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# Burial condition is the most important factor for mtDNA PCR amplification success in Palaeolithic equid remains from the Alpine foreland

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**Abstract** Faunal remains from Palaeolithic sites are important genetic sources to study preglacial and postglacial populations and to investigate the effect of climate change and human impact. Post mortem decay, resulting in fragmented and chemically modified DNA, is a key obstacle in ancient DNA analyses. In the absence of reliable methods to determine the presence of endogenous DNA in sub-fossil samples, temporal and spatial surveys of DNA survival on a regional scale may help to estimate the potential of faunal remains from a given time period and region. We therefore investigated PCR amplification success, PCR performance and post mortem damage in c. 47,000 to c. 12,000-year-old horse remains from 14 Palaeolithic sites along the Swiss Jura Mountains in relation to depositional context, tissue type, storage time and age, potentially influencing DNA preservation. The targeted 75 base pair mitochondrial DNA fragment could be amplified solely from equid remains from caves and not from any of the open dry and (temporary) wetland sites. Whether teeth are better than bones cannot be ultimately decided; however, both storage time after excavation and age significantly affect PCR amplification and performance, albeit not in a linear way. This is best explained by the—inevitable—heterogeneity of the data set. The extent of post mortem damage is not related to any of the potential impact

factors. The results encourage comprehensive investigations of Palaeolithic cave sites, even from temperate regions.

**Keywords** Ancient DNA · DNA preservation · Horse · Cave · Switzerland

## Introduction

The analysis of ancient DNA from archaeological specimens provides the unique opportunity to study genetic diversity at different time intervals of the past. Because of this, it is coveted not only in archaeology but also in other disciplines such as evolutionary, population and conservation genetics. However, progressive post mortem degradation limits the access to genetic information from sub-fossil material. The mechanisms of DNA decay, resulting in small amounts of highly fragmented and chemically modified molecules, have been intensively studied (e.g. Pääbo 1989; Lindahl 1993; Höss 1995; Hofreiter et al. 2001; Mitchell et al. 2005; Deagle et al. 2006; Hansen et al. 2006; Briggs et al. 2007; Brotherton et al. 2007; Gilbert et al. 2007; Vives et al. 2008; Lamers et al. 2009; Heyn et al. 2010; Allentoft et al. 2012; Overballe-Petersen et al. 2012; Dabney et al. 2013). Depending on burial and exposure temperature, rapid or slow sedimentation, chemical properties of the soil, pH value, the presence or absence of oxygen, water, ionic radiation and microorganisms, organic material is destroyed sooner or later. Under the most favourable conditions, such as ice cores or permafrost soil, short fragments of DNA can survive presumably up to one million years (Geigl 2002; Willerslev et al. 2007; Dabney et al. 2013; Orlando et al. 2013; Meyer et al. 2014). Yet, DNA preservation can be different in similar environments (Allentoft et al. 2012; Olalde et al. 2014), even in seemingly optimal permafrost (Campos et al. 2010). However, those environments hardly represent typical burial

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conditions. Large amounts of archaeobiological specimens from cultural layers are preserved in temperate climates. The prediction of DNA survival is difficult, and yet, as all archaeobiological remains are potentially valuable genetic resources of the past, archaeologists and museum curators need to optimally select precious samples for invasive analyses. At the same time, aDNA studies are costly—not only in terms of damaging unique samples but also in terms of labour and money—and thus, it is important to estimate the feasibility of a genetic study in a given region. Several parameters have been proposed as proxies for DNA survival, for example histology (Guarino et al. 2000), biochemical preservation, in particular amino acid racemization (Poinar et al. 1996) and the so-called thermal history (Smith et al. 2001). No consistent correlation was detected between any of the proposed proxies and DNA preservation (Götherström et al. 2002; Haynes et al. 2002; Rollo et al. 2002; Gilbert et al. 2006; Collins et al. 2009; Schwarz et al. 2009; Hoke et al. 2011). However, it has been shown that DNA degrades somewhat predictably, although with large variance, over time (Allentoft et al. 2012). Moreover, it has been suggested that burial condition substantially influences DNA preservation (Molak and Ho 2011).

Next generation sequencing (NGS) techniques can process short fragments of DNA and are thus particularly suitable for aDNA research. Some of the disadvantages of aDNA like limited amount of highly fragmented template (mainly <80 base pairs (bp); Pääbo et al. 2004; Deagle et al. 2006; Allentoft et al. 2012; Sawyer et al. 2012) could be overcome with these technologies, but the high costs and substantial labour input prior to and after NGS make it even more desirable to select suitable specimens. Conventional PCR amplification followed by Sanger sequencing is not only used to initially screen samples but we also expect that it will remain an important approach for answering many archaeologically relevant questions, such as species determination, identification of individuals and the relations between them, lineage history and genetic diversity. Large public databases for reference exist (e.g. GenBank), and there are sophisticated statistical routines at hand to evaluate the results of genetic analyses, even for short sequences (e.g. Anderson et al. 2005; Excoffier and Lischer 2010; Drummond et al. 2012). However, publicly accessible databases on DNA survival in relation to environmental, geological or depositional conditions are still limited and require further data input (e.g. <http://thermal-age.eu/>). Thus, comprehensive regional studies (e.g. Gravlund et al. 2012) and chronological surveys of aDNA preservation represent tools for local archaeologists or museum curators to select promising samples.

