



universität
wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

**“Molecular analyses of prehistoric faecal samples
for intestinal parasites”**

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the
degree of

Master of Science (MSc)

Wien, 2021 / Vienna, 2021

Studienkennzahl lt. Studienblatt / degree
programme code as it appears on the
student record sheet: UA 066 830

Studienrichtung lt. Studienblatt /
degree programme as it appears on the
student record sheet: Masterstudium Molekulare Mikrobiologie,
Mikrobielle Ökologie und Immunologie

Betreut von / Supervisor:

Assoz. Prof. Univ.-Doz. Mag. Julia
Walochnik, PhD

ACKNOWLEDGMENT

First of all, I want to thank Prof. Dr. Julia Walochnik for giving me the great opportunity to work on my master's thesis under her supervision and to grow as a scientist. I want to thank her for her time, support, and for initiating contact with our collaboration partners at the Natural History Museum in Vienna, which made the project of my master's thesis possible in the first place.

I am thankful to Mag. Christoph Hörweg and Dr. Helmut Sattmann from the Zoological Department of the Natural History Museum in Vienna for their insights and for supporting the FEMtech internship, for which I am very grateful.

Furthermore, I am of course very thankful to Dr. Kerstin Kowarik and Mag. Hans Reschreiter from the Archaeological Department of the Natural History Museum in Vienna for providing the palaeofaecal samples, for guiding me through the Hallstatt salt mine, for a lot of helpful information, and for reviewing my master's thesis.

Special thanks to my colleagues at the laboratory, who have been very kind and who made me feel welcome from day one. I also want to thank them for always offering their help and for all the fun times not only during but also after work.

My greatest gratitude I owe to my parents who supported me throughout the years of my study and who allowed me to become the person I am today.

Finally, I deeply want to thank my partner for his love and support in all my decisions. I want to thank him for always patiently listening to my problems, giving me advice, his continuous encouragement, and for making me laugh even in difficult times.

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1 INTRODUCTION

1.1 Historical background of ancient DNA studies

Over three decades ago, in November 1984, the first so-called ancient DNA study was published. In this study scientists from the University of California in Berkeley examined a 140-year-old dried muscle from a museum specimen of a quagga, a zebra-like species that became extinct in 1883. They successfully extracted DNA, and amplified and sequenced a 229 bp fragment of the mitochondrial DNA (Higuchi et al., 1984). Shortly afterwards investigations on ancient human remains showed that ancient DNA studies are not limited to relatively recent museum specimens but are also feasible with natural or artificially mummified samples that are several thousand years old (Pääbo, 1985b, 1985a, 1986). These studies represented the first successes in obtaining ancient DNA sequences.

However, in early studies, bacterial cloning was used for DNA amplification and since the amount of DNA in these old specimens is generally limited to very low concentrations of short, damaged fragments, it later was shown that this technique had many inefficient reaction kinetics (Caramelli & Lago, 2006). On the one hand, bacterial cloning has very low cloning efficiencies, and on the other hand, bacterial repair and recombination systems lead to the modification of damaged and altered molecules, resulting in cloning artefacts (Pääbo, 1989; Pääbo & Wilson, 1988; Willerslev & Cooper, 2005).

With the invention of the polymerase chain reaction in the mid-1980s, everything changed. From now on it was possible to exponentially amplify DNA sequences *in vitro* from very few or even a single copy of a “surviving” DNA sequence (Mullis & Faloona, 1987; Saiki et al., 1988). Another big advantage of this technique is that it has no capacity for repair or mis-repair, like bacterial cloning (Pääbo et al., 1989). PCR soon became the method of choice and these advances in molecular techniques significantly advanced ancient DNA studies. Ranging from ancient DNA findings in 7000-year old human brain tissue (Pääbo et al., 1988), preserved maize (Rollo et al., 1988), muscle

samples of the extinct marsupial wolf (Thomas et al., 1989), ancient human bones (Hagelberg et al., 1989), fossilized plant remains (Golenberg et al., 1990; Soltis et al., 1992), extinct moas from New Zealand (Cooper et al., 1992), insects (Cano et al., 1993; DeSalle et al., 1993) and different amber inclusions (DeSalle et al., 1992; Poinar et al., 1993). In fact, many of these ancient DNA findings were from amber-preserved organisms (Cano et al., 1993, 1994; DeSalle et al., 1992, 1993; Poinar et al., 1993). In the mid-1990s, several astonishing results on dinosaur DNA, with an age of over 80 million of years, were published (An et al., 1995; Li et al., 1995; Woodward et al., 1994). In the 2000s, two research groups reported the successful isolation and recovery of DNA sequences from ancient halite ranging in age from 11 to 445 millions of years (Fish et al., 2002; Vreeland et al., 2000).

However, many of these impressive ancient DNA reports, have been questioned, particularly the authenticity of the extracted DNA (Hedges & Schweitzer, 1995; Lindhal, 1993, 1996; Pääbo & Wilson, 1991; Poinar et al., 1996). Indeed, some of the most extravagant ancient DNA reports have been disproved or questioned. This includes publications that claim the detection of DNA sequences, which are millions of years old, in amber inclusions (Cano et al., 1993; DeSalle, 1994; DeSalle et al., 1992; Poinar et al., 1993) dinosaur bones (Woodward et al., 1994) and plant remains (Golenberg, 1991; Soltis et al., 1992). On the one hand, it has been shown that in some of these studies human or microbial contamination was a major problem due to the increased sensitivity to contamination with modern DNA (Gutierrez & Marin, 1998; Sidow et al., 1991; Willerslev & Cooper, 2005) and on the other hand, some of these studies were irreproducible (Allentoft et al., 2012; Austin, Ross, et al., 1997; Austin, Smith, et al., 1997; Sidow et al., 1991).

What always must receive attention when working on an ancient DNA study is the fulfilment of various criteria for authenticity and the evidence for the endogenous origin of the sequence. All these incidents that made some ancient DNA studies very unreliable, have led to the publication of detailed guidelines, intended to prove and guarantee the authenticity of ancient DNA (Cooper & Poinar, 2000; Gilbert et al., 2005; Pääbo et al., 2004a).

One of the most important criteria for authenticity is the reproducibility of the amplified sequences. It has to be possible to replicate the sequencing results from the same and different DNA extracts of a sample (Parducci & Bennett, 2005). Another important issue are reliable negative controls. Since every step, from extraction to PCR amplification, involves a risk of potential contamination, no ancient DNA study can be regarded as authentic unless negative controls demonstrate that the sequences do not come from modern contamination. And in the end, the results have to be reasonable and phylogenetically plausible, or unexpected and extraordinary results must be supported by transparent evidence (Austin, Ross, et al., 1997; Cooper & Poinar, 2000; Gilbert et al., 2005).

In consideration of a series of standards for ancient DNA studies, for quite a long time the oldest authentic DNA sequenced came from a 700,000-years-old horse leg bone which was frozen in permafrost at the Thistle Creek site in west-central Yukon Territory, Canada (Orlando et al., 2013). But in 2019, this report was topped by a group of scientists from the Universities of Copenhagen and Cambridge, who extracted genetic information from the tooth enamel of a rhinoceros that lived 1.77 million years ago. They used mass spectrometry-based sequencing and, in this way, an almost complete set of proteins, a proteome, in the dental enamel was identified. This means that this technique reconstructs ancient proteomes in contrast to genomes when working with ancient DNA (Cappellini et al., 2019).

The field of ancient DNA studies is permanently evolving. During the first decade, ancient DNA was sequenced using Sanger sequencing, which was the first method for precise decoding of the order of nucleotides (Sanger et al., 1977; Sanger & Coulson, 1975). This method is still used today, although there have been major improvements. At the beginning of the 21st century, a highly parallel sequencing system was described, which also revolutionized ancient DNA research completely (Margulies et al., 2005). The development of high-throughput sequencing, also known as next-generation sequencing (NGS), greatly increased the possibilities of obtaining a higher amount of genetic information from ancient remains. Shortly after this new technique was introduced, it was applied to large-scale sequencing of ancient mammoth DNA (Poinar et al., 2006) and many other ancient DNA studies followed using high-

throughput sequencing (e.g.: Green *et al.*, 2006, 2008; Maricic & Pääbo, 2009; Rasmussen *et al.*, 2010). But this was by far not the end of the development of sequencing methods, methods are developing constantly. Next-generation sequencing made it possible to sequence millions of reads in parallel saving time and money. In the end, which sequencing strategy is used for an ancient DNA study, depends on the analysed sample material. This is primarily because molecular studies with ancient remains are associated with several challenges and differ significantly from the work with modern DNA.

1.2 Challenges of ancient DNA studies

The work with ancient DNA is always associated with challenges. After the death of an organism, molecular degradation occurs since repair mechanisms, which usually maintain the integrity of DNA molecules, are no longer active (Lindahl, 1993). As a consequence, the DNA in the cells is degraded by external and also internal factors. The DNA starts to break down due to hydrolysis and it is also degraded by endogenous enzymes. Furthermore, during decomposition, the DNA is exposed to organisms that degrade or feed on macromolecules. This includes bacteria, fungi and also insects which digest the DNA (Eglinton & Logan, 1991). Additional damage can result from different environmental factors like UV light, humidity, pH, water, salinity, air or due to temperatures fluctuations (Allentoft *et al.*, 2012; Bollongino & Vigne, 2008; Collins *et al.*, 2002; Smith *et al.*, 2003). Under rare circumstances, the degradation of the DNA can be slowed down. For example, if specimens become rapidly desiccated or if the DNA gets embedded into a material that protects it from damage (Pääbo *et al.*, 2004a). But the DNA is always affected by chemical processes.

Hydrolysis is a major cause of DNA damage. When phosphodiester bonds between adjacent nucleotides are cleaved by hydrolysis single-stranded nicks can occur. Additionally, abasic sites can be the result of hydrolytic cleavage of glycosidic bonds between nitrous bases and the sugar backbone, which may lead to a chemical rearrangement that promotes strand breaks (Friedberg *et*

al., 1995; Lindahl, 1993; Shapiro, 1981). The hydrolysis of amino groups also leads to the acceleration of depurination, meaning the loss of purine residues which can also result in strand cleavage (Friedberg et al., 1995; Lindahl & Andersson, 1972; Poinar, 2003).

Different oxidative processes may result in ring fragmentation as the double bonds of both, purines and pyrimidines, are prone to oxidative attack. Additionally, the sugar rings of the deoxyribose residues are fragmented because the chemical bonds are also highly susceptible to oxidation (Friedberg et al., 1995; Lindahl, 1993). Miscoding and blocking lesions can also be induced by free radicals such as hydroxy (-OH) radicals, peroxide (O₂) radicals and hydrogen peroxide (H₂O₂) (Pääbo et al., 2004a) and other DNA damaging processes such as crosslinks can also occur (Hansen et al., 2006). Each of these processes contributes to the fragmentation and modification of DNA which make it challenging to investigate ancient DNA. It was already shown in one of the first ancient DNA studies, that the extracted DNA from ancient remains has a typical length between 40 - 500 bp (Pääbo, 1989).

It is not clear whether the rate of damage is more or less constant through time nor to what extent the damage differs between various specimens. So there is no correlation between age and DNA recovery but specimens from dry and cold climates promote slower rates of reactions and therefore seem to preserve DNA for longer periods (Allentoft et al., 2012; Pääbo, 1989).

Nevertheless, it is possible that DNA can survive for a very long time under favourable conditions, and it is precisely this DNA that can then be used for interesting studies in a wide range of research areas.

Another big challenge which ancient DNA studies face is the need to pay close attention to contamination with exogenous or modern DNA. Due to the low concentration of endogenous DNA in ancient remains, these specimens are highly susceptible to contamination. This can result in modern DNA being extracted and sequenced instead of endogenous ancient DNA. This actually represents a serious problem of ancient DNA studies which was already noted in several early ancient DNA studies (e.g.: Zischler et al. 1995; Gutierrez and Marin 1998). Especially when dealing with ancient human specimens, special

precaution is required since this DNA is ubiquitous in most excavation and laboratory environments.

It is very important that the ancient specimens are excavated quickly and then placed into separate sterile repositories to also avoid cross-contamination of the specimens. Improper storage of the samples can often be the source of contamination (Bollongino et al., 2008). The specimens should then be stored in dark, dry and cool conditions. Once the specimens are in the laboratory, they can be better protected from contamination. Tools and surfaces should always be cleaned, and protective clothing should be worn (Pääbo et al., 2004a). In general, the recommended guidelines for working with ancient DNA should be followed to ensure the authenticity of the ancient DNA. For example, negative controls should always be included to detect any possible contamination (Poinar, 2003).

Under consideration of these guidelines and especially with an improved understanding of ancient DNA damage and careful clean laboratory work, it will be exciting to see how far back researchers will be able to go with the deciphering of ancient DNA. Surely, this will also depend on the species looked at and on which sample material is available.

1.3 Palaeoparasitology

Palaeoparasitology is the study of parasites found in ancient human or animal remains recovered from palaeontological and archaeological sites or any other place where they have been preserved. Palaeoparasitology is an interdisciplinary field, which includes theoretical and practical knowledge of many other scientific fields, like archaeology, medicine, biology, geography, anthropology, evolution but above all also history (Ferreira, 2014). This research area makes it possible to study the evolution of parasites and the diseases caused by them. By studying palaeoparasitological findings in

ancient remains, the emergence, distribution and disappearance of parasites and infectious diseases over time can be investigated (Araújo et al., 2015).

Furthermore, palaeoparasitological studies can also provide insights into various other topics and research questions beyond parasitology. Through the presence of specific parasites, conclusions can be drawn about the lifestyles and habits of ancient populations, as well as on their culture (Pike, 1968). This means that information can be gained about cultural habits, hygiene or medical practices and many other aspects of life such as sanitation and waste management (LeBailly & Araújo, 2016).

Since some parasites are specific for certain animals, important information can also be obtained about eating habits in prehistoric populations. For instance, since most of the intestinal parasites are taken up orally, conclusions can be drawn about the food people used to eat. This is, for example, the case with parasites that are transmitted via raw or undercooked meat or fish like *Taenia* sp. (tapeworms) or *Diphyllobothrium* sp. (fish tapeworm), respectively (Reinhard et al., 2013). Some parasites are also transmitted by the proximity to specific animals. As an example, *Fasciola hepatica* (liver-fluke) was found in domesticated animal remains (sheep and cattle) at a 4500-year-old excavation site, where also a prehistoric human skeleton was found. From this, an ongoing infection cycle between humans and animals can be assumed (Dittmar & Teegen, 2003). Thus, by findings of certain parasites, it is also possible to infer conclusions on the domestication of certain animals. Additionally, spores and pollen are often found during the examination of coprolites, which makes it possible to draw conclusions about past environments, climates, vegetation and plant-based food of ancient populations (Bryant, 1975; Reinhard et al., 2006).

Of course, one of the main research aims in this field is to reveal the history of parasites and how parasite-host relationships have developed and changed over time. Palaeoparasitology makes it possible to give a global overview of the geographical distribution of parasites over time by comparing different palaeoparasitological studies which detected ancient parasites (LeBailly & Araújo, 2016). The emergence and spread of parasites in new regions have, among other things, something to do with the migrations of ancient populations

and the different migration routes they took. But also the disappearance of parasites in some populations can be connected to this. Parasites can be lost by the migration to other regions where different climatic conditions prevailed, which made it impossible for some parasites to maintain their life cycle. Conversely, the presence or absence of parasites provides an indication of the climate at certain places at that time. This means that infectious diseases and parasitism depend not only on the parasite and the host but also on the environment.

Those parasites which are transmitted directly between humans and which are not dependent on the external environment, like *Enterobius vermicularis* (pinworm), can be indicators for prehistoric human migrations. Other long-term coevolved parasites, that require no intermediate host but need special environmental conditions for transmission, can help to clarify migration routes of ancient humans (Araujo et al., 2008). This is, for example, the case with *Trichuris trichiura* (whipworm). Whipworms are host-specific, and they require relatively warm conditions to complete their life cycle. This means that they must have come to the New World through the migration of ancient populations but not via the Beringia route in the north as the climatic conditions would have been too cold for the survival of the parasite. Therefore, alternative migration routes to North America have been proposed (Araújo et al., 1981; Confalonieri et al., 1991; Ferreira & Araújo, 1996; K. Reinhard, 1992). Numerous different parasites have accompanied ancient populations for a very long time from Africa to all other continents and can, therefore, be found at archaeological sites around the world.

The first palaeoparasitological discovery was already made at the beginning of the 20th century, by Sir Marc Armand Ruffer, the pioneer of palaeoparasitology. He discovered calcified eggs of *Schistosoma haematobium* in the kidneys of two Egyptian mummies dated to 1250 to 1100 BC (Ruffer, 1910). Then for a while, not many palaeoparasitological studies were published. In 1944, a cooperation between parasitologists and archaeologists, reported the findings of eggs of *Trichuris trichiura* and *Ascaris lumbricoides* in a bog mummy in what was then East Prussia. Due to the low pH at the excavation site, the body was well preserved even after hundreds of years, and the eggs could be easily identified (Szidat, 1944).

A significant advancement in this research field was made in 1960, when Callen and Cameron developed a method for rehydrating desiccated ancient material using 0.5% trisodium phosphate aqueous solution. By this, it was possible to rehydrate coprolites and other desiccated remains and to investigate them with common techniques (Callen & Cameron, 1960) which is a very good approach of studying the parasites without altering the structure (Fugassa, 2014). In the next years, palaeoparasitological research continued and many studies were published investigating ancient remains, especially different coprolites. Ancient parasites were examined by several scientists in America (Faulkner et al., 1989; Ferreira et al., 1980; Heizer & Napton, 1969; Samuels, 1965; Wilke & Hall, 1975) and also in Europe researchers have been working on coprolites (Greig, 1981; Moore, 1981; Pike, 1968). In Austria, the palaeoparasitological pioneer study was conducted in 1974 by Aspöck and colleagues with ancient *Trichuris trichiura* eggs found in coprolites (Aspöck et al., 1973).

Nowadays palaeoparasitology is a well-established research field and many scientists around the world try to unravel the history of parasites by investigating ancient remains. Palaeoparasitological research can be performed on a wide spectrum of materials. The sampling strategy must be adapted according to the objective of the study and the sampling material which is available. For instance, clothing lice (*Pediculus humanus humanus*) or head lice (*Pediculus humanus capitis*) can be preserved together with ancient fabric remnants and in this way, an infestation with these parasites can be assumed (Toups et al., 2011).

If protozoa or other intestinal parasites, like helminths, are the subject of the study, coprolites are the material of choice because they contain most likely different dissemination forms of the parasites. Coprolites are fossilized faecal material and they can be collected at different places like rock shelters, latrines, archaeological layers and inside mummies (Aspöck et al., 1996; Bouchet et al., 2003; Gonçalves et al., 2003; Reinhard et al., 2008). Studies of mummified bodies have the great advantage that results can be directly linked to an individual because the exact origin of the sampling material is known. Mummified humans or animals are probably the most interesting material for palaeoparasitology because they offer the highest potential of harbouring all

kinds of parasites as different organic materials from skin to hair and even blood is often well preserved. Other specimens are more complex such as ancient material from latrines, cesspits and burial sites or any other soil sample that contains a mixture of organic matter which can stem from several humans or animals (Côté & Le Bailly, 2017).

As this study is carried out on coprolites, some additional information will be given about the preservation of ancient faecal material. Coprolites can be found, which are still easy to recognize due to their morphology. But faecal material can also be submerged into wet and swampy soil or others are completely desiccated. Many different factors play a role in how well faecal material is preserved. Desiccation, cold and also pH-value have diverse effects on coprolites and depending on that ancient parasites are either in a better or worse state of preservation (Bouchet et al., 2003). Furthermore, cultural habits regarding sanitation and waste management and other actions in correlation with hygiene also influence the state of ancient faecal material. In some cultures, it was a common practice to simply leave the excrements anywhere and not at a dedicated spot, which can make the identification more difficult. But others had latrines or other waste deposits, where the remains are often preserved much better (Ferreira, 2014).

