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Ancient DNA sequence quality is independent of fish bone weight

Lane M. Atmore [a,*], Giada Ferrari [a,b], Lourdes Martínez-García [a], Inge van der Jagt [c], Rachel Blevis [d], José Granado [e], Simone Häberle [e], Katrien Dierickx [f], Liz M. Quinlan [f], Lembi Lõugas [g], Daniel Makowiecki [h], Anne Karin Hufthammer [i], James H. Barrett [j], Bastiaan Star [a,*]

[a] Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, Norway
[b] Royal Botanic Garden Edinburgh, Scotland, United Kingdom
[c] Cultural Heritage Agency of the Netherlands, Amersfoort, Netherlands
[d] McDonald Institute for Archaeological Research, Department of Archaeology, University of Cambridge, UK
[e] Integrative Prehistory and Archaeological Science, Department of Environmental Sciences, Basel University, Basel, Switzerland
[f] BioArCh, Department of Archaeology, University of York, UK
[g] Archaeological Research Collection, Tallinn University, Tallinn, Estonia
[h] Department of Environmental Archaeology and Human Paleoecology, Institute of Archaeology, Nicolaus Copernicus University, Torun, Poland
[i] Department of Natural History, The University Museum, University of Bergen, Norway
[j] Department of Archaeology and Cultural History, NTNU University Museum, Trondheim, Norway

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ABSTRACT

The field of ancient DNA (aDNA) typically uses between 50 and 200 mg of minimum input weight of bone material for the extraction of DNA from archaeological remains. While laboratory and analysis techniques have focused on improved efficiency of extracting useable sequence data from older and poorer quality remains, bone material input requirements have rarely been critically evaluated. Here, we present the aDNA analysis of 121 size-constrained Atlantic herring remains – weighing between <10 and 70 mg – that were individually sequenced to explore the capacity of successful aDNA retrieval from small archaeological remains. We statistically evaluate the relationship between bone weight and several response variables, including library success, endogenous DNA content, and library complexity, i.e., the number of unique molecules that are obtained. Remarkably, we find no relationship between bone weight and library success, levels of endogenous DNA, or library complexity. Our results imply that – at least in the case of fish bone – even minute bones can yield positive results and that the presumed minimum sample size required should be re-evaluated. Archaeological site, instead of bone size, is the primary driver of DNA sequence quality. Our work expands the number of specimens considered suitable for aDNA analyses, and therefore facilitates efforts to minimize the destructive impact of aDNA research and mediate some of the ethical concerns surrounding destructive analysis.

1. Introduction

Ancient DNA (aDNA) analysis is constrained by access to suitable archaeological and/or palaeontological material. Research is further hampered by the inherently destructive nature of DNA extraction, meaning archaeological remains are destroyed or damaged for successful recovery of sequences. Successful DNA recovery is often unpredictable (Ferrari et al., 2021; Tin and Economo, 2014; Keighley et al., 2021), therefore workflows often involve screening large numbers of specimens from which only a small subset ultimately yields useable DNA for analysis (e.g. Star et al., 2018; Valk et al., 2021). Thus, the typical aDNA workflow is costly, in laboratory expenses, use of materials, time, and in unnecessary destruction of archaeological material. Responsible destruction and sampling of archaeological remains therefore continues to be a pressing ethical issue for the aDNA field (Pálsdóttir et al., 2019; Wagner et al., 2020).

Recent efforts in improving the aDNA workflow have focused on minimizing destruction of archaeological remains (e.g., Sirak et al., 2017; Sirak et al., 2020; Scarsbrook et al. 2022) and improving available analytical tools for using poor quality sequences (e.g., Ferrari et al.,

* Corresponding authors.
E-mail addresses: lane@palaeome.org (L.M. Atmore), bastiaan.star@ibv.uio.no (B. Star).

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2022; Boessenkool et al., 2017; Dabney and Meyer 2019; Parker et al., 2021; Damgaard et al., 2015). Yet, little research has critically evaluated the amount of archaeological material that is commonly used in aDNA workflows. Those protocols that have been developed for minimally-destructive DNA extraction have so far been focused exclusively on specific bones from human and large mammal remains (Pinhasi et al., 2015; Sirak et al., 2017; Dabney and Meyer 2019), and are not always applicable to other species.