In this paper, we investigate the preservation of mitochondrial DNA (mtDNA) and post mortem damage in 182 archaeological horse remains ranging in age from c. 47 to c. 12,000 years (ky) from different depositional contexts in the temperate, sub-oceanic/semi-continental climate of

Switzerland, typical for large areas in Europe. The 14 Palaeolithic sites are scattered along the Jura Mountains. They comprise all known locations with at least one equid remain from this time period. Detailed archaeological and depositional information is available for all specimens. We expected the degree of DNA preservation and post mortem damage-derived lesions to differ depending on burial context, tissue type, storage time after excavation, and sample age and assessed DNA preservation by polymerase chain reaction (PCR). The time frame investigated is interesting for genetic studies because it includes climatic events like the European last glacial maximum (LGM) ~23 to 19 ky BP (Hughes et al. 2013) and the subsequent warming which led to dramatic changes in the vegetation, as well as cultural developments like the arrival of anatomically modern humans in Europe and the spread of Magdalenian horse hunters, episodes that are likely to have shaped the structure of horse populations (Lorenzen et al. 2011; Orlando et al. 2013).

## Material and methods

### Archaeological samples

All samples were excavated in or close to the Jura Mountains, a limestone formation that folded up about 10 to 2 million years ago from Jurassic sediments. Today, the annual mean temperature in the area is ~10 °C with an average seasonal variation from ~0 to ~19 °C ([www.meteoschweiz.admin.ch](http://www.meteoschweiz.admin.ch)). A total of 182 horse (*Equus* sp.) teeth and bones were taken from three types of sites: (i) Caves (with a maximal depth of 15 m) and rock shelters (abris) are combined here as they have likely similar preservation conditions. Note that cultural layers—the remains of human activities—are often found in the entrance area of caves (Hahn 1983). (ii) Open-air camps with temporary waterlogged preservation due to lake transgression, and (iii) open-air finds from dry conditions not associated with cultural layers (Fig. 1, Table 1, online resource 1).

Excavations were undertaken from the 1870s to the early 1990s according to contemporary standards, which likely included washing directly at the site. The majority of samples were not treated for storage; exceptions are the teeth from Monruz which were prepared with dimethylketone-based hardener during archaeozoological analysis (W. Müller, pers. comm.). All material was stored in museums and archaeological collections since then. The age of the finds ranges between c. 47 and c. 12 ky. Twenty-seven samples with DNA preservation were <sup>14</sup>C dated using accelerator mass spectrometry (AMS) at the Ion Beam Physics laboratory of ETH Zurich, Switzerland, and calibrated with CalPal online (Danzeglocke et al. 2012). Additionally, conventional and AMS <sup>14</sup>C dates were assembled from the literature, including



**Fig. 1** Location of investigated sites along the Jura Mountain chain with equid remains in Switzerland. *Black dots* indicate sites with amplifiable mtDNA. The sites are numbered according to Table 1 and online resource 1

sites without DNA preservation. For Brügglihöhle, the age was determined by typology (Bandi et al. 1952/53) and for both Münchenstein-Steinbruch and Allschwil-Ziegelei, where cultural layers are absent, according to faunal or geological indicators (LeTensorer and Niffeler 1993, see online resource 1). Multiple typing of the same individual was avoided by choosing the same skeletal elements and/or samples from different layers or in the case of Kohlerhöhle excluded in retrospect by archaeozoological individualisation of teeth.

#### Sample preparation, DNA extraction and PCR amplification

The outer surface of teeth and bones was removed with sandpaper, and cubes of  $\sim 1 \text{ cm}^3$  cut out with a Dremel® tool. The cubes were ground with a mixer mill (Retsch MM2, Schieritz & Hauenstein, Allschwil, Switzerland). DNA extraction followed the User Developed Protocol: “Purification of total DNA from compact animal bone using the DNeasy® Blood & Tissue Kit” (Qiagen, Basel, Switzerland) for less than 100 mg. At least one mock control was performed per 20 samples. All extracts were ultra-purified with water (molecular biology grade, Eppendorf, Allschwil, Switzerland) using

30 kD filter units (Amicon/Millipore, Zug, Switzerland) to remove potential inhibitors. The final eluate was 200  $\mu\text{l}$ .