Since the start of palaeoparasitological studies at the beginning of the 20th century, microscopy is still the most usual tool used to identify preserved parasites, especially helminths and their eggs. Eggs from parasitic worms can be quite accurately determined on family or genus level by the investigation of the size, shape, and other specific characteristics like surface structures which some eggs present. For some intestinal parasites, the identification is even possible at species level. This is, for instance, the case for *Schistosoma mansoni* (blood fluke) and *Schistosoma haematobium* (urinary blood fluke) or for *Clonorchis sinensis* (Chinese liver fluke) as these eggs show shapes specifically for this species. But for most other species this is not the case, as species of the same genus often represent very similar morphological characteristics and can microscopically not be distinguished at all. For example, eggs from *Taenia saginata* (beef tapeworm) and *Taenia solium* (pork tapeworm) cannot be identified on species level microscopically as they cannot be distinguished under the microscope (Côté & Le Bailly, 2017).

Today parasites from ancient remains are more and more analysed by molecular and immunological techniques. In this way also mixed samples can be investigated and taxa with similar egg characteristics can be distinguished on species level. Furthermore, with molecular techniques parasites can also be identified even if their eggs are damaged or not identifiable anymore (Leles et al., 2008).

It could be shown that the combination of both, microscopical and molecular analysis, is a reliable way to analyse ancient parasites as this approach reduces the limitations of each method but still includes the strengths of both (Cleeland et al., 2013; Côté et al., 2016).

1.4 The history of Hallstatt

Hallstatt is a small village in Upper Austria, and it belongs to the UNESCO World Heritage Area Hallstatt-Dachstein/Salzkammergut, as it is one of the most important archaeological sites in the Old World (Figure 1 & 2). The reason why people started to settle in this specific area of the Eastern Alps can be found in the large salt deposit in the Salzbergtal, high above the Lake Hallstatt.



Figure 1: View over the Lake Hallstatt to Hallstatt with the mountains in the background (unsplash.com, 2021)



Figure 2: Hallstatt main square today. In the background the Protestant parish church of Hallstatt can be seen (unsplash.com, 2021)

The history of Hallstatt began a very long time ago when the supercontinent Pangaea broke into several continental plates in the late Permian period (about 300 million years ago) and when the Tethys Sea was formed, which extended from the Pyrenees over the Alps, Carpathians and the Himalayas to New Guinea. The Salzkammergut was located at the edge of this Tethys Sea.

An uplift or subsidence of the sea floor created a sea bay which was largely separated from the ocean. However, there was still a connection to the open sea through which salty seawater could continuously flow in. The concentration of dissolved substances in this bay increased due to strong evaporation and as a result sulphates, carbonates and salts precipitated. Later this bay was completely separated from the ocean and desiccated. In the next millions of years, the resulting salt deposit was overlaid by water-impermeable sandstone and clay layers.

Then, 240 million years ago, limestone and dolomite layers were deposited as the area above the salt deposits of the Salzkammergut was once again flooded by the sea. These marine deposits were folded, overthrust and fractured by the plate tectonic movement of the Alpine rock formations from the Mediterranean and during the later uplift of the mountains. The salt rock breccia, known as the “Haselgebirge”, was formed when the very soft and plastic salt deposits were shattered, folded and mixed with mostly interbedded, underlying or overlying rocks (Figure 3). The Salzbergthal, this narrow valley above the Hallstatt village, is one of the rare places where the Eastern alpine salt deposit is pressed near the surface (Kern et al., 2014f).

This favourable situation has attracted human presence for the last 7000 years, from the Stone Age until today (Kern et al., 2014e).

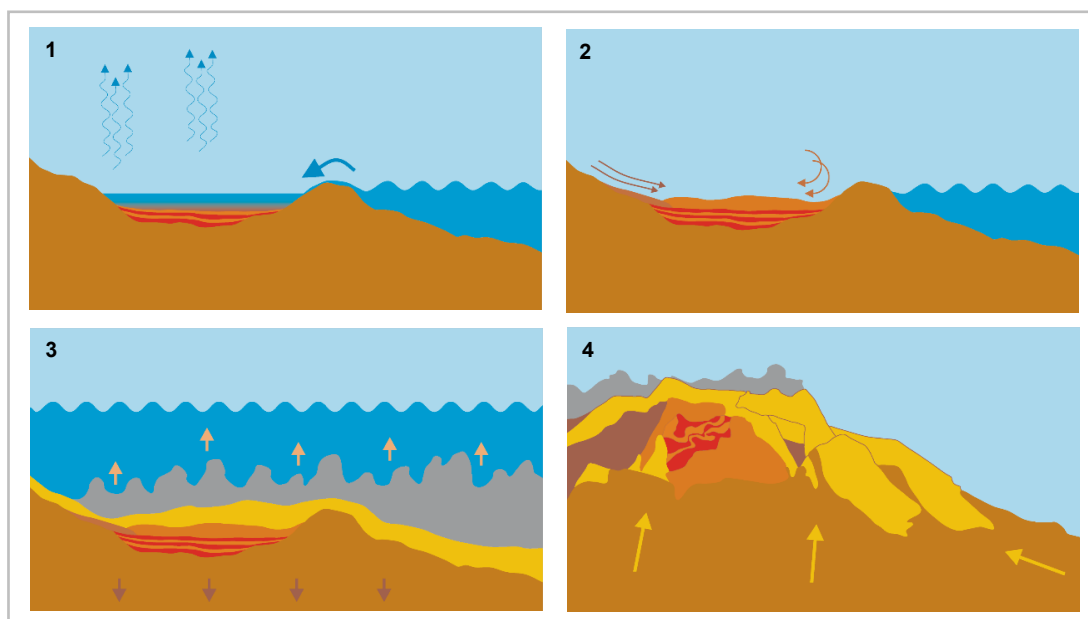


Figure 3: Illustrations showing how the salt got into the mountain, thus the origin and development of the “Haselgebirge” (Klaus Löcker by Kollmann/NHM Vienna).

Written records document well-developed medieval salt mining operations for the 14th century AD and recent palaeoecological research indicates a much earlier start of medieval mining (Festi et al., 2021). And while medieval and early modern times miners must have been well aware of the prehistoric mining traces the systematic scientific investigation of Hallstatt's prehistoric past did not begin before the 19th century. It was Johann Georg Ramsauer who first understood that the numerous prehistoric objects at the surface were the remains of a prehistoric cemetery. He really initiated the research in this area and he was also the first to carry out focused excavations in the salt mine (Kern et al., 2014a).

In 1960, the cooperation between the “Österreichischen Salinen AG” and the Natural History Museum Vienna is starting. The systematic study of the salt mine and the surrounding area is continuing until now with the support of the “Österreichischen Salinen AG” and the “Salzwelten GmbH”. The Hallstatt salt mountain is a very special location where the coexistence of prehistoric, historic and modern mining is possible (Reschreiter et al., 2019).

More than 90 prehistoric sites have been discovered so far by modern mining in the past centuries (Figure 4). Mainly because of these prehistoric mining traces, Hallstatt became UNESCO World Heritage in 1997. There is no other region in the world that has such a long history and where there is still an

ongoing production (Kern et al., 2014e). In addition, there are only three prehistoric salt mines known worldwide and the underground salt mine in Hallstatt is one of them. The other two prehistoric salt mines are in Hallein, also in Austria, and in Chehrabad in north-west Iran.

The excellent preservation conditions inside the salt mountain, the long research history and the large number of finds make it possible to reconstruct the prehistoric mining quite accurately. The earliest evidence for large scale underground mining in Hallstatt dates back to the 14th century BC, the Bronze Age, making it the oldest underground salt mine in the world. A catastrophic landslide destroyed the mines in 1,020 BC. According to archaeological evidence a fully developed mining system was back in place in the early Iron Age, in the 9th century BC, while palaeoecological research indicates that the interruption might have been shorter (Festi et al., 2021). And from then on salt mining continued, with interruptions, until today. In Central Europe the period between 800 and 450 BC is called the Hallstatt period, named after the famous cemetery in the Salzbergthal above the village of Hallstatt. The Hallstatt period is the golden age of salt production in Hallstatt. The salt made the Hallstatt communities one of the wealthiest of this era and it made this remote place to one of the most important economic centres. Many luxury goods from all over Europe were found in the grave fields and thus proved the wealth of the local people (Kern et al., 2014c).

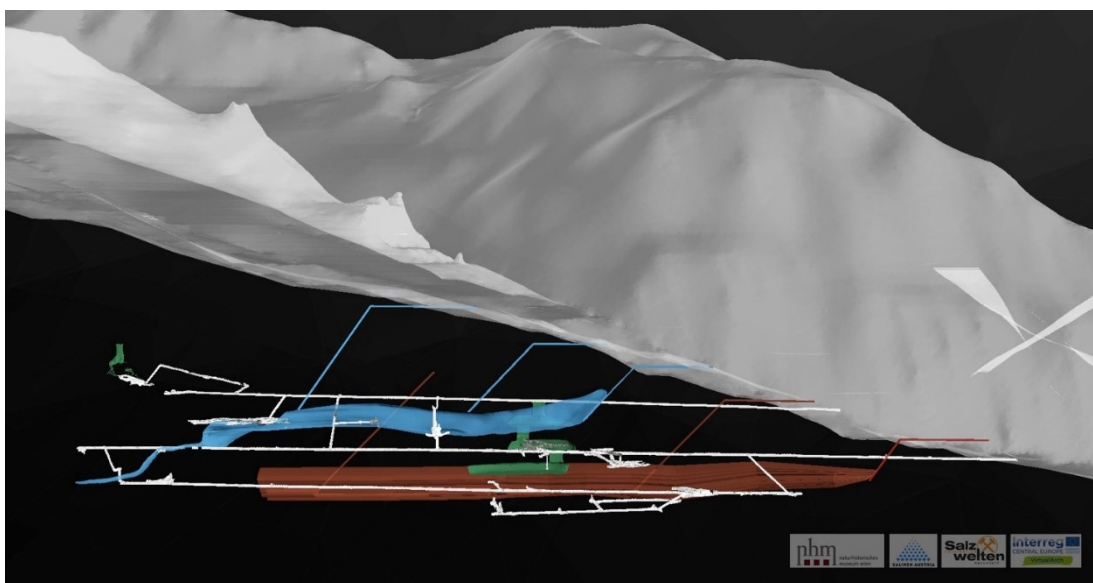


Figure 4: Cross-section through the salt deposits. The mining traces of the Bronze Age are shown in green, the early Iron Age shafts in blue and the mining of the late Iron Age in brown (D. Brandner, H. Reschreiter/NHM Vienna)

As indicated above, there are many factors making the salt mines in Hallstatt so exceptional, but one aspect is particularly relevant to the present work – the excellent preservation condition. In the large mining chambers, the miners have left the operational waste behind. Due to the pressure of the mountain cavities were re-closed, which means that all mentioned material was enclosed in the mountain (Kern et al., 2014b). Due to the preserving properties of the sodium chloride (NaCl), all organic materials inside the salt mines were preserved in almost perfect condition (Tintner et al., 2016). Over 90% of the tools and daily life products of the miners were made of organic material (Reschreiter, 2015) which usually undergoes a rapid transformation process by mineralization and humification. But the salt inhibits these processes and therefore thousands of wooden tools and pieces of equipment can be found. In addition, hundreds of leather, fur and skin items as well as bast ropes, woollen textiles and excrements have been excavated (Reschreiter et al., 2019; Tintner et al., 2016). Because everything is so well-preserved, it is possible to draw a very detailed picture of working conditions and daily life in the salt mine. Figure 5 shows an artist's illustration of life in the Hallstatt salt mine during the Iron Age (Reschreiter & Kowarik, 2019).



Figure 5: An artist's representation of the daily life at the Hallstatt salt mines during the Iron Age (Drawing: D. Gröbner, H. Reschreiter, NHM Vienna; 2006)

Due to these preservation conditions, the mining technology and working processes of that time are well known. However, other living conditions are less well known. Ancient excrements, which can also be found inside the mines, represent a vital source of information in this regard (Figure 6). Many conclusions can be deduced from ancient faeces and thus these are studied in detail.

Earlier studies on palaeofaeces from Hallstatt and the investigation of various cooking utensils and tableware have revealed dietary habits of the ancient miners (Reschreiter & Kowarik, 2019). In a sense, this has made it possible to look inside their dining bowl. A typical dish can be recognised, which is still cooked today – the "Ritschert" (Figure 7). The diet of the Iron Age miners was mainly composed of millet, unpeeled barley and broad beans, but also various bones of pigs, sheep and goats were found. The standardized pattern found in the excrements documents a monotonous, but well balanced diet (Kern et al., 2014d).



Figure 6: Human ancient excrement/coprolite which has been found in the Hallstatt salt mine (A. Rausch/NHM Vienna)



Figure 7: Look inside the dining bowl of the miners. Cooked stew, known as "Ritschert", has been their main dish for centuries (A. Rausch/NHM Vienna)

Analyses of the ancient excrements also revealed that the people from this ancient population had parasites. The first discovery of intestinal parasites, namely *Trichuris trichiura* and *Ascaris lumbricoides*, was already made in 1973 by Aspöck and colleagues (Aspöck et al., 1973). Besides different eggs of intestinal helminths, eggs of the clothes louse were found (Aspöck et al., 1999). The findings of ancient excrements and intestinal parasites are of particular interest for this study.

1.5 Humans and their parasites – a coevolution

Parasites are organisms that live in or on a host organism and benefit from it by obtaining nutrients from or at the expense of the host. They can affect the host to such an extent that the host falls ill. Generally, the less the parasite affects the health of the host, the longer and more undisturbed it is able to live with the host. This form of parasitism is in many cases the result of a long (co-) evolution of parasites with their hosts (Aspöck & Walochnik, 2002). Parasites are basically divided into three main groups: protozoa, helminths, and arthropods.

The evolution of human parasites goes hand in hand with the evolution of humans. When the first hominini, to be more precise, populations of the early *Homo erectus*, left Africa at least 1.9 million years ago, they of course took their parasites with them. Particularly these parasites that are specific for *Homo sapiens* often have a long evolution behind them (Araujo et al., 2008; Carotenuto et al., 2016). Human evolution has always had a great impact on the development of parasites. The greatest impact on human parasitology had certainly the domestication of animals and later the settling of humans (Figure 8).

About 1.2 million years ago, the extensive loss of hair covering meant that hairless skin was much less exposed to parasites especially stationary ectoparasites (Rogers et al., 2016). This most likely resulted in the loss of some parasites. However, where hair remained, stationary parasites also remained, such as *Pediculus humanus capitis* (the head louse) (Pagel & Bodmer, 2003). In contrast, the body louse (*Pediculus humanus humanus*) is linked to clothing, thus, the body louse could only emerge after humans began to cover their bodies with skins, hides and, later, textiles (Aspöck & Walochnik, 2007).

Then, about 300,000 years ago *Homo sapiens* appeared in Africa (Richter et al., 2017) and from there humans spread out and migrated basically all around the world (Templeton, 2002). Thereby, humans brought their parasites, which they had inherited from their primate ancestors, with them. But they also acquired new parasites from animals with which they came into contact on

their way (Cox, 2002). This does not (yet) include parasites with complex life cycles, which depend on specific climatic conditions, or parasites which are adapted to a specific intermediate hosts or vectors, since the climatic conditions vary in different regions of the world. In general, the distribution of parasites depends on the locally available host species and on the regional climatic conditions.

The parasite spectrum of humans once again changed significantly with the settling of ancient populations. When humans changed their nomadic, hunter-gatherer way of life to a settled, agricultural lifestyle, the transmission of parasites (and also other pathogens) between humans was facilitated. Population density and constant exposure to the same habitat also made the development of more complex life cycles possible (this includes water-borne and vector-borne diseases). Furthermore, the domestication of animals led to the acquisition of new parasites, as humans came into close and permanent physical contact with animals (Cox, 2002; Steverding, 2020). This was the beginning of a restructuring of the human parasite spectrum (Figure 8). The domestication of pack and riding animals (donkeys, horses), of animals bred for meat production (goats, sheep, pigs, cattle) and of companion animals (dogs, cats) increased the exposure to novel parasites.

The first domestic animal was the dog, it companioned humans long before they started to settle down. The domestication of dogs began around 15,000 years ago, bringing humans into contact with parasites that did not affect them before. Humans can be accidental hosts (e.g.: *Toxocara canis*) or accidental intermediate hosts (e.g.: *Echinococcus granulosus*) for parasites of dogs, which can lead to severe diseases in humans. With the intensification of livestock breeding and the development of agriculture the parasite spectrum of humans expanded more and more. Of course, many other human activities, such as trade, international shipping and wars have also led to the dispersal of many parasites. Especially in the 16th, 17th and 18th centuries, when many hundreds of thousands of slaves were displaced from Africa to America, many pathogens, including several parasites, were introduced to the New World. This included, for example, *Plasmodium falciparum*, *Onchocerca volvulus* and *Schistosoma mansoni*.

Today, travel and globalization are important drivers for the dispersal of pathogens, including parasites. (Aspöck & Walochnik, 2007; Steverding, 2020).

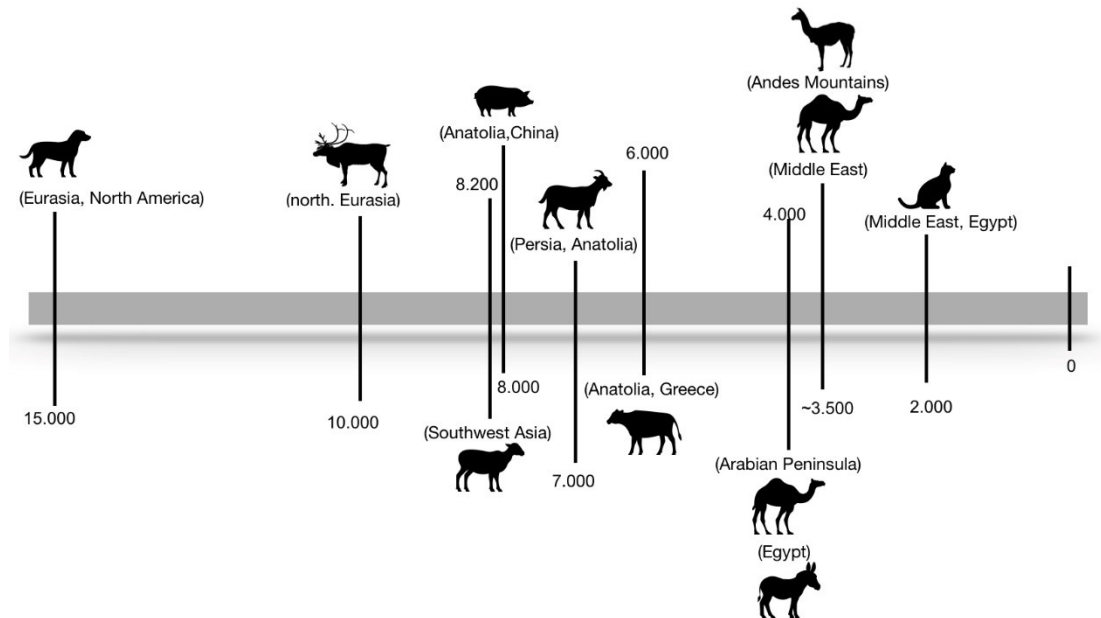


Figure 8: Timeline of the domestication of animals. The dates are all given in BP (before present). Modified from: Aspöck & Walochnik 2007

1.5.1 Human intestinal parasites

Human intestinal parasites are still a major cause of morbidity and mortality all around the world. In many developing countries they are among the most prevalent infections in humans. Gastrointestinal parasites include protozoa and helminths. Diseases such as giardiasis, amoebiasis and cryptosporidiosis, caused by unicellular intestinal parasites, are usually associated with diarrhoea and are always called infections (Haque, 2007). An infection is characterized, among other things, by invasion and multiplication of the pathogen. Although all intestinal helminths invade the host (actively or passively), very few can multiply within humans and this is then correctly called an infestation (Aspöck, 2006). Since this project focuses on intestinal worms, these will now be discussed in more detail.

