reinhard, 2003; Reinhard, Ambler & McGuffie, 1985). As an example of the latter, Reinhard, Hevly & Anderson (1987) discuss the use of anthelmintics by ancient populations given the low occurrence of parasites and the presence of *Chenopodium* sp. seeds in coprolites that have been analyzed.

Sediments and coprolites also display multiple macrofossils that are objects of study (charcoal, wood, bones, feathers, hairs, seeds, fly pupae, scatophagous insects, etc.). Entomological, zoological, botanical, and anthracological studies have produced important knowledge, thus reinforcing interdisciplinary collaboration as a valuable characteristic of modern paleoparasitology.

Although paleoparasitology promises to produce valuable information in the coming years, the extent of such information is limited by the quality of both the available techniques and the material for study. This limitation relates closely to the fluidity of relations between archaeologists, paleontologists, and paleoparasitologists. The dissemination of paleoparasitology in the archaeological and paleontological arena is indispensable for excavations to include the recovery and preservation of organic sediments.

A comparison of the first published review of paleoparasitological techniques (Reinhard et al., 1988) and the review conducted ten years later (Araújo et al., 1998) highlights the important strides made in the field. Gonçalves, Araújo & Ferreira (2003) conducted a new review on human paleoparasitology and incorporated new findings from 894 coprolites, offering an overview of the parasites found on the different continents, with the European colonization of the Americas as the timeline.

In short, when planning a new review of another “paleoparasitological decade”, the most striking differences may be the incorporation of new research groups, molecular studies in routine paleoparasitological work, and lasting interdisciplinary collaboration in the field’s approach. For an overview of new research groups, see *Memórias do Instituto Oswaldo Cruz*, 98, suppl. I, 2003 and 101, suppl. II, 2006, focusing on paleoparasitological and paleopathological studies.

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