One 75 bp target of the mt d-loop covering nucleotide positions 15,696–15,730 (except primers, Xu and Anarson 1994) was PCR amplified in 25  $\mu\text{l}$  volumes with primers Ec5f (5’ACCCATCCAAGTCAAATCA) and Eac1r (5’GGCTTGGTGATTAAGCTCGT) containing 1.5 U AmpliTaq Gold, 1 $\times$  GeneAmp 10 $\times$  PCR Gold Buffer (150 mM Tris-HCl, 500 mM KCl, pH 8.0) and 2 mM MgCl<sub>2</sub> (all Applied Biosystems, Hombrechtikon, Switzerland); 0.4 mM dNTP Mix (Promega, Dübendorf, Switzerland); 0.2  $\mu\text{M}$  of each primer; 20  $\mu\text{g}/\mu\text{l}$  bovine serum albumin (BSA, Roche, Basel, Switzerland), and up to 5  $\mu\text{l}$  template DNA on a Mastercycler ProS (Eppendorf, Allschwil, Switzerland). The cycling conditions were 12 min initial denaturation, followed by 50 cycles of denaturation at 95 °C for 40 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 60 s at 72 °C. Non-template controls were performed alongside all amplifications. To overcome any potential PCR inhibition, DNA extracts were diluted 1:10 (Kemp et al. 2014). Firstly, 0.3 and 3  $\mu\text{l}$  template DNA for each sample and extraction were PCR targeted. If not successful, the amplification was repeated with 0.5 and 5  $\mu\text{l}$

**Table 1** Overview of archaeological sites (numbers correspond to Fig. 1), age bins, type of sites, years of excavation, storage time bins, tissue types, mtDNA d-loop amplification, and PCR performance of positive samples expressed in percentage of total PCR

Site	Date range of site (ky BP)	Age bin (ky BP)	Type of site	Year(s) of excavation	Storage time bin (y)	Samples with mt d-loop amplification (total samples)				PCR performance: Positive PCR (total PCR)	
						Teeth		Bones		n	%
						n	%	n	%		
1 Riehen-Ausserberg	47–45	47–37	Open, dry	1967	20–50	–	–	–(8)	0	–	–
2 Münchenstein-Steinbruch	Pre-LGM	47–37	Open, dry	1919 <i>et seq.</i>	70–90	–(4)	0	–	–	–	–
3 Allschwil-Ziegelei	Pre-LGM	47–37	Open, dry	1923	70–90	–(3)	0	–	–	–	–
4 Schalberghöhle	41–37	47–37	Cave	1926–1927	70–90	3 (6) <sup>a</sup>	67	–	–	10 (31)	32
	12.5	17–12				1	–	–	3 (10)	30	
5 Kohlerhöhle	24–19	23	Cave	1934–38	70–90	11 (11)	100	–	–	36 (44)	82
	15–13	17–12				12 (15)	80	–	–	40 (90)	44
6 Kesslerloch	18–13	17–12	Cave	1874, 1883, 1898–1999, 1902–1903	110–140	29 (48)	60	14 (19)	74	75 (105)/28 (58)	71/48
7 Hauterive-Champréveyres	18–13	17–12	Open, temporally wetland	1983–1986	20–50	–(6)	0	–	–	–	–
8 Käsloch	17–15	17–12	Cave	1905	110–140	3 (4)	75	–	–	6 (22)	27
9 Monruz	17–15	17–12	Open, temporally wetland	1989–1992	20–50	–(10)	0	–(19)	0	–	–
10 Schweizersbild	17–13.5	17–12	Abri	1891–1893	110–140	6 (11)	55	–	–	19 (25)	76
11 Abri Neumühle	14.5–12	17–12	Abri	1965	20–50	1 (1)	100	–	–	3 (4)	75
12 Birseck-Ermitage	14	17–12	Cave	1910, 1914–1922	110–140	–(3)	0	–	–	–	–
13 Rislisberghöhle	16–12.5	17–12	Cave	1971, 1973	20–50	4 (13)	31	–	–	9 (29)	31
14 Brügglihöhle	Magdalenian?	17–12	Cave	1940, 1943, 1951–1952	70–90	–	–	–(1)	0	–	–
Totals						70 (135)	52	14 (47)	30	229 (418)	55

For detailed locations, dates and references, see online resource 1

<sup>a</sup>Two samples without preserved mtDNA have not been <sup>14</sup>C dated

template DNA; in case of only negative results, the sample was deemed failed. Further targets were amplified for positive specimens (data not shown). All PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen, Zug, Switzerland) following the manufacturer's protocol, except that the reaction volume was halved. Generally, two clones of each PCR product were Sanger sequenced by Microsynth (Balgach, Switzerland), but the number varied from one to eight.