Our earliest ancestors must have already been aware of several parasites, simply due to their large size. The Ebers papyrus discovered at Thebes dating to 1500 BC, a period of Egyptian medicine, is probably the first written record of human parasites (Bryan, 1930) and refers to intestinal worms. Hippocrates (460 to 375 BC) already knew about different intestinal parasites from humans and domesticated animals (Cox, 2002).

Later, after the resurgence of science and scholarship during the Renaissance, the science of helminthology really started in the 17th and 18th centuries (Cox, 2002). Six helminths were named and described by Linnaeus: *Fasciola hepatica*, *Taenia solium*, *Taenia lata* (= *Dibothriocephalus latus*), *Gordius medinensis* (= *Dracunculus medinensis*), *Ascaris vermicularis* (= *Enterobius vermicularis*) and *Ascaris lumbricoides* (Linnaeus, 1758).

Meanwhile, a total of around 350 helminth species have been identified as parasites of humans worldwide. Only a very small number of these (less than 30 species) are dependent on the host *Homo sapiens* (Aspöck, 2006). In accordance with their systematic heterogeneity, human intestinal helminths are anatomically and morphologically extremely diverse. The smallest are smaller than 1 mm (larvae) or a few millimetres (e.g.: *Echinococcus* species), the longest are more than 10 m long, in exceptional cases even around 15 m long (fish tapeworm). With up to more than 10 mm, the thickest helminths in humans, are the roundworm and the kidney worm (Aspöck & Walochnik, 2002).

The medically important helminths belong to three different groups: the trematodes (flatworms), the cestodes (tapeworms) and the nematodes (roundworms).

1.5.1.1 Trematodes (flatworms)

Most trematode species have an oval to lanceolate shape and are dorsoventrally flattened, but there are also some exceptions like the filamentous schistosomes. The most distinctive external feature of trematodes are their two suckers as adhesive organs, namely an oral sucker and a

ventrally located sucker. Most species are hermaphrodites (exception schistosomes) with a complex life cycle including at least two hosts. Molluscs or snails are usually the first intermediate hosts, and in some species, arthropods or fish are the second intermediate hosts (Galaktionov & Dobrovolskij, 2003). Humans can be infested through many different ways. The most important representatives of the trematodes are the schistosomes (blood flukes), *Fasciola hepatica* (common liver fluke) and *Fasciola gigantica* (giant liver fluke), *Dicrocoelium dendriticum* (lancet liver fluke), *Opisthorchis* and *Clonorchis* species (Southeast Asian/cat liver fluke and Chinese liver fluke) and *Paragonimus* spp. (lung flukes) (Jones & Cappello, 2004).

Worldwide, the most common human trematodes are the schistosomes, causing schistosomiasis, which is an acute and chronic parasitic disease (WHO, 2020b). Schistosomiasis mainly affects poor communities in tropical and subtropical areas, with Africa being the most severely affected continent, without access to clean drinking water and poor hygiene/sanitation (Verjee, 2019). As estimated by the WHO (World Health Organisation), over 290 million people would have required preventive treatment in 2018 and that in sub-Saharan Africa, at least 200,000 deaths per year are due to schistosomiasis (WHO, 2020b, 2021a). In Central Europe, only a few species are considered as medically relevant, including *F. hepatica*, *D. dendriticum*, *Trichobilharzia* spp. and *Bilharziella polonica* and other *Bilharziellinae* (Auer & Aspöck, 2014c).

1.5.1.2 Cestodes (tapeworms)

Adult cestodes typically have a flat, ribbon-shaped body, with a length of several metres, consisting of a head (scolex), with which the worm attaches to the intestinal wall, and a series of segments (proglottids). They do not have a gut or a mouth, nutrients are absorbed via their specialised neodermal cuticle or tegument. The reproductive organs are hermaphroditic, and development usually proceeds via one or two intermediate hosts. The proglottids, containing the eggs, are shed with the hosts faeces. Infestation of the host occurs through ingestion of raw or undercooked infected meat. Humans can harbour the adult

worms in their intestines, but also the larval stages of certain cestode species can inhabit various organs of humans, causing severe diseases (Saari et al., 2019). There are three species for which humans are the only definitive host: *T. saginata* (beef tapeworm), *T. solium* (pork tapeworm) and *T. asiatica* (Asian tapeworm). The infestations (taeniasis) are usually inapparent or with mild abdominal symptoms. However, humans can also function as accidental intermediate hosts for the pork tapeworm, resulting in cysticercosis, which is a life-threatening parasitosis caused by the larvae of *T. solium*. The so-called metacestodes can develop in different organs, including the muscles, skin, eyes and also the CNS (central nervous system), which leads to a serious condition known as neurocysticercosis. This is still a major cause of adult-onset seizures in most low-income countries. *T. saginata* and *T. solium* are distributed worldwide, and *T. asiatica* is limited to Asia (CDC, 2021e; Cobo, 2014). Other important cestodes are *Echinococcus granulosus* (definitive host: mainly dog) and *Echinococcus multilocularis* (definitive hosts: mainly fox and dog), the causative agents of cystic echinococcosis and alveolar echinococcosis, respectively. Human infection occurs through peroral uptake of *Echinococcus* eggs (Deplazes et al., 2017). According to the WHO, over 1 million people are affected with echinococcosis worldwide (WHO, 2020a). Less pathogenic parasites of this group are *Hymenolepis nana* (dwarf tapeworm) and *Dibothriocephalus latus* (fish tapeworm). *H. nana* is transmitted directly (eggs) or indirectly (accidental ingestion of infected insects), especially in warm countries and is the most common cestode infestation worldwide (CDC, 2017; Mega et al., 2012). Fish tapeworm infections are relatively wide distributed but generally occur in the Northern Hemisphere in inland lake areas of Europe, Asia, and the Americas (CDC, 2019, 2020). *D. latus* is the longest intestinal worm of humans with a length of up to 20 m. Definitive hosts are humans and fish-eating mammals. Humans become infected by eating raw or insufficiently heated fish (Auer & Aspöck, 2014b).

1.5.1.3 Nematodes (roundworms)

Nematodes are characterized by a spindle-shaped and unsegmented body with sizes ranging from a few millimetres to 1 m. They are dioecious, with the males being smaller than the females. The development starts with eggs through four larval stages to adult nematodes (Seesao et al., 2017). The species with the greatest medical importance are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), hookworms (*Ancylostoma* spp. and *Necator* spp.), *Enterobius vermicularis* (pinworm) and *Strongyloides stercoralis* (dwarf nematode) (Stepek et al., 2006). In Central Europe, the most prevalent helminth of the gastrointestinal tract is *E. vermicularis* (Auer & Aspöck, 2014a). However, according to the WHO, more than 1.5 billion people are infested with one or more of the so-called soil-transmitted helminths worldwide. The main species that infest humans are the roundworm (approximately 807–1,121 million infestations), the whipworm (approximately 604–795 infestations) and the hookworms (approximately 576–740 infestations) (CDC, 2021c; WHO, 2021b). The eggs of these parasites are passed with the faeces of infested humans. In countries with poor sanitation or where human faeces are used as fertilizers, soil-transmitted helminths are particularly common. *Trichuris* and *Ascaris* eggs are shed as immature eggs and only become infective under exposure to oxygen and the appropriate environmental conditions (temperature and moisture dependent) (Wang & Davis, 2020). Humans are infested by oral uptake of the infective eggs, either via contaminated water, vegetables or fruits, or soil-contaminated hands. The life cycle of the most common soil-transmitted helminth, *A. lumbricoides*, is shown in Figure 9. Hookworm transmission is different as the eggs are not infective, but larvae hatch in the soil, mature and then actively penetrate the skin (CDC, 2021d, 2021b, 2021a). People with light soil-transmitted helminth infestations usually have no symptoms. Severe infestations, however, can cause a range of symptoms, including abdominal pain and diarrhoea, but also malnutrition and rectal prolapse. In children, severe worm infestations can lead to physical and cognitive growth retardation.

Soil-transmitted helminthiases are classified as "Neglected Tropical Disease" (NTD) because they cause tremendous suffering worldwide, especially in poor countries, although they are generally easily treatable (CDC, 2021c; WHO, 2021b).

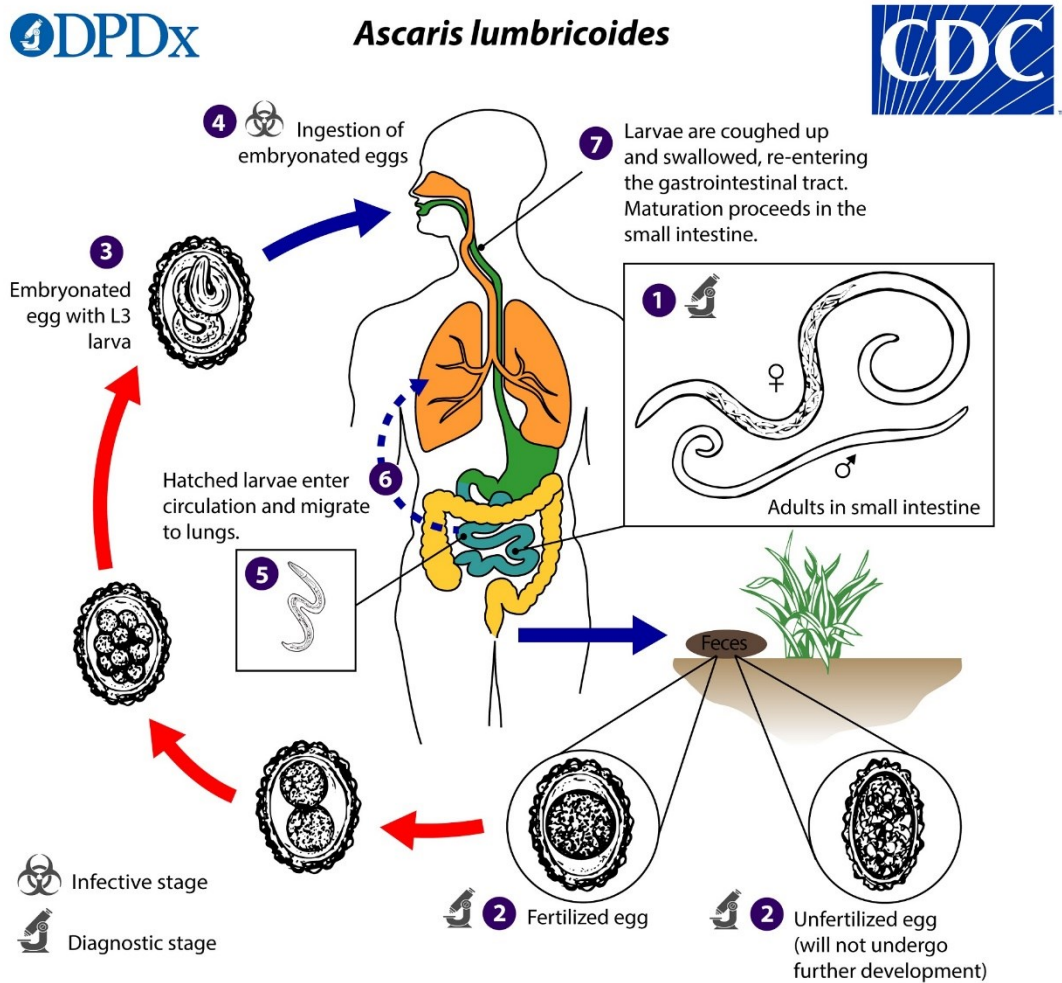


Figure 9: Life cycle of *Ascaris lumbricoides*, a typical soil-transmitted helminth (CDC 2021).

2 PURPOSE OF THE STUDY

This interdisciplinary project was conducted by the cooperation with the Natural History Museum Vienna. The Natural History Museum Vienna started the systematic study of the ancient salt mine in Hallstatt and the surrounding area in 1960 in cooperation with the “Österreichische Salinen AG”. This research is continuing with additional support of the “Salzwelten GmbH”. Due to the exceptional preservation conditions inside the salt mines, not only remains of food, clothing and different tools have been found, but also ancient excrements of the miners. These are a vital source of information on the health status of the ancient local population. Already in previous studies it was shown that the people of the Hallstatt period, like the people before and after them, had parasites.

Palaeoparasitological studies can provide information on the distribution of parasitic infections during a particular time in the past. Furthermore, the emergence and elimination of parasites in time can be investigated which in turn can help to place the archaeological findings into a historical perspective (Meigouni et al., 2020). Although the study of ancient DNA (aDNA) from palaeoparasitological samples is very challenging, it represents an approach that can provide further information for certain research questions (Wood, 2018). Particularly intestinal helminths are well suited for palaeoparasitological studies as their eggs are remarkably resistant to environmental impacts and provide extraordinary preservation ability (Søe et al., 2015).

The main objective of this study was to recover ancient DNA of intestinal helminths present in the coprolites from the Hallstatt salt mine.

The first aim was to morphologically identify the helminth eggs by light microscopy. Previous studies have already proven the presence of *Ascaris lumbricoides* and *Trichuris trichiura* eggs in palaeofaecal samples from the Hallstatt salt mine (Aspöck et al., 1973; Rodler, 2019). In the current study, a special focus was also on the detection of parasites that require a specific intermediate host for transmission (*Taenia saginata*, *Taenia solium* and *Dibothriocephalus latus*), as the presence of these parasites would reveal

more information about the dietary habits of the ancient population and the history of animal husbandry.

The second aim was to extract ancient parasitic DNA from the coprolites. For this, a method had to be established which is suitable for the extraction of ancient parasitic DNA from palaeofaecal samples preserved in a salty environment.

Finally, the third aim was to amplify and sequence ancient parasitic DNA using species-specific primers which target different genes. This is essential in order to confirm the origin of the ancient parasitic DNA in the coprolites.

3 MATERIALS AND METHODS

3.1 Samples

In total, 35 coprolite samples from the ancient Hallstatt salt mine were analysed in this study. Four samples date to the Bronze Age, 31 samples date to the Iron Age. The samples were taken on five different dates several years apart (1989, 1993, 2003, 2015, 2018 & 2019). Twenty-six samples were from the museum collection and four samples were taken in 2018 for a previous study on coprolites from the Hallstatt salt mine (Rodler, 2019), and additional five samples were collected in 2019 for this project. Only the samples from 2018 and 2019 were taken under sterile conditions.

The coprolites had a variety of colours, from greyish-light brown to dark brown (Figure 10). The samples from the museum collection were completely dried out, thus they had a hard and friable texture. The samples from 2018 and 2019 were moist and had a softer, clay-like texture.



Figure 10: Examples of coprolites from the Hallstatt salt mine (A. Rausch/ NHM Vienna).

3.1.1 Sampling sites

The sampling sites included in this study are the “Kernverwässerungswerk” site, the “Christian von Tuschwerk” site, the “Kübeck” site and the “Josef Ritschner Werk” site in the Hallstatt salt mine (Figure 11). Narrow tunnels lead through the ancient mining chambers (Figure 12), as they have been completely buried by debris. The coprolites are embedded in this debris material and can be found at various sites of these narrow passages (Figures 13 & 14).

Temperature and humidity are relatively constant all year long and there is no natural light in the mines.

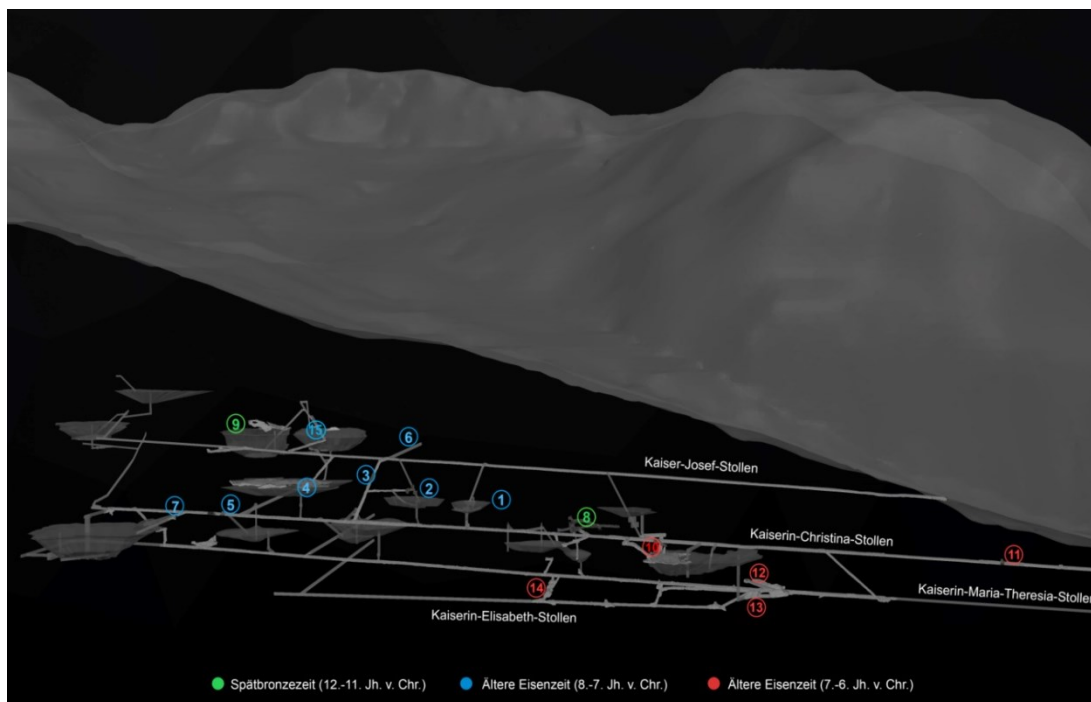


Figure 11: Cross-section showing the distribution of prehistoric mining sites in the Hallstatt salt mine. Green – Bronze Age, Blue – late Iron Age, Red – early Iron Age. 15 - Kübeck (Aufdeckungsschlag) site, 5 - Josef Ritschner Werk (Sinkwerksebentel) site, 2 – Kernverwässerungswerk site, 8 - Christian von Tuschwerk site (D. Brandner/NHM Vienna)



Figure 12: Narrow corridor leading through the ancient mining chambers.



Figure 13: The coprolites are embedded in debris material. Orange circles show three palaeofaecal samples.



Figure 14: This picture shows the position of the coprolite samples taken at the “Josef Ritschner Werk” site. The samples are located on the ceiling of the narrow corridor.



Figure 15: These two pictures give a closer view of the coprolites, which can be distinguished from the surrounding material by their brownish colour.

3.1.2 Sample handling

To avoid contamination, the five fresh samples were taken under sterile conditions. Therefore, sterile scalpels, sterile disposable gloves, a mask and sterile tubes were used for sampling. The outermost layer was first scraped off and then the ancient faecal material was directly sampled into a tube with a sterile scalpel. During the transport to the laboratory, the samples were refrigerated.

In the laboratory, the samples were handled with highest caution to avoid any contamination. The samples were processed inside a laminar air flow cabinet (Heraeus HERAsafe® HSP12, Kendro Laboratory Products GmbH, Hanau, Germany). The workspace was always cleaned with 70% ethanol before and after processing the samples and decontaminated by UV irradiation (30 min). A laboratory coat, sterile gloves and a mask were used during the work.

For the establishment of the molecular methods, recent stool samples from the institute's archive containing eggs of intestinal helminths were used.

3.2 Microscopic detection

Initially, all samples were microscopically screened for the presence of intestinal helminths.