#### Data analysis

The potential effects of study sites (cave/abri, open dry and wetland sites), tissue type (tooth or bone), storage time, and sample age on PCR amplification and performance and the amount of post mortem damage were analysed with linear models using R (R Development Core Team 2014). Due to a clustering of excavations in certain years, storage time was

assigned to three categories: 20–50 years ( $n=57$ ), 70–90 years ( $n=40$ ), and 110–140 years ( $n=85$ ). Accordingly, age bins were defined: 17–12 ky ( $n=151$ ), 23 ky ( $n=11$ ), and 47–37 ky ( $n=20$ ). Data were described with bar and boxplot charts. First, general amplification success, i.e. whether mtDNA was amplifiable from a sample or not, was evaluated with the aforementioned factors and with binomial distributed errors using a generalised linear model (GLM) and a  $\chi^2$  test. From then on, only positive samples were considered. Second, PCR performance per individual sample was recorded by concatenating the number of positive and negative PCR attempts and evaluated as mentioned but with quasibinomial distributed errors. Third, for evaluation of miscoding lesions, sequences were edited and aligned by eye with BioEdit (Hall 1999), and base modifications that could not be reproduced were considered inconsistent. We determined the number of miscoding lesions per sample and position as follows: identical clones from the same amplification were counted as one

sequence, and in case further clones from the same amplification differed, they were counted separately each. Because the estimation of the *true* rate of damage is virtually impossible with a conventional PCR approach, C→T substitutions were used as a *proxy* for DNA damage (Brotherton et al. 2007; Vives et al. 2008). For standardisation, the counted misincorporated thymines were divided by the total number of cytosines for each specimen. To examine the influence of the mentioned factors on the amount of damage in individual specimens, we applied the GLM model with inverse Gaussian distributed errors.

### Precautions and authenticity

Established standards in aDNA research at the Integrative Prehistory and Archaeological Science (IPAS) were adhered to (e.g. Schlumbaum et al. 2010). In detail: All ancient DNA work (pre-PCR) was performed in dedicated, physically separated laboratories for sample preparation, DNA extraction and PCR setup in a different building than the post-PCR laboratory following a strict one-way policy. Experiments were performed freshly showered and wearing dedicated freshly washed clothes. Gloves and sleeves were changed regularly. Bones and teeth were cut in an acrylic glass box equipped with an UV lamp and a vacuum cleaner to remove bone dust. After each working step, surfaces and tools were cleaned with soap and commercial bleach (Javel-Wasser, Migros, Zurich, Switzerland) and UV irradiated for at least 30 min. Diamond cutting disks were cleaned with soap and ethanol followed by 30 min UV irradiation from each side. Mixer mill beakers were cleaned with soap, quartz sand and 30 min bleach incubation. PCR was set up in a laminar flow cabinet equipped with an UV lamp. Plastic ware was UV irradiated prior to use. We did not perform sample preparation, extraction and PCR on the same day. No modern horse DNA was analysed in the laboratories, and none of the coworkers had contact with living horses. At the same time, archaeological samples of other species were processed in the aDNA laboratories, but cross-contamination was never documented. PCR products in the negative controls were always either microorganisms or unidentifiable according to GenBank Blast search. Each target was validated with at least two independent extractions and three PCR products; all products were cloned and Sanger sequenced.

## Results

### PCR amplification success

We investigated equid remains from 14 Palaeolithic sites comprising caves, rock shelters (abri deposits) and open dry

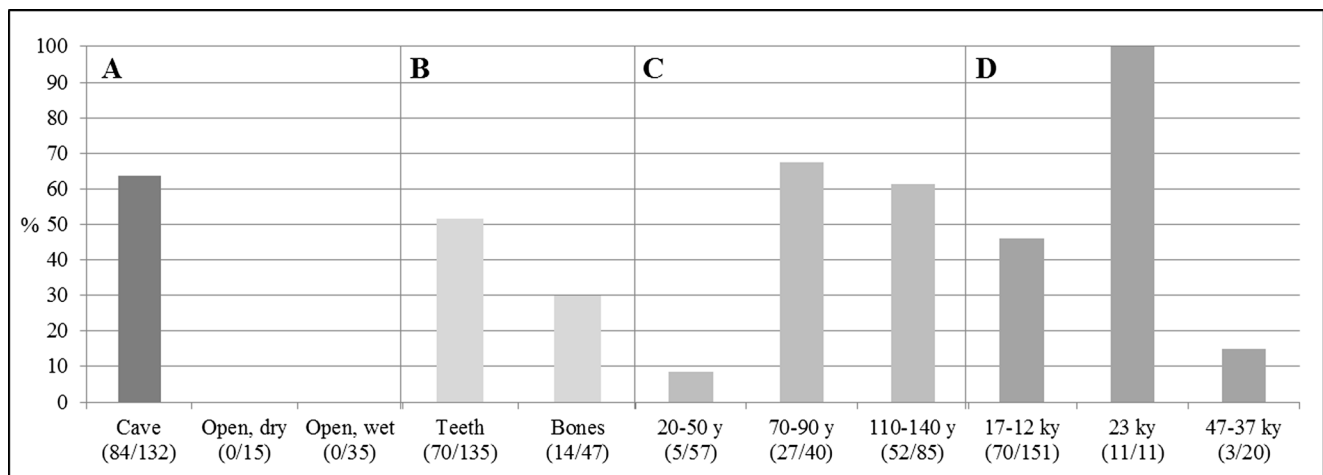
and wetland sites in Switzerland (Fig. 1, Table 1, online resource 1). Eighty four out of 182 remains (46 %) from seven sites have yielded equine mtDNA. DNA amplification of the 75 bp target was strongly related to depositional condition; all positive samples stem from cave and abri deposits (Fig. 2a, Table 2). This results in a success rate for cave/abri sites of 64 % (84/132 remains). Amplification success varied between the cave/abri sites; 23/26 samples (88 %) from Kohlerhöhle but only 4/13 samples (31 %) from Rislisberghöhle and none from both Brügglihöhle ( $n=3$ ) and Birseck-Ermitage ( $n=1$ ) had amplifiable mtDNA preserved (online resource 2). No mtDNA was obtained from any of the tested samples from five Palaeolithic open dry and temporally wetland sites. A higher percentage of teeth than bones had amplifiable mtDNA preserved; however, the difference is not significant (Fig. 2b). Both storage time after excavation and age had a significant relation to DNA survival, albeit not in a linear way (Fig. 2c, d). For example, the sites excavated most recently include all temporally wetland sites, considerably lowering the success rate within the 20–50 years bin. Moreover, the temporally waterlogged samples in the 17–12 ky age bin and the dry open sites in the 47–37 ky age bin obviously complicate the interpretation of the results.

### PCR performance

For a detailed analysis of those samples that yielded mtDNA, we tested whether the factors tissue type, storage time and sample age influenced PCR performance. We did not detect differences in the amplification performance between teeth and bones (Fig. 3a, Table 3). Storage time had an inverse relationship to PCR performance: Specimens that had been unearthed before or around 1900 performed better than those excavated later (Fig. 3b). Sample age has a significant influence on PCR performance (Fig. 3c, see also online resource 2B). As expected, the oldest specimens amplified generally worse, but note that the 47–37 ky bin is only represented by three positive samples from Schalberghöhle. The performance differences between the individual cave sites were more pronounced than the differences between the age bins.

### Miscoding lesions

We evaluated a total of 7,980 nucleotides from 228 sequences (see “Methods”) and identified 62 C→T substitutions (Table 4) resulting in a deamination rate of 2 % for cytosines. In agreement with a previous study (Vives et al. 2008), we found only eight of G→A substitutions (Table 4), suggesting preferential amplification of the light strand. Based solely on C→T changes, none of the factors influenced the pattern of misincorporations. We observed large variation among individuals. More than half of the samples (47/84) exhibited no damage at all; 36 specimens were deaminated between 1 and



**Fig. 2** Percentage of Palaeolithic equid samples with 75 bp target of amplifiable mtDNA in Switzerland. **a** In relation to original site (cave/abri, open and dry, open and wet); **b** in relation to tissue type (tooth,

bone); **c** in relation to storage time after excavation (20–50 years, 70–90 years, 110–140 years); and **d** in relation to age (17–12 ky, 23 ky, 47–37 ky)

7.5 %, and two had damage rates as high as 13 and 15 %, respectively. The three oldest samples show on average more deaminations, but the difference is not significant (Fig. 4a–c, Table 5).

#### Intra-site comparison

We expected differences in mtDNA preservation, individual PCR amplification success and post mortem damage patterns to be linked with finds buried in different positions within the cave, with tissue type and age. The caves Kesslerloch and Kohlerhöhle allow investigating any potential effect of these parameters in the absence of among-site variation (online resource 2). Kesslerloch is the only cave where both equid bones and teeth were unearthed and genetically analysed; all samples are in the same age bin. Kohlerhöhle specimens can be assigned to two age bins: 23 ky and 17–12 ky; these are all teeth. For both sites, samples were deposited in different parts of the cave. Fourteen samples from Kesslerloch were excavated near the entrance (area s) and 27 more than 6 m distance from the two openings (area m–n and c; see online resource 3; Merk 1876; Nüesch et al. 1904; Heierli 1907). DNA

preservation, PCR performance and damage rate were not different throughout the cave. Whereas mtDNA was preserved in a higher percentage of bones than teeth, in contrast to the overall observations in the entire data set, the performance of the 29 positive teeth was significantly better than of the 14 positive bones. Both tissues showed similar damage patterns (online resource 4).