Prior to microscopy, approximately 30 mg of each sample were transferred into a 1.5 ml Eppendorf tube and rehydrated in 0.5 ml 10% NaOH at room temperature by gentle shaking on a rocking platform overnight. On the following day, the samples were vortexed, and 40 µl of the suspension were placed onto a microscopic slide, covered with a cover glass (18 x 18 mm) and examined at 100x and 400x magnification by light microscopy using a Nikon Eclipse E800 microscope (Nikon Instruments Inc., USA). Three slides per sample were analysed. All findings were recorded and samples for further molecular analyses were selected based on egg morphology and density. The rehydrated samples were stored at 4°C.

3.2.1 Photomicroscopy

For comparison, several images were taken of recent intestinal helminth eggs and the ancient eggs using 100x, 400x and 1000x magnification. In this way, the morphological appearance of the ancient eggs can be compared to the morphology of recent eggs. The micrographs were taken with a microscope camera, which offers fast, high resolution image capture (Nikon DS-Fi2, Nikon Metrology, Germany). All the images were analysed and edited using the computer program NIS-Elements BR (Nikon Metrology, Germany).

3.2.2 Single-egg isolation

A method for the isolation of single eggs was established. This was necessary, on the one hand, to establish a DNA extraction method for samples with a very low DNA content, and on the other hand, to test the sensitivity of the different polymerase chain reactions. Furthermore, this method allows to investigate single eggs.

For this method, an inverted microscope (Nikon TMS, Nikon Instruments Inc., USA) was used, 100 µl dH₂O were placed in a culture dish and 25 µl of the sample were added and compounded with the water. The sample was then microscopically analysed at 40x–100x magnification. Single eggs were isolated with a micropipette (volume 5 µl–10 µl). To achieve this, the pipette tip was placed at the edge of the droplet and then slowly moved to the eggs. Single eggs were picked and transferred to a 1.5 ml Eppendorf tube containing 50 µl of 70% ethanol or dH₂O, depending on how long the eggs were stored afterwards.

3.3 DNA Extraction

Altogether, 33 samples were selected for molecular analyses.

Four different DNA extraction methods were tested for their ability to isolate and purify ancient DNA. The DNA extraction with the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), the QIAamp® DNA Stool Kit (QIAGEN, Hilden, Germany) and the GeneClean® Kit for ancient DNA (MP Biomedicals, USA) resulted in low DNA yield and negative PCR results despite positivity in the microscopical analysis. Only with the DNeasy® PowerSoil® Kit (QIAGEN, Hilden, Germany) DNA in the required quality could be extracted and thus this kit was used for the ancient samples. Therefore, this procedure will be described in more detail below. Initial tests to establish the molecular methods were performed using recent stool samples positive for respectively, *Ascaris* and *Trichuris*, preserved in 70% ethanol from the institute's archive. These recent samples were never processed at the same place or time as the ancient samples.

3.3.1 Homogenization

In order to extract DNA, it is important to break the eggshells to release the genetic material which is enclosed in the eggs. Therefore, the rehydrated coprolite samples were processed with a Precellys® 24 homogenizer (Bertin Instruments, France).

Four different bead-beating procedures were tested for their ability to break the hard eggshells — 0.5 mm glass beads, 0.1 mm zirconium ceramic oxide beads, a mix of both beads mentioned before and 0.7 mm garnet beads.

The best results were obtained using the garnet beads, which are a component of the DNeasy® PowerSoil® Kit (QIAGEN, Hilden, Germany). Prior to homogenization the rehydrated samples need to be washed to remove as much NaOH as possible. Therefore, the contents (beads + solution) were

removed from the PowerBead tubes and transferred into a fresh microcentrifuge tube. Subsequently, 400 µl dH₂O were added to the PowerBead tubes and approximately 30 mg of rehydrated coprolite sample. The samples were vortexed and then centrifuged at room temperature for 5 minutes at 20,000 x g. The supernatant was discarded. Again, 200 µl dH₂O were added to the sample, vortexed and centrifuged at room temperature for 5 minutes at 20,000 x g. This washing step was repeated a third time. Now as much liquid as possible was removed and beads and solution were added back to the PowerBead tubes. The samples were gently vortexed. 60 µl of solution C1 (DNeasy® PowerSoil® Kit) were added to the sample and inverted several times. The samples were subjected to 3 cycles of homogenization at 5,800 rpm for 15 seconds with 60 seconds break in between.

Afterwards the samples were incubated overnight at 56°C and 400 rpm.

3.3.2 DNA extraction with the DNeasy® PowerSoil® Kit

After homogenization and overnight incubation, the samples were vortexed and centrifuged for 30 seconds at 10,000 x g. The supernatant was transferred to a clean 2 ml Collection Tube. The following steps were performed according to the DNeasy® PowerSoil® Kit manual (QIAGEN, Hilden, Germany). Finally, the DNA was eluted in 20–30 µl Elution buffer, depending on the number of eggs in the sample. The DNA was stored at –20°C.

This kit was especially designed to remove inhibitors which are commonly found in soil and environmental samples using a special Inhibitor Removal Technology® (IRT). In combination with mechanical and chemical cell lysis this method yields high-quality DNA.

3.4 Primer design

Several primers from the literature, in-house primers and the primers that were used in a previous study (Rodler, 2019), were tested and compared. However, none of these primers were suitable for the specific detection of *Ascaris lumbricoides* and *Trichuris trichiura* DNA in the palaeofaeces samples of this study.

Therefore, new primers for each species were designed for this project. Briefly, sequences from each species and from different genes were downloaded as FASTA files from the NCBI database (<https://www.ncbi.nlm.nih.gov/nuccore/?term=>). Different genes were chosen for the primer design to enhance sensitivity and specificity. The sequences were aligned using GeneDoc (National Resource for Biomedical Supercomputing, Pittsburgh, USA) and ClustalX (Conway Institute, Dublin, Ireland). Appropriate primer binding sites were selected for forward and reverse primers. The primers were designed to have a length between 19–22 bases and a G/C content between 40–55%. The forward and the reverse primers have approximately the same melting temperature (between 50–60 °C) and end with at least one G/C pair. To ensure that the primers do not contain internal secondary structures, they were checked for their self-complementarity using OligoCalc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>).

Furthermore, the primer sequences were blasted against the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure their specificity. The primers were designed to amplify short sequences with a length between 97 and 246 base pairs (bp), as ancient DNA usually is highly fragmented.

All primers were ordered at Microsynth AG (<https://srvweb.microsynth.ch/>).

3.4.1 Specific primers for *Ascaris lumbricoides*

Four specific primers pairs, amplifying fragments of three different genes, were designed for the detection of *Ascaris lumbricoides*.

Two primer pairs are located in the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*), which result in overlapping amplicons. The first primer pair was slightly modified from literature (ACF2 & ACR2, Sørensen et al. 2015) and flanks an approximately 182 bp long sequence. The second primer pair amplifies an approximately 172 bp long sequence. The third primer pair targets a sequence with a length of approximately 193 bp within the mitochondrial NADH dehydrogenase subunit 1 gene. The primer pair targeting the mitochondrial cytochrome B gene results in relatively short sequences with a length of approximately 138 bp.

More detailed information on the *A. lumbricoides* primers is given in Table 1 below.

Table 1: Specific primers for the identification of *Ascaris lumbricoides*.

Locus	Primer	Primer name	Primer sequence (5'–3')	Melting temperature (°C)	Amplicon size (bp)
<i>cox1</i>	Forward	COX1FW_asc_EB	GGGTCTTTGGGTATGGTTTA	56.4	182
	Reverse	COX1RW_asc_EB	CCAAACAAGGTAGCCAACCA	58.4	
<i>cox1</i>	Forward	COX1FW2_asc_EB	CTGTTGGTATGGATCTTGA	53	172
	Reverse	COX1RW2_asc_EB	CGGTTAACCCACCAATAGTA	56.4	
NADH1	Forward	NADH1FW_asc_EB	GACTCCTTTGAATTCTTCGG	56.4	193
	Reverse	NADH1RW_asc_EB	GACTTTCTTACCATACTACT	54.3	
CytB	Forward	cytBFW_asc_EB	GTTAGGTTACCGTCTAGTAAG	57.5	138
	Reverse	cytBRW_asc_EB	CAAAAAGGCCAAAGCACCAT	56.4	

3.4.2 Specific primers for *Trichuris trichiura*

To detect and identify *Trichuris trichiura* five specific primer pairs were designed targeting short, small subunit ribosomal nucleic acid (SSU rRNA) and internal transcribed spacer 2 (ITS2) sequences.

Two primer pairs, targeting fragments of the 18S rRNA gene, result in two overlapping PCR amplicons with a length of 180 bp and 239 bp, respectively. The third primer pair targets a 230 bp fragment within the 18S rRNA gene.

The other two primer pairs amplify overlapping sequences of the internal transcribed spacer 2 region with a fragment length of 170 bp and 201 bp, respectively.

More detailed information on the *T. trichiura* primers is given in Table 2.

Table 2: Specific primers for the identification of *Trichuris trichiura*.

Locus	Primer	Primer name	Primer sequence (5'-3')	Melting temperature (°C)	Amplicon size (bp)
SSU rRNA	Forward	18SFW_tri_EB	CTCGTAGTTGGATTGCGGAT	58.4	180
	Reverse	18SRW_tri_EB	CACCACGGTTCAAGCACTA	57.5	
SSU rRNA	Forward	18SFW2_tri_EB	GGAACGTTTCTCCATGAGAC	58.4	239
	Reverse	18SRW2_tri_EB	GAGCATCCAGGGCAATCTT	57.5	
SSU rRNA	Forward	18SFW3_tri_EB	CTAGTTGTGACCGTAAACGA	56.4	230
	Reverse	18SRW3_tri_EB	GATCTGTCAATCCTCGCAGT	58.4	
ITS2	Forward	ITS2FW_tri_EB	GTTGAAGAACGACGTGACAC	58.4	170
	Reverse	ITS2RW_tri_EB	CACCAACCTCAACCTGTAG	57.5	
ITS2	Forward	ITS2FW2_tri_EB	CTAGTAGCATTGCGAACTGCT	56.4	201
	Reverse	ITS2RW2_tri_EB	GCTTAACGACCCTCAGACA	57.5	

3.5 Polymerase chain reaction (PCR)

All amplifications were carried out in a 50 µl reaction volume composed of 32.25 µl master mix and distilled water to fill up the reaction volume. All samples were tested in independent setups with DNA concentrations between 3 and 6 µl. The master mix for one reaction consisted of 11 µl dH₂O, 5 µl Reaction buffer B (Mg²⁺ free, Solis BioDyne, Tartu, Estonia), 5 µl MgCl₂ (25 mM, Solis BioDyne, Tartu, Estonia), 5 µl forward primer (diluted 1:10), 5 µl reverse primer (diluted 1:10), 1 µl dNTP mix (20 µmol, 20 mM of each, Solis BioDyne, Tartu, Estonia), 0.25 µl Hot FIREPol DNA polymerase (5 U/µl, Solis BioDyne, Tartu, Estonia).

The PCRs were performed in a thermo cycler (Eppendorf, Hamburg, Germany). The annealing temperature for each primer pair was calculated to be approximately 2–5°C lower than the melting temperature of the primers. The highest possible annealing temperature was determined by several runs, because the higher the temperature, the more specific the PCR. All PCR programmes started with an initial denaturation step (hot start) at 95°C for 15 min, followed by 35 cycles of 1 min at 95°C denaturation, 45–50 sec annealing between 52–54°C, elongation at 72°C for 50 sec and at the end a final elongation step at 72°C for 7 min. The cycling conditions for all primer pairs can be seen in Table 3. After PCR, the samples were stored at 4°C until use.

Table 3: Cycler conditions for all PCR reactions

Programme name	PCR step	Temperature	Time	Primers
ebascaris	Initial Denaturation	95 °C	15 min	COX1FW_asc_EB COX1RW_asc_EB NADH1FW_asc_EB NADH1RW_asc_EB
	Denaturation	95°C	1 min	
	Annealing	54°C	50 sec	
	Elongation	72°C	50 sec	
	Final Elongation	72°C	7 min	
ebascaris2	Initial Denaturation	95 °C	15 min	COX1FW2_asc_EB COX1RW2_asc_EB cytBFW_asc_EB cytBRW_asc_EB
	Denaturation	95°C	1 min	
	Annealing	54°C	50 sec	
	Elongation	72°C	50 sec	
	Final Elongation	72°C	7 min	

ebtrichuris	Initial Denaturation	95 °C	15 min	All <i>Trichuris</i> primers listed in Table 2
	Denaturation	95°C	1 min	
	Annealing	54°C	50 sec	
	Elongation	72°C	50 sec	
	Final Elongation	72°C	7 min	

3.5.1 Primer specificity

The specificities of the designed primers were tested with DNA extracted specifically for the positive control samples for *A. lumbricoides* and *T. trichiura*. For the preparation of a positive control sample for *Trichuris trichiura*, a small piece of parasite tissue of an adult helminth was used and processed with the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. A positive control for *Ascaris lumbricoides* was already available in the laboratory. All primers produced amplicons of the expected length (Figure 16 & 17). To verify that the correct DNA was amplified, the PCR products were excised from the gels, purified and finally confirmed by sequencing.

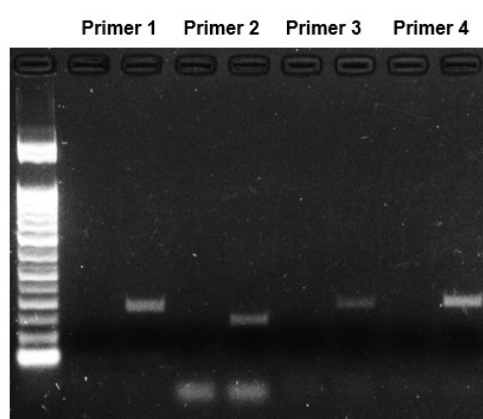


Figure 16: *Ascaris* primers specificity testing.
 Primer 1=COX1FW_asc_EB&COX1RW_asc_EB,
 Primer 2=cytBFW_asc_EB&cytBRW_asc_EB,
 Primer 3=COX1FW2_asc_EB&COX1RW2_asc_EB,
 Primer 4=NADH1FW_asc_EB&NADH1RW_asc_EB,
 M=Marker

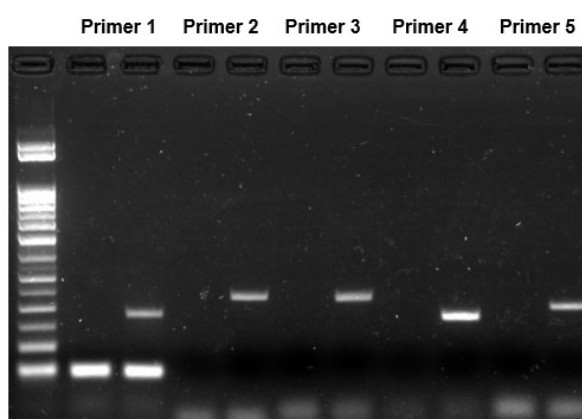


Figure 17: *Trichuris* primers specificity testing.
 Primer 1=18SFW_tri_EB&18SRW_tri_EB,
 Primer 2=18SFW2_tri_EB&18SRW2_tri_EB,
 Primer 3=18SFW_tri_EB&18SRW_tri_EB,
 Primer 4=ITS2FW_tri_EB&ITS2RW_tri_EB,
 Primer 5=ITS2FW2_tri_EB&ITS2RW2_tri_EB,
 M=Marker

3.5.2 Enhancement of sensitivity

The amount of DNA in ancient samples is usually very low. For samples with particularly low DNA content or incomplete amplification (very weak bands), the sensitivity can be enhanced by a second amplification step under the same cyclor conditions as in the first PCR. Therefore, 2 μ l and 5 μ l respectively of the PCR products from the first PCR were used and amplified again with the same PCR programme. Positive and negative control samples were treated as usual.

This method allows to amplify even the smallest amount of DNA. Figure 18 shows the gel after the first amplification step. There are no visible amplicons. Figure 19 shows the gel after the second amplification step. It demonstrates that all samples analysed were positive even though no visible PCR product could be observed after the first PCR.

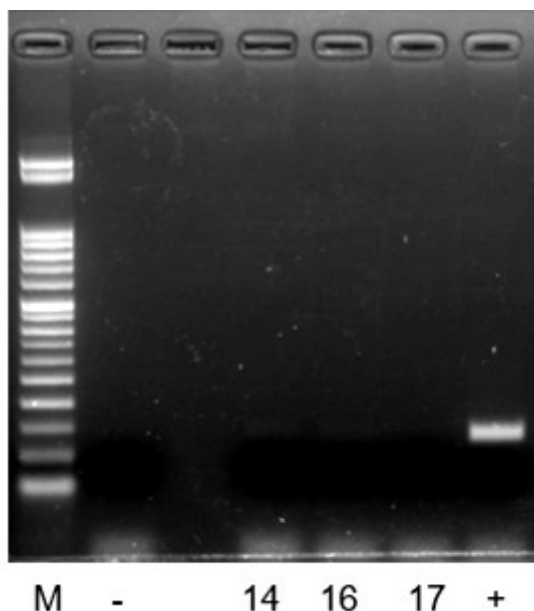


Figure 18: after the first PCR no visible PCR products can be seen on the gel. M=Marker, - = negative control, + = positive control

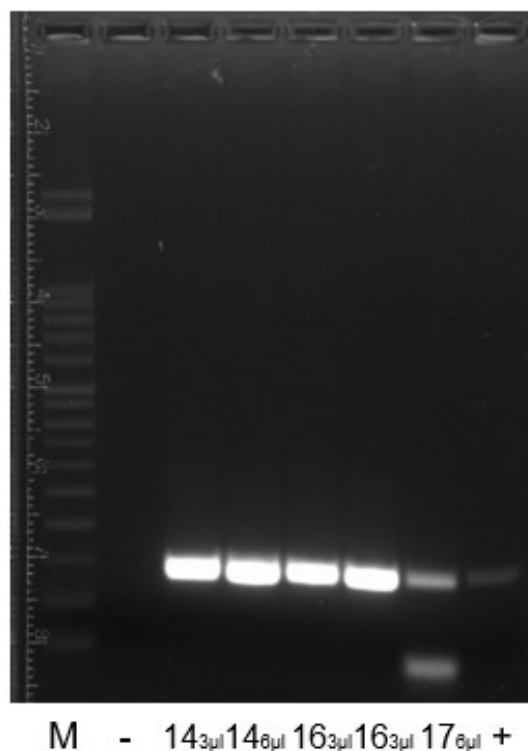


Figure 19: the same samples as in Figure 18 after the second amplification step. All samples show visible PCR products. M=Marker, - = negative control, + = positive control

3.5.3 Identification of PCR inhibitors in the aDNA extracts

The polymerase chain reaction is prone to inhibiting compounds, which may be present in the DNA extract. PCR inhibitors can originate from the sample or may be introduced during sample processing. This can affect the sensitivity or even lead to false-negative results (Schrader et al., 2012). Especially when working with ancient samples, and particularly with faecal/soil samples preserved in salt, special attention must be paid. It is always advisable to include an inhibition control to the first PCR to detect the possible presence of PCR inhibitors.

After every DNA extraction, an inhibition control was added to the first PCR. For this control, 1 µl positive control DNA plus 1 µl aDNA were added to the master mix. If the positive control is inhibited, PCR inhibitors are present in the DNA extract which prevent the PCR from working.

Figure 20 shows a gel with the PCR results of two different DNA extraction methods. For both extraction methods the same samples were used, but only one method shows visible PCR products on the gel. The other method triggered the inhibition control which means that no band can be seen on the gel. This in turn indicates that the DNA extract contains PCR inhibitors.