The cave Kohlerhöhle is almost 15 m long with an additional 3 m abri area (online resource 5; Lüdén 1938). The 23 ky old specimens ( $n=11$ ) were all unearthed from the rear 6 m of the cave, some sticking within the maxilla (foramina apicale not protected); mtDNA was preserved in all teeth. In contrast, the 15 younger samples (15 to 13 ky old) were dispersed all over the cave and abri area, but albeit three of the teeth had no mtDNA preserved (one from the hall, one from the abri and one of unknown deposit), we also did not detect differences in preservation, PCR performance and deamination rate between interior and entrance areas. Comparison of the age bins confirmed the observation that the 23 ky specimens performed better than the younger samples in the same cave (online resource 6).

**Table 2** Effect of study sites (cave/abri, open and dry, open and wet); tissue type (tooth, bone); storage time after excavation (20–50 years, 70–90 years, 110–140 years); and sample age (17–12 ky, 23 ky, 47–37 ky) on mtDNA preservation (PCR success) of Palaeolithic equid remains from Switzerland

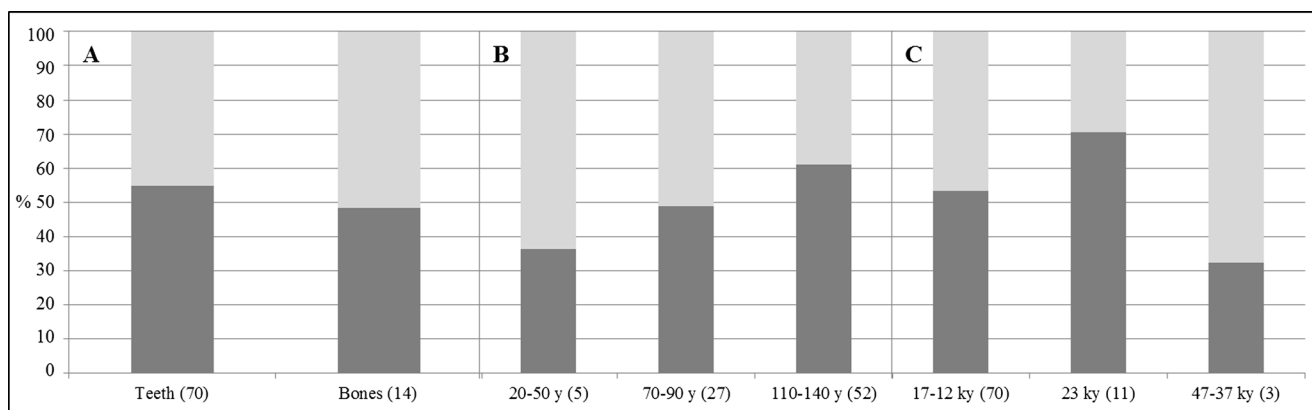
	Df	$\chi^2$	<i>p</i>
Site	2	79.414	<0.0001***
Tissue type	1	0.422	0.52
Storage time	2	10.213	0.006**
Sample age	2	6.056	0.05*

Significance codes: 0.001=\*\*\*, 0.01=\*\*, 0.05=\*

#### Discussion

Long-term DNA survival in archaeological contexts is complex and influenced by a number of parameters, mainly probably by temperature (Smith et al. 2003; Lamers et al. 2009; Allentoft et al. 2012; Sawyer et al. 2012). The present data set, though comprising all known Palaeolithic sites in Switzerland with more than one horse remain, is heterogeneous: All open dry sites predate the European LGM, waterlogged open sites are both spatially and temporally close to each other, and the amount of pre-Magdalenian specimens from caves is low.





**Fig. 3** Proportion of positive (dark grey) to negative (light grey) PCR products obtained from positive Palaeolithic equid samples from cave and abri sites in Switzerland (PCR performance). **a** Effect of tissue type

(tooth, bone); **b** effect of age (17–12 ky, 23 ky, 47–37 ky); and **c** effect of storage time after excavation (20–50 years, 70–90 years, 110–140 years)

Teeth were overrepresented in our study, mirroring the archaeological record concerning horses from the Aurignacian to the Magdalenian in the region Swabian/Swiss Jura (Niven 2003; Napierala 2008). Moreover, the often scarce documentation and ambiguous definition of depositional contexts in published data makes it difficult to compare our results. However, in archaeology, and in particular archaeogenetics, representativeness is hardly expected. Despite the heterogeneity of the data set, our results support the finding that within the time frame discussed here, survival of mtDNA is mainly determined by in situ burial conditions. We demonstrate for the first time that Palaeolithic cave depositions are a much better source of ancient DNA than open dry and wetland sites from the same time period, even when morphological and in some cases also collagen preservation is good. The majority of caves and abris provided preservation conditions good enough to allow PCR amplification of mitochondrial DNA in more than 50 % of specimens. One reason for the superior DNA preservation may be the fact that remains at the rock shelters as well as at the cave entrance areas were rapidly sedimented with chalky soil protecting against hydrolytic and oxidative damage. Inside the caves, cultural layers were also covered by sinter (Bally 1908; Sedlmeier 1993; Napierala 2008), a calcareous deposit that develops when water dilutes calcium carbonate from limestone. These speleothems can form thick layers and seem to protect organic residues largely from

microbes. In general, DNA preservation in the caves investigated was comparable to studies on large as well as small mammals from other European cave sites (e.g. Edwards et al. 2010; Stiller et al. 2010; Münzel et al. 2011; Pruvost et al. 2011; Brace et al. 2012). Two caves did not preserve equid mtDNA. This result might be explained by the destruction of cultural layers at Birseck-Ermitage to make the cave publicly accessible in the nineteenth century (Sarasin et al. 1918). Similarly, the roof of Brügglihöhle had collapsed, possibly amid the 1356 earthquake (Lambert et al. 2005), exposing the archaeological layer (Bandi et al. 1952/53); these factors probably caused the general poor preservation status of the faunal remains.

The open dry sites are comparatively old based on <sup>14</sup>C dated samples from Riehen Ausserberg (>45 ky BP) and additional geological and faunal indicators (LeTensorer and Niffeler 1993), but equine mtDNA of similar age has been obtained from a cave in the Swabian Jura (Germany, Weinstock et al. 2005). Thus, it seems plausible that the dynamics of the soil itself (root penetration, pedogenesis, eluviation), soil-dwelling microorganisms as well as seasonal temperature and humidity fluctuation have promoted DNA decay in open sites. The Magdalenian remains at Champréveyres and Monruz were temporarily waterlogged due to transgression of Lake Neuchâtel (Coope and Elias 2000) which might explain the failed amplifications because

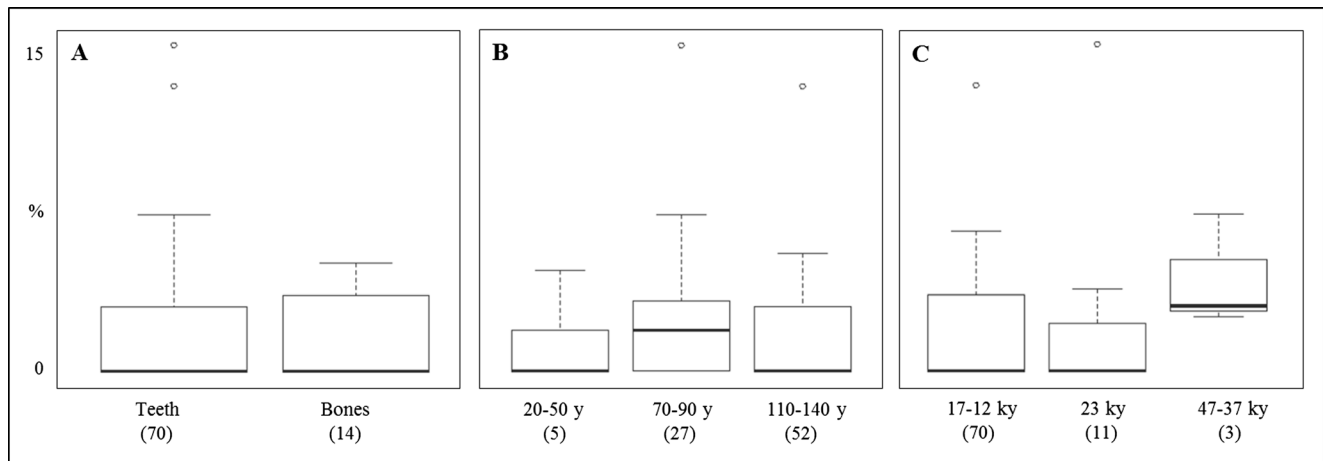
**Table 3** Effect of tissue type (tooth, bone), storage time after excavation (20–50 years, 70–90 years, 110–140 years); and sample age (17–12 ky, 23 ky, 47–37 ky) on PCR performance of Palaeolithic equid samples from cave/abri sites in Switzerland

	Df	$\chi^2$	<i>p</i>
Tissue type	1	0.847	0.44
Storage	2	14.651	0.006**
Sample age	2	14.778	0.005**

Significance codes: 0.001=\*\*\*, 0.01=\*\*, 0.05=\*

**Table 4** Number (n) of adenines (A), cytosines (C), guanines (G), and thymines (T) in all sequences and miscoding lesions (transitions) observed in a 75 bp mtDNA d-loop fragment from Palaeolithic equid samples from cave/abri sites in Switzerland

	n	Transitions
A	2,273	4
C	2,969	62
G	691	8
T	2,047	3



**Fig. 4** mtDNA damage as represented by percentage of C→T substitutions obtained from positive Palaeolithic equid samples from cave and abri sites in Switzerland. **a** Effect of tissue type (tooth, bone); **b** effect of

storage time after excavation (20–50 years, 70–90 years, 110–140 years); and **c** effect of age (17–12 ky, 23 ky, 47–37 ky)

the alternation between wet and dry environment is not beneficial for preservation in general (Reiche et al. 2003; Schlumbaum and Edwards 2013). In this context particularly, PCR inhibition can be a serious problem for the amplification of ancient DNA (Kemp et al. 2014). We took a range of measures to overcome potential inhibitors (ultra-purification and dilution of extracts, adding BSA to the PCR), but we cannot rule out that it affected our overall results.