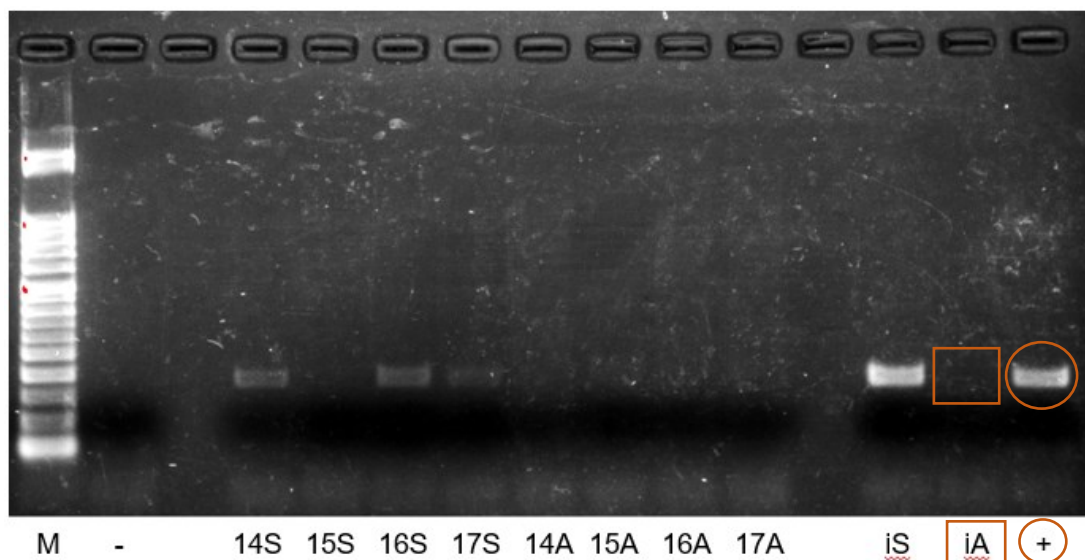


Figure 20: PCR results of two different DNA extraction methods. The second DNA extraction method triggered the inhibition control. No band can be seen here. M=marker, - = negative control, + = positive control (orange circle), iS = Inhibition control Soil Kit, iA = Inhibition control Ancient Kit (orange square)

3.6 Gel electrophoresis

The PCR results were evaluated by agarose gel electrophoresis. Briefly, 25 µl of each PCR product were mixed with 5 µl gel loading buffer (Sigma Aldrich, St. Louis, USA). The mixture was loaded onto a 2% agarose gel which was stained with GelRed™ (Biotium, Fremont, USA). To evaluate the amplicon length, 30 µl of a 50 bp DNA step ladder (Sigma Aldrich, St. Louis, USA) were loaded into the first slot. The electrophoresis system was run at 125 V and 300 mA (Bio-Rad Laboratories, Inc., California, USA).

Subsequently, the gels were evaluated under UV light using a transilluminator (Gel Doc XR+, Bio-Rad Laboratories, Inc., California, USA) which visualizes the bands on the gel.

3.6.1 Purification of bands

All bands of appropriate sizes were excised for DNA sequencing from the gel with a sterile scalpel. The excised bands were put into a 1.5 ml Eppendorf tube containing 300 µl capture buffer and the DNA was purified with the GFX™ PCR DNA and Gel Band Purification Kit (Cytiva Europe GmbH, Wien, Austria) according to the manufacturer's instructions. The DNA was eluted in 10–50 µl elution buffer depending on the intensity of the bands. The purified DNA was stored at –20°C.

3.7 DNA Sequencing

Sanger sequencing was performed in-house to verify the specificity of the designed primers and to identify the ancient parasitic DNA detected by PCR. All positive samples were sequenced in both directions at different concentrations in at least two independent setups.

3.7.1 Sequencing PCR

Sequences were obtained from both strands.

The sequencing PCR was performed with only one primer of a primer pair at a time with the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, ThermoFisher, Waltham, USA). The Master mix contained 1 µl, 2 µl or 3 µl purified aDNA, 2 µl AB Mix (BigDye™ Terminator v1.1 Cycle Sequencing RR-100, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), 1 µl 5X Sequencing Buffer (BigDye® Terminator v1.1, v.3.1, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), 2 µl forward or reverse primer and was then filled up with dH₂O to a reaction volume of 10 µl.

The cycler conditions for the sequencing PCR are listed in Table 4.

Table 4: Cycler conditions for the sequencing PCR reactions

Initial Denaturation	96 °C	30 sec
X 30 cycles		
Denaturation	96 °C	10 sec
Annealing	50 °C	5 sec
Elongation	60 °C	4 min
Storage	4 °C	until use

3.7.2 Purification of the sequencing PCR product

The resulting PCR products were added to 0.5 ml tubes containing 1 µl sodium acetate (NaAc). Then, 40 µl ethanol (100%) were added, the mixture was vortexed and incubated on ice for 20 min. The samples were then centrifuged at 13,800 x g at 4°C for 30 min (Heraeus Fresco™ 17 Centrifuge, Thermo Fisher Scientific, Waltham, USA). After centrifugation, the supernatant was carefully discarded and 90 µl of 70% ethanol were added without resuspending the pellet. The samples were centrifuged again at 13,800 x g at 4°C for 10 min.

Again, the ethanol was discarded and, in order for all ethanol to evaporate, the tubes were left open for another 3 min. Subsequently, 20 µl HiDi™ formamide (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) were added and the tubes were again left with open lids for another 5 min. After this step, the samples were first incubated at 94°C for 4 min in a Thermomixer (Thermomixer comfort 1.5 ml, Eppendorf AG, Hamburg, Germany) and then on ice for 10 min.

Sequencing was performed with an SeqStudio Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA).

3.7.3 Analysis of the sequencing results

The obtained forward and reverse sequences were first checked by blasting against sequences in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All reliable sequences of both strands were aligned to a consensus sequence using GeneDoc (National Resource for Biomedical Supercomputing, Pittsburgh, USA) and compared to reference sequences using ClustalX (Conway Institute, Dublin, Ireland). To ensure the correct order of the bases in the consensus sequence the chromatogram files from the sequencer were analysed using Chromas (Technelysium Pty Ltd., Australia) and matched with the sequences. MEGA-X (Molecular Evolutionary Genetics Analysis, Pennsylvania State University, USA) was used for visualisation of the sequencing results.

4 RESULTS

4.1 Microscopical analysis

Altogether, 35 coprolite samples were analysed by microscopy after rehydration. Four samples date back to the Bronze Age and 31 samples date back to the Iron Age.

Of the 35 samples analysed, 32 (91%) were positive for intestinal helminth eggs (Figure 21). Eggs from *T. trichiura* were detected most frequently, namely in 31 of the positive samples. Of these, 14 samples contained only eggs of *T. trichiura* and 17 samples additionally contained eggs of *A. lumbricoides*. One additional sample contained only eggs of *A. lumbricoides* (Figure 22). No eggs of other intestinal helminths were detected. An overview of the microscopical results is given in Table 5.

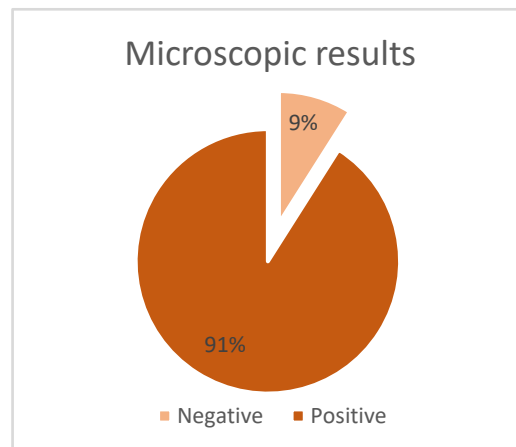


Figure 21: Microscopic results of intestinal helminth eggs in the coprolite samples in percent.

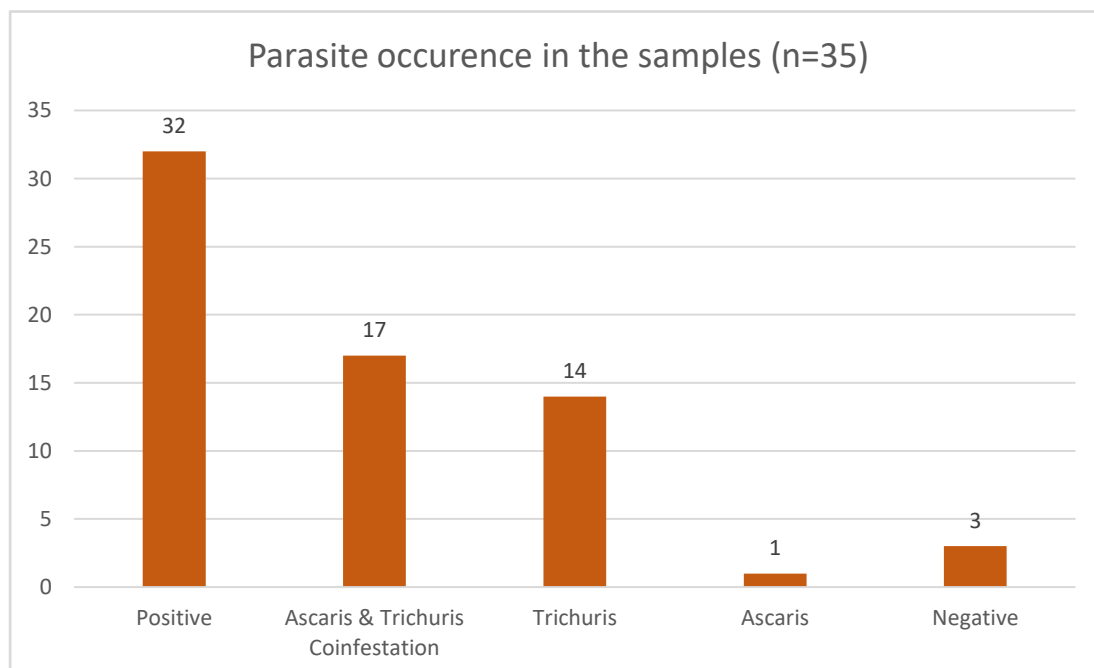


Figure 22: Intestinal helminth occurrence in the coprolite samples. Coinfestations refer to samples containing *A. lumbricoides* and *T. trichiura* eggs.

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Table 5: List of coprolite samples with an overview of the microscopical results. KW = Kernverwässerungswerk, TW = Christian von Tuschwerk, KB = Kübeck, JRW = Josef Ritschner Werk

#	ID	Sampling site	Sampling date	Time period	<i>Ascaris lumbricoides</i>	<i>Trichuris trichiura</i>
1	89.826/5	KW	1989	Iron Age	●	●
2	89.826/6	KW	1989	Iron Age	●	●
3	90.213/1	KW	1993	Iron Age		●
4	90.213/2	KW	1993	Iron Age	●	●
5	90.213/3	KW	1993	Iron Age	●	●
6	90.213/4	KW	1993	Iron Age		●
7	90.213/8	KW	1993	Iron Age	●	●
8	90.213/9	KW	1993	Iron Age	●	●
9	90.213/10	KW	1993	Iron Age		●
10	90.245/1	KW	1993	Iron Age		●
11	3.041	TW	2003	Bronze Age		●
12	OST 3.22	TW	2003	Bronze Age		
13	121.28 3	KW	2015	Iron Age		●
14	121.30 2	KW	2015	Iron Age	●	●
15	121.81	KW	2015	Iron Age	●	●
16	121.86 6	KW	2015	Iron Age	●	●
17.1	90.213 A	KW	1993	Iron Age	●	●
17.2	90.213 B	KW	1993	Iron Age		●
17.3	90.213 C	KW	1993	Iron Age		
17.4	90.213 D	KW	1993	Iron Age		●
17.5	90.213 E	KW	1993	Iron Age		●
17.6	90.213 F	KW	1993	Iron Age		●
17.7	90.213 G	KW	1993	Iron Age	●	●
17.8	90.213 H	KW	1993	Iron Age	●	●
17.9	90.213 I	KW	1993	Iron Age		●
17.10	90.213 J	KW	1993	Iron Age		●
A	NN	KW	2018	Iron Age	●	●
B	18.215	TW	2018	Bronze Age	●	●
C	NN	KW	2018	Iron Age	●	●
D	NN	KB	2018	Iron Age	●	●
E	2019/1	TW	2019	Bronze Age		●
F	2019/2	JRW	2019	Iron Age	●	
G	2019/3	JRW	2019	Iron Age	●	●
H	2019/4	KW	2019	Iron Age		
I	2019/5	KW	2019	Iron Age		●

4.1.1 Micrographs

In general, the parasite eggs were very well preserved and the eggs of *Ascaris lumbricoides* (roundworm) and *Trichuris trichiura* (whipworm) could be clearly identified based on their morphological characteristics. The eggs even retained their yellow-brownish (bile-stained) colour.

4.1.1.1 Ancient *Ascaris lumbricoides* eggs

The oval-shape and the mamillated coat of the roundworm eggs were intact and most of the eggs did not have any visible damage on the outer layers (Figure 23). Differences can be seen between the internal structures of the ancient roundworm eggs and the recent eggs (Figure 24). In many ancient eggs, the internal structures had contracted and were no longer connected to the outer layers (Figures 25 & 26). The exceptional preservation of the eggs also makes it possible to distinguish different developmental stages of the eggs (e.g.: Figures 27 and 28). Figures 29 and 30 are showing micrographs of roundworm eggs from the Bronze Age.



Figure 23: Ancient *Ascaris lumbricoides* egg from sample 2019/2 dated to the Iron Age. Magnification: 400x



Figure 24: Recent *Ascaris lumbricoides* egg for comparison. Magnification: 400x



Figure 25: Ancient *Ascaris lumbricoides* egg with contracted internal structures. The photo was taken under 400x magnification from a sample from the Iron Age (sample ID 2019/3).



Figure 26: Ancient *Ascaris lumbricoides* egg with contracted internal structures. The photo was taken under 1000x magnification from a sample from the Iron Age (2019/3).



Figure 27: Ancient fertilized and decorticated *Ascaris lumbricoides* egg from a sample (2019/3) from the Iron Age. Magnification: 400x



Figure 28: Ancient *Ascaris lumbricoides* egg from a sample (2019/3) from the Iron Age. The internal structures could indicate a developing larva. Magnification: 400x



Figure 29: Ancient *Ascaris lumbricoides* egg from a sample (18.215) from the Bronze Age. Magnification: 400x

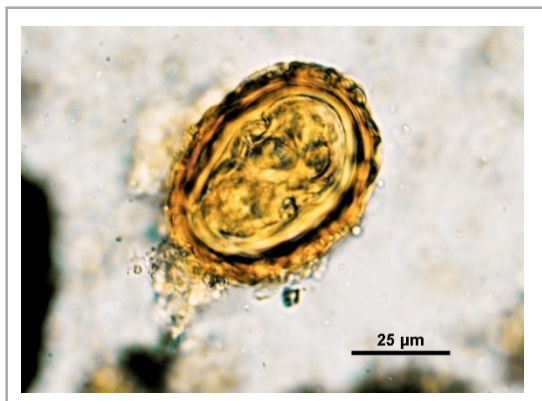


Figure 30: Ancient *Ascaris lumbricoides* egg from a sample (18.215) from the Bronze Age. Magnification: 400x

4.1.1.2 Ancient *Trichuris trichiura* eggs

The ancient *Trichuris trichiura* eggs had retained their barrel-shaped appearance but most of the eggs had lost the translucent hyaline polar plugs (Figure 31), which are typical for the whipworm eggs (Figure 32). The polar plugs are essential to protect the inner content from the environment. Many eggshells were well preserved, but just as many exhibited damages on the outer layers (Figures 32–35). Some eggs were empty, and others probably got filled with debris from the environment (Figure 36). Figures 37 and 38 are showing micrographs of whipworm eggs from the Bronze Age.



Figure 31: Ancient *Trichuris trichiura* egg from a sample (90.213/9) from the Iron Age. Arrows indicate the lost polar plugs. Magnification: 400x



Figure 32: Recent *Trichuris trichiura* egg for comparison. Magnification: 400x

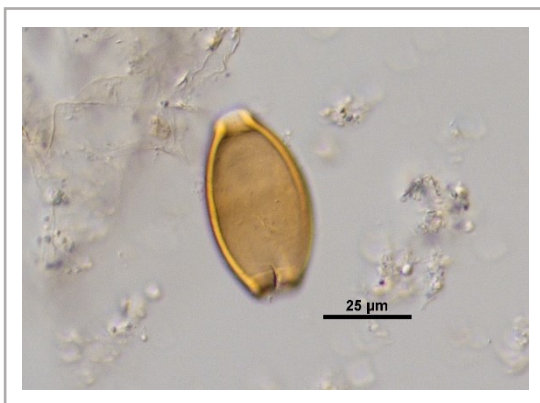


Figure 33: Ancient *Trichuris trichiura* egg with damages on the shell wall. Interference contrast image was taken from a sample (89.826/5) dated to the Iron Age. Magnification: 400x

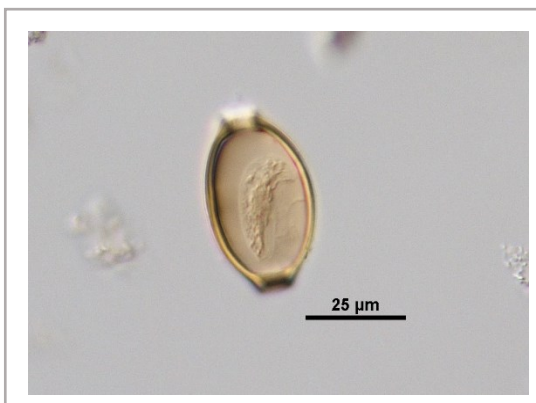


Figure 34: Ancient *Trichuris trichiura* egg with damages on the shell wall. Interference contrast image was taken from a sample (90.245/1) dated to the Iron Age. Magnification: 400x

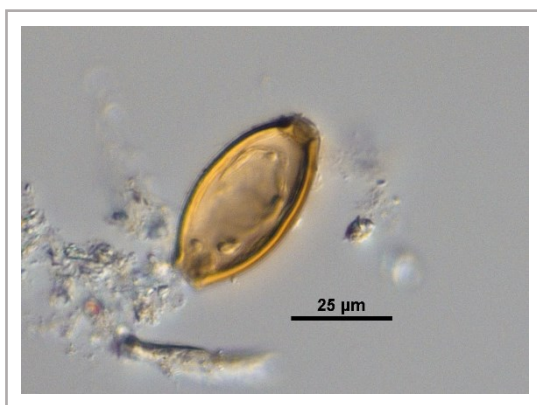


Figure 35: Ancient *Trichuris trichiura* egg with damages on the eggshell. Interference contrast micrograph taken from a sample (89.826/6) from the Iron Age. Magnification: 400x

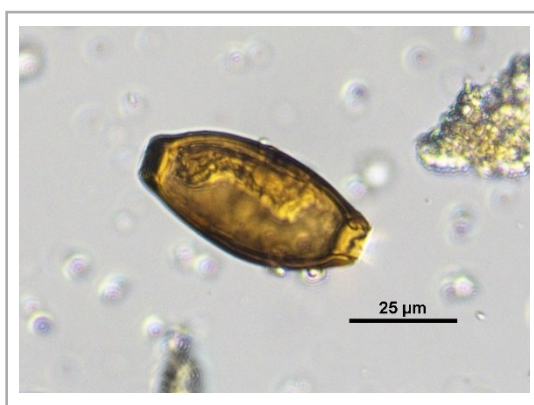


Figure 36: Ancient *Trichuris trichiura* egg without polar plugs. Debris may have gotten into the egg. Interference contrast micrograph taken from a sample (90.213/3) from the Iron Age. Magnification: 400x



Figure 37: Ancient *Trichuris trichiura* egg from a sample (18.215) from the Bronze Age. Magnification: 400x



Figure 38: ancient *Trichuris trichiura* egg from a sample (18.215) from the Bronze Age. Magnification: 400x

4.2 Molecular analyses

4.2.1 PCR results

Specific PCRs were run with all 18 samples, that were microscopically positive for *Ascaris lumbricoides*, and with 10 samples, microscopically positive for *Trichuris trichiura*. From 16 *Ascaris* samples amplicons of the expected length were achieved, in some cases even of different genes. Two samples did not produce amplicons. Of the *Trichuris* samples, three did give amplicons, however not of the appropriate size. In all other *Trichuris* samples the amplification of ancient DNA was unsuccessful. All visible PCR products were excised from the respective gels for DNA sequencing. An overview of the PCR results is given in Table 6.