It is commonly assumed that teeth are preferable for ancient DNA analyses. Their composition makes them less prone to contamination, protects DNA against deamination and leads to better preservation (Lindahl 1993; Gilbert et al. 2005; Adler et al. 2011; Campos et al. 2012), notably if articulated to the jaw bones (Higgins and Austin 2013). In the complete data set, we did not detect significant preservation and performance differences between teeth and bones; note that all teeth had to be considered loose because their roots were exposed. However, bones were only amplifiable from one cave site (Kesslerloch) and are generally underrepresented in the archaeological record. Thus, any differences in preservation between skeletal elements (bone vs teeth) may have been masked by this imbalance. Previous publications have been contradictory on this topic (e.g. Götherström et al. 2002;

Ricaud et al. 2005; Miloš et al. 2007; Pruvost et al. 2008; Higgins and Austin 2013).

The samples we analysed have been stored in archaeological collections up to 140 years. Pruvost et al. (2007) have pointed out that freshly excavated bones would be the first choice for ancient DNA analyses, but in fact, most archaeogenetic studies rely on museum specimens. Similar to Gravlund et al. (2012), we can confirm the hypothesis that many stored samples still have genetic information preserved although they might not have been treated with the precautions that are recommended today for aDNA studies (e.g. Bollongino et al. 2008; Pruvost et al. 2008; Matisoo-Smith and Horsburgh 2012).

The observation that specimens from the 23 ky age bin were better preserved than those from the 17–12 ky age bin even when compared within the same site (Kohlerhöhle) might reflect the influence of temperature at the time of deposition (Smith et al. 2003) which was considerably lower during the LGM (Buoncristiani and Campy 2011; Cupillard et al. 2014).

We did not observe a significant increase in the amount of C→T substitutions in older samples in contrast to the observation from NGS data (Sawyer et al. 2012), probably because PCR does not allow capturing the ends of DNA template strands, where the majority of deamination events occur (Briggs et al. 2007; Brotherton et al. 2007).

In contrast to genomic studies that investigate single samples and can afford specially tailored extraction and sequencing methods (e.g. Meyer et al. 2012; Orlando et al. 2013; Meyer et al. 2014; Olalde et al. 2014), many archaeological studies require routine access to aDNA from a larger number of samples (e.g. Edwards et al. 2007; Stiller et al. 2010; Münzel et al. 2011; Brace et al. 2012). On this note, we scored successful PCR amplification and PCR performance in positive samples as key variables rather than applying more sophisticated measures of DNA preservation such as quantitative PCR. In many archaeological studies aiming to apply

**Table 5** Effect of tissue type (tooth, bone), storage time after excavation (20–50 years, 70–90 years, 110–140 years); and sample age (17–12 ky, 23 ky, 47–37 ky) on mtDNA damage as represented by C→T substitutions in Palaeolithic equid samples from cave and abri sites in Switzerland

	Df	F	p
Tissue type	1	5.8527e-06	0.3808
Storage	2	7.6108e-06	0.6054
Sample age	2	1.3813e-05	0.4041

Significance codes: 0.001=\*\*\*, 0.01=\*\*, 0.05=\*

aDNA analyses, assessing the chances for a successful amplification using traditional PCR is the first important parameter to justify the damage inflicted on the sample as well as the amount of working time and money invested.

## Conclusion

By evaluating mtDNA d-loop amplification, performance and damage patterns in 182 Palaeolithic horse remains up to 47 ky in age from different depositional contexts in Switzerland, we found that the type of depositional environment is the most important factor affecting DNA preservation. Cave sites provide more favourable environments for DNA preservation than open sites. Moreover, both equid teeth and bones are suitable sources of mtDNA. In our data set, the factors storage time after excavation and sample age were of influence but not in a linear way. In the future, a systematic evaluation of younger samples with different depositional contexts and excavation biographies should reveal the eligibility for ancient DNA analyses of further site types, e.g. the famous Neolithic lacustrine sites, or Celtic and Roman food waste for genetic analyses. Finally, by saying that Palaeolithic faunal remains from caves offer better chances to DNA preservation in temperate areas, the fast development in technology may come up with solutions for samples from other burial conditions in the future, in particular for specimen of interest not preserved in caves.

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