RESULTS

Table 6: Overview of the PCR results. Dots (●) indicate successful amplification of aDNA by PCR and crosses (x) indicate no amplification.

#	ID	Sampling site	Sampling date	Time period	Ascaris lumbricoides PCR			Trichuris trichiura PCR
					cox1	cytB	NADH1	
1	89.826/5	KW	1989	Iron Age	●	x	x	
2	89.826/6	KW	1989	Iron Age	●	●	x	
3	90.213/1	KW	1993	Iron Age				x
4	90.213/2	KW	1993	Iron Age		x	●	x
5	90.213/3	KW	1993	Iron Age	●	●	x	x
6	90.213/4	KW	1993	Iron Age				
7	90.213/8	KW	1993	Iron Age	●	x	x	
8	90.213/9	KW	1993	Iron Age	x	x	x	
9	90.213/10	KW	1993	Iron Age				
10	90.245/1	KW	1993	Iron Age				
11	3.041	TW	2003	Bronze Age				
12	OST 3.22	TW	2003	Bronze Age				
13	121.28 3	KW	2015	Iron Age				x
14	121.30 2	KW	2015	Iron Age	●	●		x
15	121.81	KW	2015	Iron Age	x			
16	121.86 6	KW	2015	Iron Age	●	●		x
17.1	90.213 A	KW	1993	Iron Age	●	●		
17.2	90.213 B	KW	1993	Iron Age				
17.3	90.213 C	KW	1993	Iron Age				
17.4	90.213 D	KW	1993	Iron Age				
17.5	90.213 E	KW	1993	Iron Age				
17.6	90.213 F	KW	1993	Iron Age				
17.7	90.213 G	KW	1993	Iron Age	●	x	●	
17.8	90.213 H	KW	1993	Iron Age	●	●	●	
17.9	90.213 I	KW	1993	Iron Age				
17.10	90.213 J	KW	1993	Iron Age				
A	NN	KW	2018	Iron Age	●	●	x	x
B	18.215	TW	2018	Bronze Age	●	x	●	
C	NN	KW	2018	Iron Age	●	x	x	
D	NN	KB	2018	Iron Age	●	x	●	
E	2019/1	TW	2019	Bronze Age				x
F	2019/2	JRW	2019	Iron Age	●	●	●	
G	2019/3	JRW	2019	Iron Age	●	●	●	
H	2019/4	KW	2019	Iron Age				x
I	2019/5	KW	2019	Iron Age				x

4.2.1.1 *Ascaris lumbricoides* specific PCRs

In total, 31 PCR runs resulted in amplicons of the correct length. As shown in Figure 39, cytochrome c oxidase subunit 1 amplicons were obtained from 15 samples, cytochrome B amplicons from 9 samples and NADH dehydrogenase subunit 1 amplicons from 7 samples. All visible PCR products were excised from the respective gels for DNA sequencing.

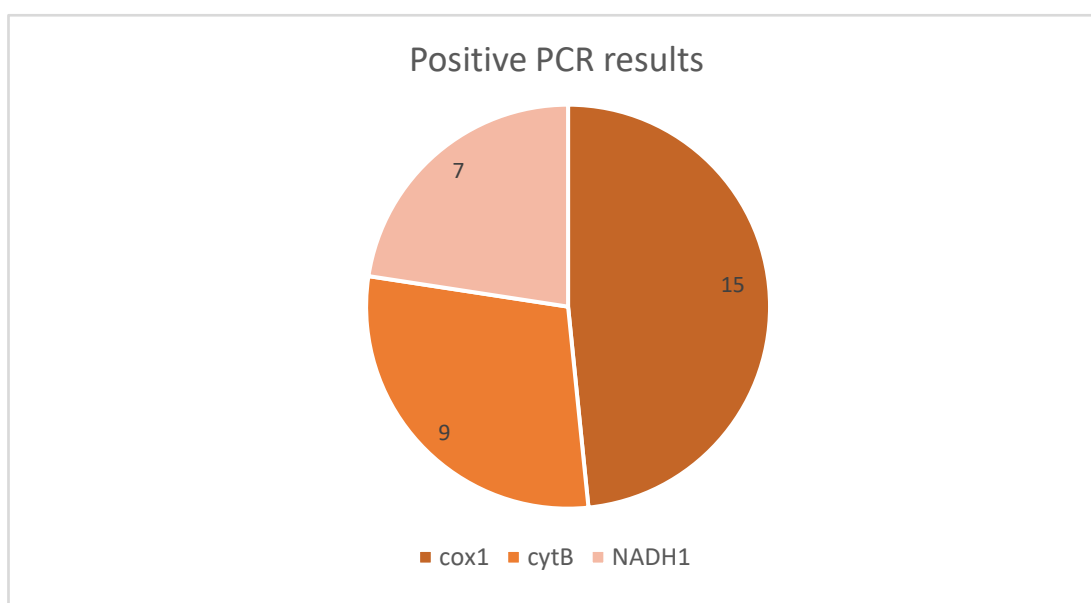


Figure 39: *Ascaris lumbricoides* PCR results with the number of samples from which amplicons were obtained. cox1 = mitochondrial cytochrome c oxidase subunit 1 gene, cytB = mitochondrial cytochrome oxidase gene, NADH1 = mitochondrial NADH dehydrogenase subunit 1 gene

In Figures 40–46, several examples of successful PCR amplifications are shown. Figure 40 gives the results of the PCR with the cox1 primers with successful amplifications for all samples investigated. Particularly worth highlighting is the band of sample B in lane 5, which represents the successful amplification of ancient parasite DNA from the Bronze Age.

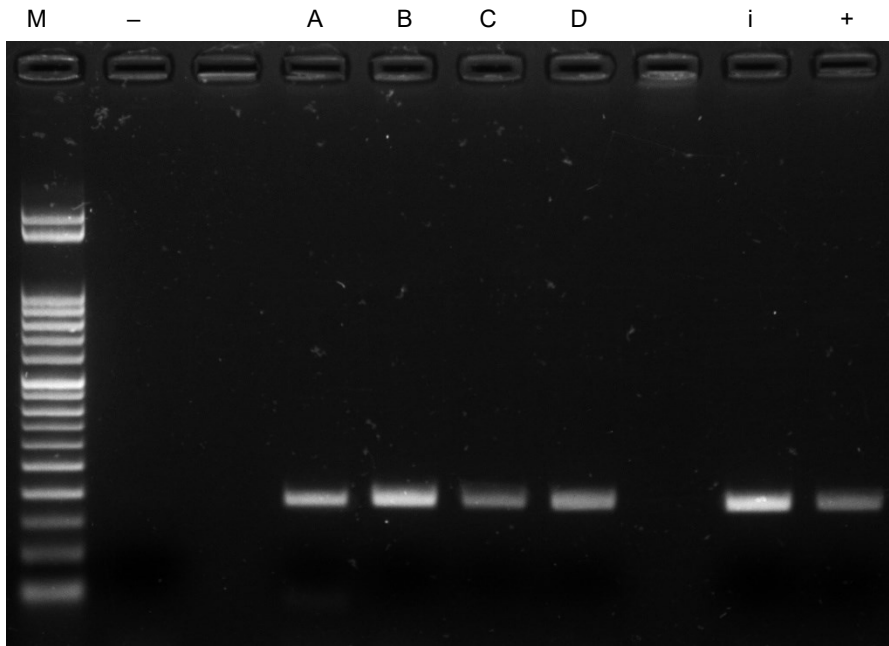


Figure 40: *Ascaris lumbricoides* cox1 PCR results. Slots are labelled with the sample numbers. Slots between the samples and the controls were left blank. M = Marker, - = negative control, + = positive control, i = Inhibition control

Figures 41 and 42 are examples of successful amplifications after PCR from the PCR products with the *cytB* and *NADH1* primers, respectively, when no visible PCR products were present after the first PCR (Figure 43). Figure 42 includes the positive PCR results of a sample (B) from the Bronze Age.

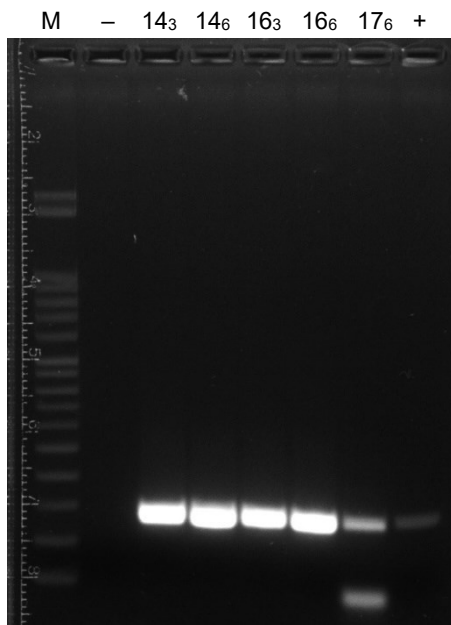


Figure 41 (copy of Figure 19): *Ascaris lumbricoides* *cytB* PCR results after the second round of amplification. Slots are labelled with the sample numbers. M = marker, - = negative control, + = positive control, X3 = 3 µl PCR product, X6 = 6 µl PCR product

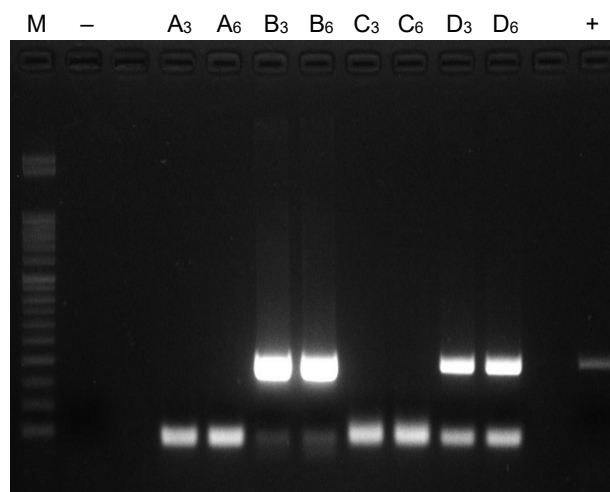


Figure 42: *Ascaris lumbricoides* *NADH1* PCR results after the second round of amplification. Slots are labelled with the sample numbers. M = marker, - = negative control, + = positive control, X3 = 3 µl PCR product, X6 = 6 µl PCR product

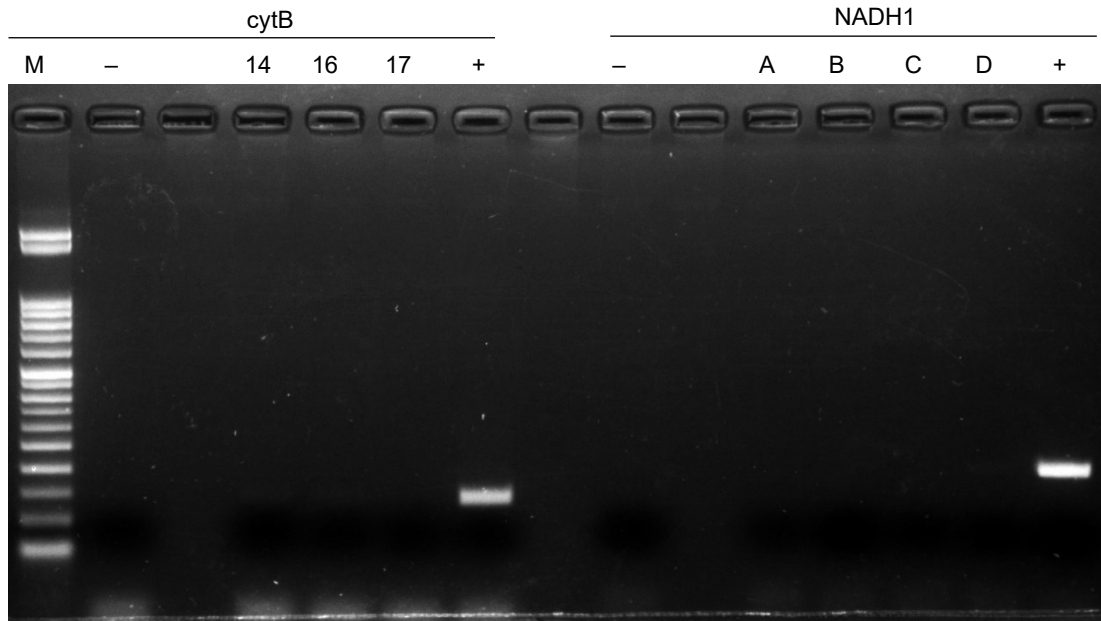


Figure 43: *Ascaris lumbricoides* cytB and NADH1 PCR results after the first round of amplification. Slots labelled with sample numbers. Slots between the samples and the negative controls were left blank. M = marker, – = negative control, + = positive control

The PCR results of the samples (sample IDs F and G) collected under sterile conditions specifically for this study are shown in Figures 44–46. Amplicons of the correct sizes were obtained for all three targeted genes (*cox1*, *cytB* and NADH1).

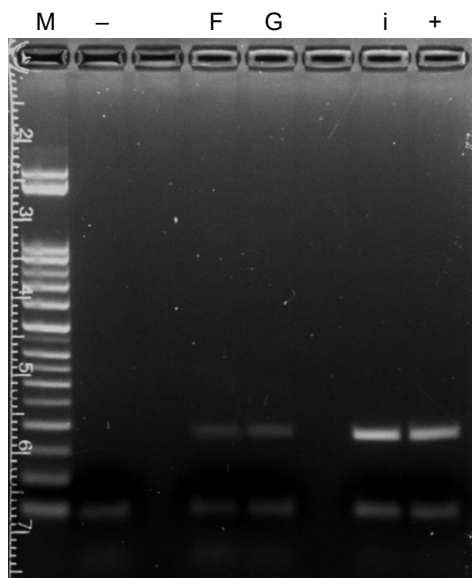


Figure 44: *Ascaris lumbricoides* *cox1* PCR results of samples F and G. Slots are labelled with the sample numbers. M = marker, – = negative control, + = positive control

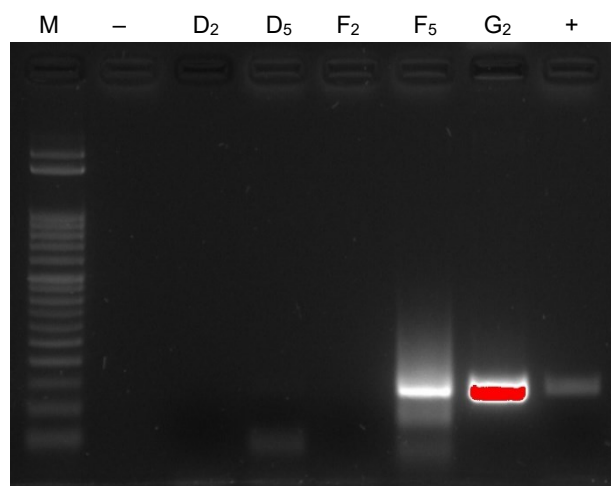


Figure 45: *Ascaris lumbricoides* *cytB* PCR results, including the samples F and G taken for this study. Slots are labelled with the sample numbers. M = marker, – = negative control, + = positive control, X2 = 2 µl PCR product, X5 = 5 µl PCR product

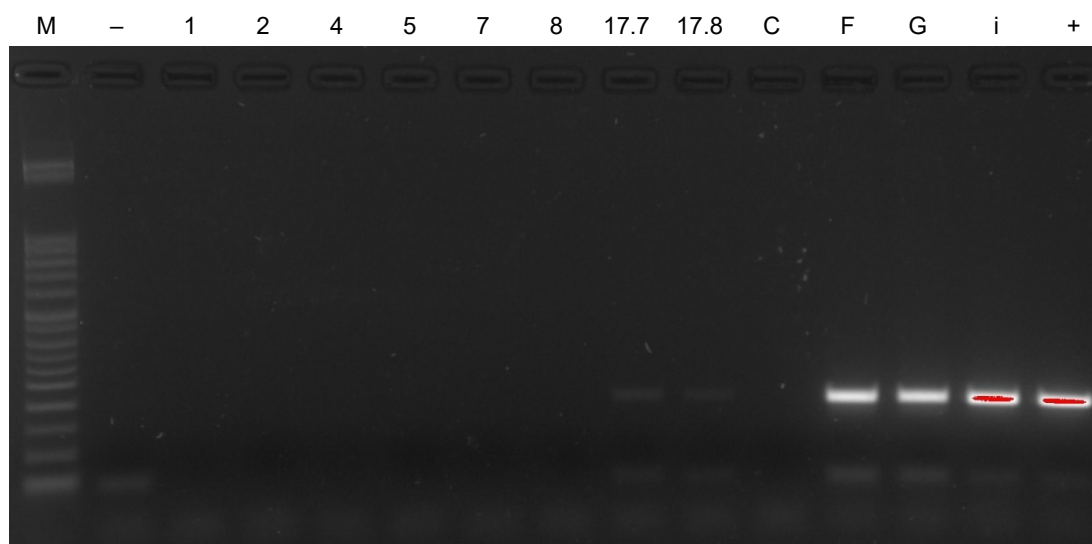


Figure 46: *Ascaris lumbricoides* NADH1 PCR results, including the samples F and G specifically taken for this study. Slots are labelled with the sample numbers. M = marker, – = negative control, + = positive control, i = Inhibition control

4.2.2 Sequencing results

The 31 positive *A. lumbricoides* PCR results provide proof of principle for the successful amplification of ancient parasite DNA. For detailed evaluation and specific identification, all amplicons were subjected to Sanger sequencing. Reliable sequences were obtained from 29 PCR amplicons. Of these, 15 were of the *cox1*, eight were of the *cytB* and six were of the *NADH1* gene.

An overview of the results is given in Table 7 and a summary of the sequencing results in Figure 47.

Table 7: Overview of the obtained sequences. Dots (●) indicate consensus sequences with high query cover and high sequence identity and circles (○) indicate obtained forward and reverse sequences with lower query coverage and lower sequence identity with *Ascaris* sequences in the NCBI database.

#	ID	Sampling site	Sampling date	Time period	Ascaris lumbricoides sequences		
					cox1	cytB	NADH1
1	89.826/5	KW	1989	Iron Age	○		
2	89.826/6	KW	1989	Iron Age	○		
3	90.213/1	KW	1993	Iron Age			
4	90.213/2	KW	1993	Iron Age			○
5	90.213/3	KW	1993	Iron Age	●	○	
6	90.213/4	KW	1993	Iron Age			
7	90.213/8	KW	1993	Iron Age	●		
8	90.213/9	KW	1993	Iron Age			
9	90.213/10	KW	1993	Iron Age			
10	90.245/1	KW	1993	Iron Age			
11	3.041	TW	2003	Bronze Age			
12	OST 3.22	TW	2003	Bronze Age			
13	121.28 3	KW	2015	Iron Age			
14	121.30 2	KW	2015	Iron Age	●	●	
15	121.81	KW	2015	Iron Age			
16	121.86 6	KW	2015	Iron Age	●	●	
17.1	90.213 A	KW	1993	Iron Age	●	○	
17.2	90.213 B	KW	1993	Iron Age			
17.3	90.213 C	KW	1993	Iron Age			
17.4	90.213 D	KW	1993	Iron Age			
17.5	90.213 E	KW	1993	Iron Age			
17.6	90.213 F	KW	1993	Iron Age			
17.7	90.213 G	KW	1993	Iron Age	●		
17.8	90.213 H	KW	1993	Iron Age	●	●	●
17.9	90.213 I	KW	1993	Iron Age			
17.10	90.213 J	KW	1993	Iron Age			
A	NN	KW	2018	Iron Age	○	○	
B	18.215	TW	2018	Bronze Age	●		●
C	NN	KW	2018	Iron Age	○		
D	NN	KB	2018	Iron Age	●		●
E	2019/1	TW	2019	Bronze Age			
F	2019/2	JRW	2019	Iron Age	●	●	●
G	2019/3	JRW	2019	Iron Age	●	●	●
H	2019/4	KW	2019	Iron Age			
I	2019/5	KW	2019	Iron Age			

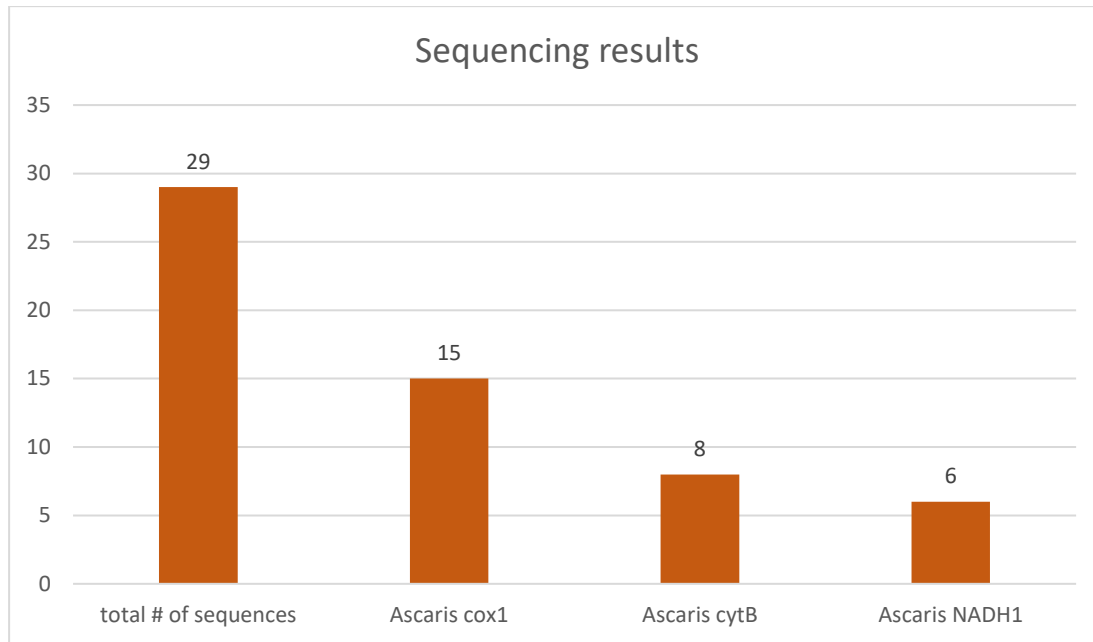


Figure 47: Summary of sequencing results.

4.2.2.1 *Ascaris lumbricoides* *cox1* sequences

Sequences of a fragment of the *cox1* gene of *A. lumbricoides* were obtained from 15 coprolite samples. Sequences of 11 samples were of high quality and consensus sequences were generated. The sequences of the remaining four PCR amplicons were too short and/or had too many ambiguous bases and were therefor excluded from further analyses.

Full amplicons were obtained, and primer sites were excised. The obtained *cox1* consensus sequences have a length of 142 bp and were blasted against sequences available in GenBank. All 11 consensus sequences were confirmed to be of *Ascaris lumbricoides*. Sequence identities ranged from 129–141/142 bp (92.81% to 99.30%), with query coverages between 97% and 100%.

The obtained sequences were aligned to six reference sequences of *A. lumbricoides* from GenBank belonging to the established three different clades. In this fragment of *cox1*, clade A and clade B are not clearly

It is noticeable that the freshly obtained samples (sample IDs F & G) and the samples, that had been stored for a shorter period of time (sample IDs B & D), resulted in better sequences. The *cox1* sequence of sample B (ID: 18.215) from the Bronze Age has 137/142 bp (96.48%) sequence identity with 100% query coverage to the *A. lumbricoides* isolate (KY045803) of clade A, listed first in the alignment in Figure 48.

[illegible]

RESULTS

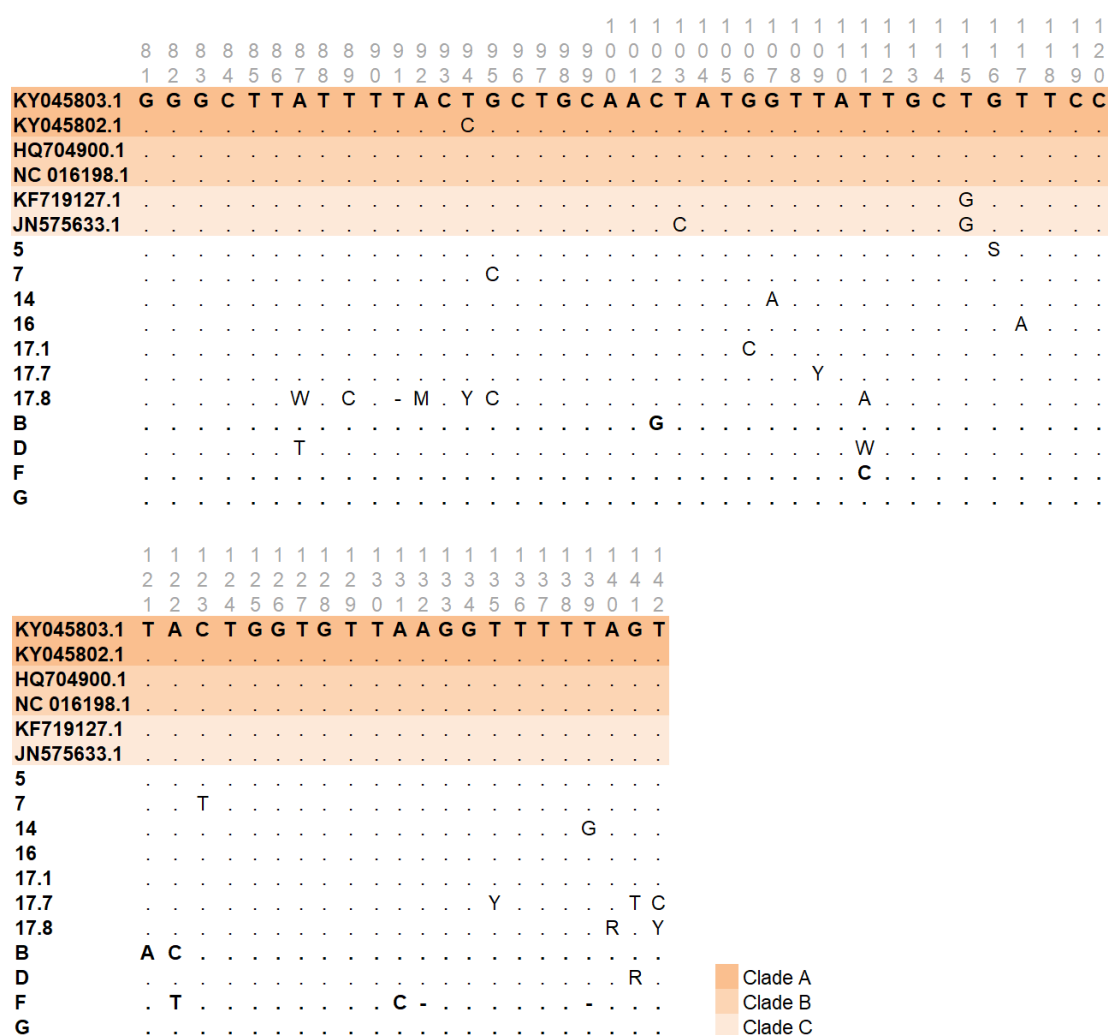


Figure 48: Multiple alignment and comparison of all obtained high quality ancient *cox1* sequences to reference sequences, of different *A. lumbricoides* isolates belonging to different clades (A, B and C), from GenBank. Note, sample B is from the Bronze Age and sample F and G are the freshly obtained samples specifically for this study.

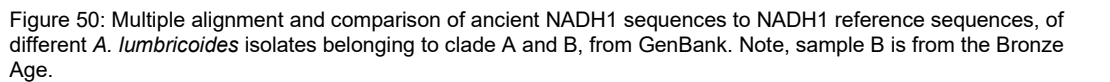
4.2.2.2 *Ascaris lumbricoides* *cytB* sequences

Sequences of a fragment of the *cytB* gene of *A. lumbricoides* were obtained from eight coprolite samples. Of five of these samples reliable consensus sequences could be generated. The sequences of the remaining three PCR amplicons were too short and/or had too many ambiguous bases and were therefor excluded from further analyses.

Full amplicons were obtained, and primer sites were excised. The obtained sequences were aligned to reference sequences, of different *A. lumbricoides* isolates belonging to clade A and B, from GenBank. For this gene, no reference sequences are available of *A. lumbricoides* isolates belonging to clade C. In this fragment of *cytB*, clade A and clade B are not distinguishable, but haplotypes within clade A and B do show variabilities, as shown in Figure 49.

Figure 49 shows a multiple alignment of the obtained ancient *cytB* sequences with *cytB* reference sequences of different *A. lumbricoides* isolates belonging to clade A and B from GenBank.

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5 DISCUSSION

This study provides the first two successfully amplified, and sequenced, ancient DNA sequences of the soil-transmitted intestinal parasite *Ascaris lumbricoides* of the Bronze Age. Furthermore, several sequences of different genes of *A. lumbricoides* of coprolites from the Hallstatt salt mine dated to the Iron Age were obtained. These sequences represent the first molecular data on *A. lumbricoides* of the Hallstatt period in Austria. Altogether, the 35 palaeofaecal samples investigated in this study showed a high prevalence of soil-transmitted intestinal helminths with a limited diversity of helminth species. Eggs of *T. trichiura* and *A. lumbricoides* were exceptionally well preserved and could be clearly identified by microscopy based on their morphological characteristics. Moreover, the first micrographs of intestinal helminths of the Bronze Age in Austria were taken.

5.1 First ancient DNA of *A. lumbricoides* from the Bronze Age

Altogether, 35 palaeofaecal samples were investigated in this study of which four were dated back to the Bronze Age. The investigation of the coprolites was performed with two different approaches: microscopy and molecular analysis. Microscopical analysis revealed the presence of intestinal helminths in three of the four coprolites from the Bronze Age. Two samples (sample IDs: 3.4041 and 2019/1) contained only eggs of *T. trichiura* and one sample (ID: 18.215) contained eggs of *T. trichiura* together with eggs of *A. lumbricoides*. These results of the microscopical analysis provided a guideline for the molecular methods. Three of these samples (IDs: 3.4041, OST 3.22 & 18.215) have been investigated previously and yielded the same microscopical results. However, molecular data were not obtained from these samples in that previous study (Rodler, 2019).

Molecular analyses of ancient remains are usually associated with several challenges, primarily because of the damaged and fragmented nature of the ancient DNA. After the death of an organism, molecular degradation begins

and the rate of degradation depends on external and internal factors (Lindahl, 1993), which makes the recovery of ancient DNA very challenging. However, under rare circumstances and special preservation conditions (Pääbo et al., 2004b), like in the interior of a particularly thick-walled helminth egg preserved without the access to oxygen, the degradation of the DNA can be slowed down. This led to the successful amplification and sequencing of two fragments of ancient DNA of *A. lumbricoides* from a coprolite which was dated to the Bronze Age between 1158 and 1063 BC (Grabner et al., 2021). To the best of my knowledge, these two sequences of *A. lumbricoides* are the world's first recovered ancient DNA sequences of this species dated from the Bronze Age.

First, a 142 bp long *cox1* sequence was obtained. The sequence identity was 137/142 bp (96.48%) with 100% query coverage compared to a reference sequence, of an *A. lumbricoides* isolate belonging to clade A, available in GenBank. Additionally, a 152 bp long fragment of the NADH1 gene was successfully sequenced. The BLASTN analysis for this sequence revealed 145/150 bp (96.67%) sequence identity with 98% query coverage to an *A. lumbricoides* sequences, of an isolate belonging to clade A, in GenBank. By obtaining sequences from two different genes, the presence of specific DNA was corroborated. Of course, ancient DNA results must always be regarded with caution as ancient remains are highly susceptible to contamination with exogenous or modern DNA (Pääbo et al., 2004b). In this case, however, the authenticity of the ancient DNA sequences can be confidently confirmed for several reasons. The samples were taken under sterile conditions and stored separately from other samples in the laboratory. Tools and surfaces were sterilized, and protective clothing was worn during coprolite analysis. The presence of eggs of *A. lumbricoides* was confirmed by microscopical analysis. Furthermore, all PCRs were run with negative controls confirming that no contamination occurred during the molecular analysis. Also, in contrast to working with ancient human DNA or ancient bacterial DNA, it is highly unlikely that the samples were contaminated with intestinal helminths or their eggs in Austria, where *A. lumbricoides* is since many years not endemic anymore.

There is only a limited number of reports on gastrointestinal helminth remains from the Bronze Age worldwide, and even fewer molecular data of parasites are available from that period. The most recent larger palaeoparasitological

project in Europe, besides this one, was conducted at the Late Bronze Age archaeological site of Must Farm; a pile-dwelling settlement located in wetland in Cambridgeshire, England, UK. Several sediment samples and coprolites were collected from the occupation deposits for parasite analysis by digital light microscopy. This led to the discovery of helminth eggs of five different taxa: fish tapeworm (*Diphyllbothrium* sp.), whipworm (*Trichuris* sp.), the giant kidney worm (*Diocotophyme renale*, a dog parasite, only very rarely affecting humans), *Echinostoma* sp. and *Capillaria* sp. (Ledger et al., 2019). On the island of Kea (Greece), the analysis of several soil sediment samples taken from different burials in the sacrum and iliac area, revealed eggs of intestinal parasitic worms, namely the whipworm (*Trichuris*) and the large roundworm (*Ascaris*), dating back to the Neolithic to Byzantine period (Anastasiou et al., 2018). Other archaeological sites that have revealed evidence for intestinal helminths dated to the Bronze Age in Europe are, for example: Grésine (France) (Le Bailly & Bouchet, 2010, 2013) and Hulín (Czech Republic) (Šebela et al., 1990), where *Ascaris* sp., *T. trichiura* and *Ancylostoma duodenale* (one of the two human hookworms) and *Dicrocoelium dendriticum* (the lancet liver fluke) were found.

Outside of Europe, parasite eggs from archaeological excavations dated to the Bronze Age were detected in Iran. Close to Yasuj city, in a burial of a Bronze Age cemetery (2600–2200 BC), eggs of *D. dendriticum* were found, which represent the oldest finding of *D. dendriticum* in the Near East. These findings enabled the researchers to draw conclusions about the close relationship between humans and herbivores in this area, as the life cycle of this parasite typically includes sheep, snails and ants (Mowlavi et al., 2015). Furthermore, by the investigation of sheep and carnivore coprolites obtained from an archaeological site dating back to about 3200–1800 BC in Shahr-e Sukhteh (Iran), *Taenia* spp., *Capillaria* spp. and *D. dendriticum* eggs were found. Evidence for a rare human helminthiasis was reported from a grave of the Shahr-e Sukhteh archaeological site dating back to the Bronze Age by the finding of *Physaloptera* spp. eggs in a soil sample collected in the pelvic and sacrum bones area of a skeleton. These nematodes have insects as intermediate hosts and are transmitted to mammals including humans by oral uptake of the insects (or of paratenic hosts) (Makki et al., 2017). In Tel Meggido

(Israel), a poorly preserved whipworm egg was detected during conventional microscopic observations of faecal material which was found near the remains of a large palace dated to the Late Bronze Age (mid-14th century BC) (Langgut et al., 2016).

Occasionally, parasite remains of the Bronze Age are found by chance. This was, for example, the case in a study from Sardinia (Italy) where numerous Bronze Age pits and wells were analysed in an archaeobotanical approach. The authors reported the findings of one *Ascaris* spp. egg and a high prevalence of *T. trichiura* eggs during pollen identification in the samples of a well. These findings allowed them to make inferences about the possible use of the well as a cesspit (Sabato et al., 2015). This again demonstrates that palaeoparasitological findings can help to draw new conclusions in other research areas.

However, all these studies were only on a morphological and microscopical basis and no molecular analyses were performed. Molecular data on intestinal helminths and parasites in general from the Bronze Age and earlier periods is only very rarely available.

In 2015, Côté et al. performed a PCR-based approach coupled with next-generation sequencing for the identification of gastrointestinal helminths directly from archaeological sediments. A variety of archaeological samples, with an age of 100 to 7,200 years, from various geographical and cultural origins were investigated. This led to the successful recovery of *Ascaris* DNA from sediment samples dated back to about 5000 BC, although no parasitic remains could be detected during microscopical analysis. Furthermore, DNA of the nematodes *Ascaris* spp., *Trichuris trichiura*, *Enterobius vermicularis*, the cestode *Taenia saginata* and the trematode *Dicrocoelium dendriticum* could be obtained from a sample taken at an archaeological site in Spain, dated to 5234–5034 BC (Côté et al., 2016). Genetic analysis was also performed in a palaeoparasitological study with sediment samples from the Neolithic (5320–4980 BC) lakeside settlement of La Draga in Spain. Multiplex polymerase chain reactions were performed with several samples to target different gastrointestinal helminths. Ancient DNA of *Ascaris* sp., *T. trichiura*, *E. vermicularis*, *T. saginata* and *D. dendriticum* could be obtained. Interestingly,

two of these taxa (*D. dendriticum* and *E. vermicularis*) were not observed during microscopical examination. However, during microscopical analysis additional parasites were found, namely *Diphyllbothrium* sp., *Capillaria* sp., *Paramphistomum* sp. (parasite of ruminants) and *Macracanthorhynchus* sp. (animal parasite, affecting mostly pigs and wild boar). These results enhance the knowledge on the lifestyle of the inhabitants of this archaeological site (Maicher et al., 2017). In 2018, palaeoparasitological analyses were performed with a mammalian carnivore coprolite from an archaeological site in Argentina dated to 14,981–14,552 BC with the aim of exploring ancient ecological relationships. The morphological characteristics of the parasite eggs found in the coprolite referred to *Toxascaris* sp. These results were confirmed by ancient mitochondrial DNA analysis which identified the parasite eggs as *Toxascaris leonina*, a parasite of dogs, cats and other carnivores that can also infest humans. This represents the oldest molecular record worldwide (Petrigh et al., 2019).

The recovery of ancient parasite DNA from the Bronze Age and even older periods is very difficult; on one hand, because of the very limited number of well-preserved parasitic remains and on the other, due to the mostly damaged nature of ancient DNA. However, studying this less-investigated period is very exciting and new discoveries will help to clarify many questions concerning not only the evolution of parasites and their co-evolution with humans, but also the history of mankind. With palaeoparasitological findings and the successful recovery of ancient parasite DNA, new conclusions can be drawn on the distribution of parasites and the way of life of ancient populations.

5.2 First detailed molecular data of *A. lumbricoides* from the Hallstatt period in Austria

Molecular data of *A. lumbricoides* dated to the Iron Age, between approximately 750 and 662 BC (Grabner et al., 2021), could be obtained from 16 coprolites. A total of 19 *A. lumbricoides* specific sequences from different genes were successfully amplified and sequenced. This is the first time that ancient DNA of *A. lumbricoides* has been recovered from coprolites dated to

the Hallstatt period in Austria. In a previous study, bands in PCR could be detected but no reliable sequence data was obtained (Rodler, 2019).

For the molecular approach, three suitable mitochondrial marker genes for species identification were used and novel primers designed. Mitochondrial markers were selected as the mitochondrial DNA (mtDNA) is present in a much higher copy number than the genomic DNA. This facilitates the amplification, as the amount of ancient DNA is mostly extremely limited (Loreille et al., 2001). The mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*) was chosen as it is a commonly used genetic marker for species identification (Hebert et al., 2003), and because the sequence availability in GenBank is high. Specific primers targeting short sequences of the mitochondrial cytochrome B gene (*cytB*), and the mitochondrial NADH dehydrogenase subunit 1 gene (*NADH1*), were designed and used to confirm the obtained ancient *cox1* sequences. *CytB* and *NADH1* have been used in previous palaeoparasitological studies as well (Wood, 2018).

For the *cox1* gene, 10 sequences with a length of 142 bp were obtained which were confirmed to be of *A. lumbricoides*, with sequence identities of 92.81% to 99.30% to reference sequences available in GenBank. *CytB* sequences were obtained from five coprolites with a fragment length of 97 bp. The sequence identities ranged from 93.75% to 98.97% to *A. lumbricoides* reference sequences available in GenBank. The four *NADH1* sequences obtained from the Iron Age samples had a length of 152 bp and their sequence identities ranged from 90.67% to 98.00% to reference sequences in GenBank. To summarise, this means that for six samples sequences of at least two different genes were obtained and for four samples sequences of one gene. The differences observed in the obtained ancient sequences were mainly bases that could not be reliably identified, probably due to the old age of the DNA.

Reliable phylogenetic analyses below the species level are not possible with such short sequences, as obtained in this study. However, the alignment of the obtained ancient sequences to reference sequences of different isolates of *A. lumbricoides* belonging to three different clades (A, B and C), revealed that the ancient sequences show highest similarity to reference strains of clade A and highest dissimilarity to isolates of clade C.

All PCRs with *T. trichiura* species-specific primers targeting different genes (SSU rRNA & ITS2) were unsuccessful. This indicates that the ancient DNA of *T. trichiura* might have been severely damaged. The preservation of the ancient DNA was probably strongly affected by the lack of protection inside the eggs. Morphologically, the eggs of *T. trichiura* showed more damage than the eggs of *A. lumbricoides*. Particularly important is probably the loss of the polar plugs that *T. trichiura* eggs typically possess. These polar plugs keep the eggs closed and protect the internal structures from environmental influences, which might have had a damaging effect on the DNA. However, it must be mentioned that ancient DNA of parasites sometimes can be detected even in the absence of microscopically visible parasitic structures, as shown in previous studies (Côté et al., 2016; Guedes et al., 2020; Maicher et al., 2017). In the current study, however, much older samples were investigated and the taphonomic processes may have had too much impact on the preservation of the *T. trichiura* DNA, making ancient DNA recovery very difficult or even impossible.

A few other studies report the recovery of ancient parasitic DNA from archaeological sites dated to the Iron Age. For instance, different intestinal helminths were detected in the bottom layer of a man-made pond in Hobby (Denmark). DNA shotgun sequencing resulted in read assignments for *Ascaris* spp., *T. trichiura*, *T. suis* (whipworm of pigs), *Taenia saginata* and *T. hydatigena* (tapeworm of dogs and cats). The results indicate that the pond had been used for human excrements, animal dung and kitchen waste (Tams et al., 2018). Ancient DNA of *A. lumbricoides* and *T. trichiura* were also detected in a latrine/cesspit sample from the archaeological site Qal'at al-Bahrain (Kingdom of Bahrain) which is dated to 500–400 BC. In that study, the parasite eggs were concentrated prior to DNA shotgun sequencing (Søe et al., 2018). The two nematodes, *A. lumbricoides* and *T. trichiura*, are the most common parasites found in archaeological sites. Even the first ancient DNA studies on intestinal helminths reported the successful amplification and sequencing of two fragments from two different genes of 600-year-old *Ascaris* eggs from 'Places d'Armes' in Namur (Belgium). In that study, fragments of the 18S rRNA gene and cytochrome B gene were targeted (Loreille et al., 2001). Most of the ancient DNA reports come from palaeoparasitological findings dating from the last 2000 years (Flammer et al., 2020; Søe et al., 2018; Wood,

2018). The main reason for this is that organic material is only preserved under certain conditions for several thousands of years and therefore, in most cases, no ancient remains of parasites can be found. The most interesting material for ancient DNA studies are probably mummified humans or animals, as the results can be directly linked to an individual. Furthermore, they offer the highest potential of harbouring all kinds of parasites as hair, skin and blood are often well-preserved (Côté & Le Bailly, 2017; Pääbo, 1985b; Seo et al., 2014). In fact, the first palaeoparasitological detection was made in two Egyptian mummies dated to 1250 to 1100 BC at the beginning of the 20th century, in which calcified *Schistosoma haematobium* eggs were found (Ruffer, 1910). Interestingly, in 1734, a great discovery was made of a well-preserved corpse of a prehistoric miner in Hallstatt, who died in a catastrophe around 350 BC that devastated the entire Salzberg Valley and brought mining to a halt at this site. But the body of the “man in the salt” was buried in the cemetery in Hallstatt and is therefore no longer available for research (Reschreiter & Kowarik, 2015). However, there is still the chance of finding preserved bodies in the salt mine. This would allow for extensive palaeoparasitological studies and novel approaches for ancient DNA analyses.

5.3 High prevalence of intestinal helminths in the population of Hallstatt during the Bronze Age and Iron Age

Palaeoparasitology is an increasingly important research field which allows the comparison between parasitic infections of the past and the present, and it can help to predict the impact of parasites in the future (Makki et al., 2017). In this study, 91% of the palaeofaecal samples (32 of 35) were positive for intestinal helminth eggs. *T. trichiura* eggs were most abundant and detected in 31 palaeofaecal samples. Additionally, 14 samples also contained eggs of *A. lumbricoides* and one sample contained only *A. lumbricoides* eggs. Importantly, eggs of both species, thus, were detected in coprolites dated to the Iron Age and the Bronze Age. Based on the current archaeological dating of the sampling sites (Grabner et al., 2021), it can be stated with certainty that the human whipworm and the human roundworm were present among the

miners in Hallstatt, at least in the period between 1158 and 662 BC. The exact extent of the infestations of the miners or the prehistoric population of Hallstatt is difficult to assess. The results, however, indicate a high burden of *T. trichiura* and *A. lumbricoides* and lead to the assumption that at least these two intestinal helminths were very common in this region during both, the Iron Age and the Bronze Age. Both nematodes detected in this study are soil-transmitted helminths (STH), which means that the transmission is faecal-oral between humans through contaminated soil without an intermediate host. *A. lumbricoides* worms live in the small intestine of infested humans, and thousands of eggs are produced daily by the adult female worms, which are then passed in the stool. Depending on the environmental conditions, the eggs mature in the soil into infective eggs containing a larva. After infective eggs are swallowed, the larvae hatch and migrate through the body, reaching the lungs and being coughed up and swallowed again, before the adult worms develop and establish in the small intestine. The life cycle of *T. trichiura* is similar but unlike *A. lumbricoides*, the whipworm larvae do not migrate through organs. The larvae hatch in the small intestine, mature and establish themselves in the caecum and ascending colon (Holland, 2010; Jourdan et al., 2017). In both cases, the eggs are deposited on soil if an infested person defecates on soil, or if human faeces are used as fertilizers. Depending on the external conditions, it takes around three to six weeks for the eggs to become infective. The eggs are taken up orally via hands (contaminated with soil) or contaminated water or food, including vegetables that have not been washed carefully or cooked (CDC, 2021c). The presence of roundworms and whipworms is always an indication for poor sanitation and hygiene (Flammer & Smith, 2020). In the waste layers of the mines, evidence has been found that, especially during the Iron Age, working and living went hand in hand and there were no divided activity zones in the mines. This means that besides the extraction of salt, basic needs of the miners were also fulfilled everywhere inside the mines. This includes cooking, eating and defecation (Reschreiter & Kowarik, 2019). Only at the “Kernverwässerungswerk” site one bigger pile of palaeofaeces was found so far (Stöllner, 2003), which could indicate that this spot was used by a miner more than once or by several people. All other palaeofaecal samples were found distributed in the salt mines. Generally, the

development of *Ascaris* eggs requires oxygen and is temperature dependent. For example, while development at 26°C takes only around two weeks, at 16°C it takes around three months (Kim et al., 2012). In the mines, the normal temperature is around 8°C (Reschreiter & Kowarik, 2019), however the salt mines were rather crowded, and the miners were heating with fires and cooking (like shown in the illustration in Figure 5) and these circumstances for sure led to higher temperatures which facilitates the development of the intestinal helminths. Moreover, it can be assumed that the hygiene standard outside the mines was similar and these living conditions surely have promoted the transmission of these intestinal worms, since they do not require an intermediate host. Today, soil-transmitted helminths are considered as neglected tropical pathogens, as a large part of the world's population is still infested with those worms, although they could be controlled or even eliminated rather easily. In countries with warm and moist climates, and parts of the world where hygiene and sanitation are poor, they cause tremendous suffering to many people. According to the CDC, approximately 807–1,121 million people are infested with *Ascaris* and approximately 604–795 million with the whipworm (CDC, 2021c). Light infestations usually cause no symptoms but people with heavy infestations can have a range of health problems. These include abdominal pain, diarrhoea and in the worst case rectal prolapse. In children, who often have heavier infestations, intestinal helminths can cause cognitive and physical growth retardation (CDC, 2021c; Drake & Bundy, 2001). Since the worms can cause severe symptoms and because the worms are excreted when they die, it can be assumed that the miners knew about them. There is also evidence that the miners tried to relieve their stomach pains. Several preserved leaves of butterbur (*Petasites officinalis* and *Petasites paradoxus*) were found in the mines, which are used in folk medicine to treat stomach aches (Kromer, 1985). Nevertheless, the prehistoric inhabitants of Hallstatt were certainly not aware how they acquired the helminths.

Interestingly, remains of other intestinal parasites were not found in the palaeofaecal samples from Hallstatt, although a larger diversity of helminths has been detected in coprolites of the same time period in other archaeological sites. For example, in France, *Fasciola* sp., *Taenia* spp. and *Diphyllbothrium*

sp. were found in coprolites from the lakeside of Chalain (Dommelier et al., 1998). *Dicrocoelium dendriticum*, *Capillaria* sp. and *Taenia* sp. were identified in coprolites from Shar-e Sukhteh, which is an archaeological site dated back to 3,200–1,800 BC (Makki et al., 2017). At the Molawi street archaeological site (5000 BC) south of Tehran, *Enterobius vermicularis* was detected in soil samples taken around a female skeleton (Paknazhad et al., 2016). Various parasitic helminths of humans and animals were also found in soil samples of the Chehrabad salt mine (dating back to 500 BC) in north-west Iran, including whipworms, roundworms, tapeworms, lancet liver flukes and horse and human pinworms (Nezamabadi et al., 2013). Furthermore, *Trichosomoides crassicauda*, *Trichuris* sp. and *Syphacia* sp. eggs were detected in rodent coprolites (Mowlavi et al., 2014), *Macracanthorhynchus hirudinaceus* eggs in canine palaeofaeces (Mowlavi et al., 2015), and *Taenia* sp. in a mummified miner (Nezamabadi et al., 2013) at this salt mine. Even in Austria, eggs of *Fasciola hepatica*, *Dicrocoelium dendriticum* and *Taenia* sp. were found together with *Ascaris lumbricoides* and *Trichuris trichiura* in coprolites from the ancient salt mine in Dürrnberg near Hallein, which is around 60 km from Hallstatt (Aspöck et al., 1973, 1998; Stöllner, 2003). In 1991, in the Ötztal Alps on the border between Austria and Italy, the frozen mummy of a man (known as “Ötzi”) was found, who lived between 3,400–3,100 BC. During palaeoparasitological investigations, of several samples of the sacral-gluteal region, eggs of *Trichuris trichiura* were detected which indicates that the prehistoric man was infested with human whipworms (Aspöck et al., 1995).

Another, special focus of this study was on the detection of parasites that require a specific, intermediate host for transmission, as the presence of these parasites would reveal more information on the dietary habits of the ancient population and the history of animal husbandry. Especially, *Taenia* spp. and *Diphyllobothrium* sp. were expected to be found in the palaeofaecal samples from the Hallstatt salt mine due to several reasons. On one hand, different structures resembling eggs of *Taenia* spp. and *Dibothriocephalus latus* (= *Diphyllobothrium latum*) were found in a previous study with coprolites from the Hallstatt salt mine (Rodler, 2019), but could not be reliably identified at that time. On the other hand, thousands of animal bones were found in the Salzberg Valley. During the Bronze Age, salt production was not the only

economic branch in Hallstatt. Meat processing and conservation was probably just as important, as hundreds of pig carcasses were salted in log basins every year (Reschreiter & Kowarik, 2019). It is also well known that, at least during the Iron Age, large quantities of meat from different animals (pork, goat, beef and mutton) were consumed (Pucher, 2009). Eggs of *Taenia* spp. dated back to the Iron Age have also been found in Austria before during palaeoparasitological investigations of several coprolites from the salt mine in Dürrnberg (Aspöck et al., 1998). However, in this study none of the intestinal parasites that are associated with the consumption of meat or fish were found. Both, *Taenia* spp. and *Dibothriocephalus latus* are transmitted via infected meat (pork/beef) or fish consumed either raw or undercooked. The absence of these parasites in the Hallstatt palaeofaecal samples does not necessarily mean that these parasites were not present. It could also indicate that the meat and fish (if fish were also consumed) were well-cooked and thus the parasite larvae were killed. Earlier studies on palaeofaeces and the investigation of various cooking utensils and tableware have revealed the dietary habits of the ancient miners during the Iron Age (Reschreiter & Kowarik, 2019). It was even possible to show, through many analyses and experiments, that the typical stew, which was consumed during the Iron Age, has cooked for many hours. Consequently, the meat in the stew was properly cooked. As it has been proven that heating the meat to an internal temperature of 70°C kills these parasites (Allen, 1947), potential cysticerci in the Hallstatt meat would have no longer been infectious after cooking.

The two intestinal worm species detected in this study are, however, not the only parasites found inside the Hallstatt salt mine. In an early study, the resistant eggshells of the body louse were detected in the clothing of the miners (Hundt, 1960). This is interesting from a medical point of view, as the body louse can transmit various pathogens including the causative agents of epidemic typhus (Kern et al., 2014e; Schöffel et al., 2019). Such findings are possible due to the very special conditions in the salt mine. The exceptional preservation of the parasite eggs found was already emphasised in 1971, in the Austrian palaeoparasitological pioneer study conducted by Aspöck and colleagues, when eggs of *T. trichiura* and *A. lumbricoides* were found in several coprolite samples from the salt mines in Hallstatt and Hallein (Aspöck

et al., 1973). All organic material including the excrements of the miners, left behind in the mines, have been preserved in a near perfect condition (Tintner et al., 2016). The ancient salt mines represent the only sites, besides burial sites, where materials of all types are perfectly conserved. Worldwide, there are only three prehistoric salt mines with such exceptional preservation conditions in salt – two are in Austria, in Hallstatt and Hallein/Dürrenberg, and the third is in north-west Iran in Chehrabad (Reschreiter & Kowarik, 2019).

The Hallstatt salt mine is certainly one of the rare places in the world where remains of human parasites have been preserved in almost perfect condition. As archaeological research continues, several more palaeofaecal remains will be discovered and perhaps even other interesting remains. This will enable new collaborations between archaeologists and parasitologists which will bring even deeper insights into the status of parasitic infections in Hallstatt during the Bronze Age and the Iron Age. One thing is certain: there is still much information hidden in the salt mines of Hallstatt, just waiting to be discovered.

6 CONCLUSION

In conclusion, this study includes the first successfully amplified and sequenced ancient DNA sequences of *A. lumbricoides* dated to the Bronze Age. Additionally, several sequences of different genes of *A. lumbricoides* were obtained from coprolites dated back to the Iron Age, which represent the first molecular data of this soil-transmitted helminth of the Hallstatt period in Austria. Microscopically, *A. lumbricoides* and *T. trichiura* were detected in 91% of the palaeofaecal samples, indicating a high prevalence of these parasites in the prehistoric population. Interestingly, however, only a limited number of parasite species were found. Due to the exceptional preservation conditions in the salt mine, this study includes the first micrographs of intestinal helminth eggs from the Bronze Age in Austria. Altogether, this project shows that palaeoparasitological aDNA studies have the potential to enhance archaeological studies, and it underlines the importance of interdisciplinary research.

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8 APPENDIX

8.1 Abstract

Molecular analyses of prehistoric faecal samples for intestinal parasites

Palaeoparasitological studies analyse remains of parasites detected in archaeological sites and can provide valuable information on the emergence, distribution, and elimination of parasites during a particular time in the past. In the prehistoric salt mines of Hallstatt, located in the Austrian Alps, human coprolites are embedded in salt. The main aim of this interdisciplinary study, performed in collaboration with the Natural History Museum of Vienna, was to recover ancient DNA of intestinal parasites. Altogether, 35 coprolite samples from the Hallstatt salt mine, dating back to the Bronze Age (1,158–1,063 BC) and the Iron Age (750–662 BC), respectively, were analysed by microscopical and molecular methods. In 91% of the coprolite samples, eggs of soil-transmitted helminths (STH), namely of *Trichuris trichiura* and/or *Ascaris lumbricoides*, were detected by light microscopy. The eggs of *A. lumbricoides* were exceptionally well preserved. For further analysis, a method for the extraction of ancient parasitic DNA from palaeofaecal samples preserved in a salty environment was established and species-specific primers targeting different genes were designed. Altogether, sequence data of *A. lumbricoides* from 11 coprolites were obtained, including three different genes, the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*), the mitochondrial cytochrome B gene (*cytB*) and the mitochondrial NADH dehydrogenase subunit 1 gene (*NADH1*). The most important results are two ancient DNA sequences of *A. lumbricoides* from a coprolite dating back to the Bronze Age, which represent the world's first molecular data of this species from this period.

8.2 Zusammenfassung

Molekulare Analysen von prähistorischen Stuhlproben auf Darmparasiten

Paläoparasitologische Studien untersuchen Überreste von Parasiten, welche in archäologischen Fundstätten gefunden werden, und können wertvolle Informationen über das Auftreten, die Verbreitung und das Verschwinden von Parasiten während einer bestimmten Zeit in der Vergangenheit liefern. In dem prähistorischen Salzbergwerk von Hallstatt in den österreichischen Alpen, sind menschliche Koprolithen in das Salz eingebettet. Das Hauptziel dieser interdisziplinären Studie, die in Zusammenarbeit mit dem Naturhistorischen Museum Wien durchgeführt wurde, war die Gewinnung vorzeitlicher DNA von intestinalen Parasiten. Insgesamt wurden 35 Koprolithen aus dem Salzbergwerk in Hallstatt untersucht, von denen 4 in die Bronzezeit (1.158–1.063 v. Chr.) und 31 in die Eisenzeit (750–662 v. Chr.) datiert wurden. In 91% der Koprolithen konnten Eier von sogenannten „soil-transmitted helminths“ (STH) nachgewiesen werden, nämlich von *Trichuris trichiura* und/ oder *Ascaris lumbricoides*. Die Eier von *A. lumbricoides* waren außergewöhnlich gut erhalten. Zur weiteren Analyse wurde eine Methode zur Extraktion von vorzeitlicher parasitärer DNA aus in Salz konservierten Paläofäkalproben etabliert und spezies-spezifische Primer zur Amplifizierung verschiedene Gene entworfen. Insgesamt konnten aus 11 Koprolithen Sequenzdaten von *A. lumbricoides*, und zwar des mitochondrialen Cytochrom c Oxidase-Untereinheit 1-Gens (*cox1*), des mitochondrialen Cytochrom B-Gens (*cytB*) und des mitochondrialen NADH Dehydrogenase-Untereinheit 1-Gens (*NADH1*) gewonnen werden. Die wichtigsten Ergebnisse sind zwei DNA-Sequenzen von *A. lumbricoides* aus einem Koprolithen aus der Bronzezeit. Dies sind die weltweit ersten molekularen Daten von *A. lumbricoides* aus dieser Zeitperiode.