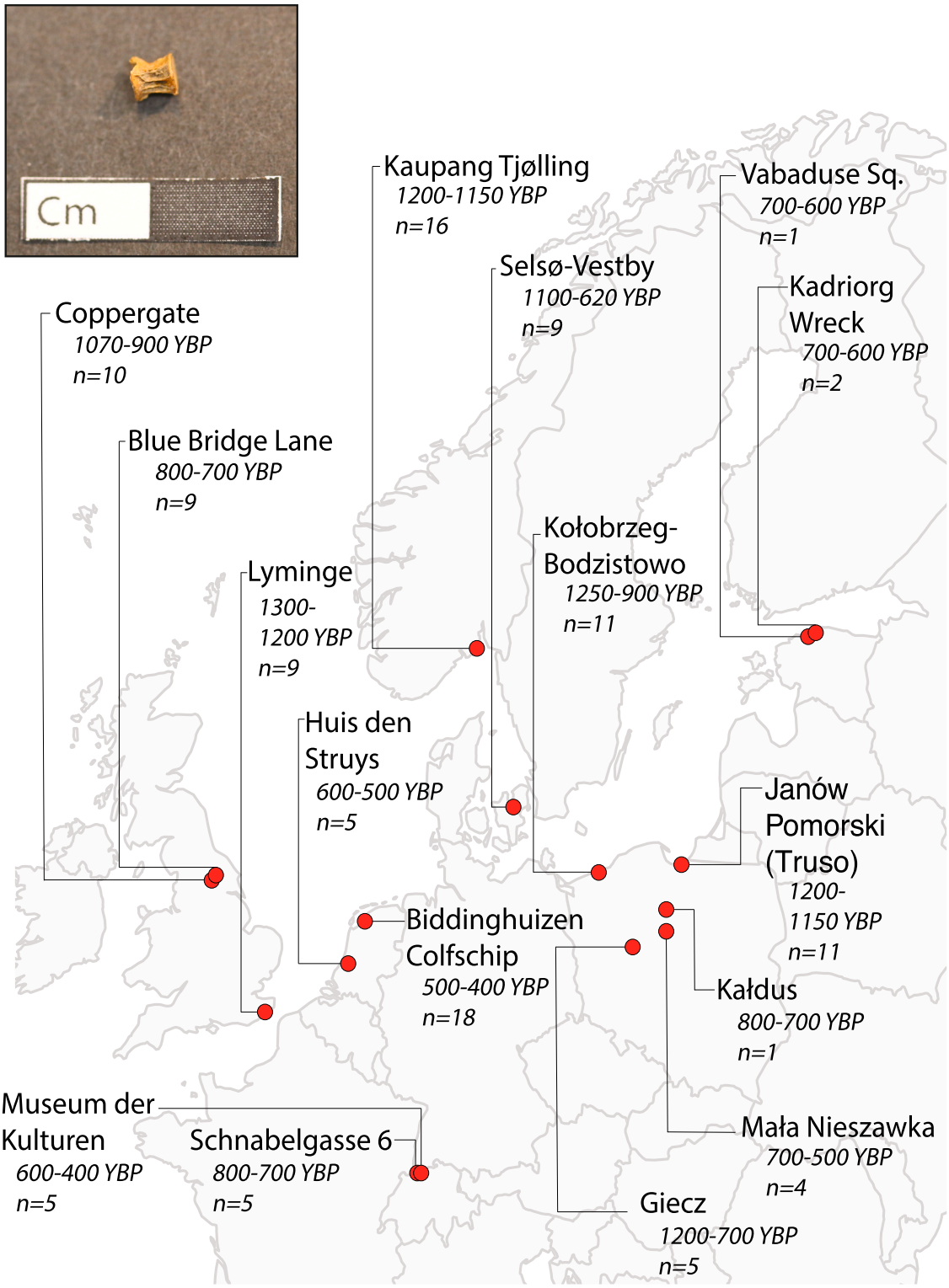

Fig. 1. – Sampling distribution of archaeological Atlantic herring bones. Sampling was conducted across 16 sites throughout Europe, ranging from 1300 to 400 YBP. A total of 121 herring bones were processed in the ancient DNA lab. The inset photo shows the small size of herring vertebrae, which were the most commonly sampled skeletal elements.

The field of aDNA typically uses 50–200 mg of minimum input weight of bone material for the extraction of DNA from archaeological remains (Dabney et al., 2013; Dabney and Meyer 2019; Dalén et al., 2007; Palkopoulou et al., 2015). It has been shown that more material – crushed bone with some upper limit, e.g. 200 mg – can improve complexity, and help successful extraction of DNA from archaeological bones (Boessenkool et al., 2017; Sirak et al., 2017); although DNA from some mammal bones (e.g. petrous bone) has been successfully extracted from smaller quantities of bone powder (Dabney and Meyer 2019; Parker et al., 2021). Moreover, given the unpredictability of success and often time-limited access to samples, researchers often take sufficient material from a bone to be able to run multiple extractions, resulting in significant quantities that are removed from individual remains.

Nonetheless, for some species, there are no bones large enough to yield such amounts of bone, leading researchers to resort to bulk bone approaches (e.g., Grealy et al., 2015; Seersholm et al., 2021), or to avoid these species altogether. For example, small fish bones may often be assumed to be of insufficient quantity for ancient DNA extraction for high-throughput sequencing even if uncovering the historical ecology of the oceans could retrieve a wealth of information (Oosting et al., 2019; Atmore et al., 2021). Given the ethical issues surrounding the destruction of irreplaceable archaeological remains and the possibility of sequencing previously-unexplored ancient specimens, the quantity of bone material required for successful aDNA retrieval must be interrogated, particularly for species which are not human and receive less research attention and ethical consideration (Pálsdóttir et al., 2019).

Here, we evaluate the impact of bone quantity on the success of ancient DNA retrieval of Atlantic herring (*Clupea harengus*), a species with particularly small bones, which is prevalent in many European archaeological sites. Although DNA has been successfully amplified from ancient herring bone before (Speller et al., 2012; Moss et al., 2016), their suitability for whole genome sequencing has yet not been systematically explored. We sampled 121 individual skeletal elements found in 16 archaeological sites around Europe dating from 700 to 1600 CE (see Fig. 1). Each sampled herring bone weighed between <10 and 70 mg. The smallest amount used for mammal bones in recommended protocols has so far been limited to a minimum of 10 mg and then only in cases with uniquely well-preserved bone (Dabney and Meyer, 2019). DNA was extracted from each bone separately, sequenced, and assessed for quality using the proxy measures of endogenous DNA content, percent clonal reads, total reads, mapped nuclear reads, and DNA extract concentration.

2. Materials and methods

2.1. Archaeological material

Individual herring bones were sampled from 16 archaeological sites around Europe dated between 1300 and 400 years ago (YBP) (Fig. 1, Table 1). 121 bones were collected with weights ranging from <10 to 70 mg. Each bone was photographed and initially weighed on a scale with precision of 0.01 g. All ancient DNA lab work was conducted in the designated ancient DNA clean lab at the University of Oslo following established protocols for minimizing contamination (Gilbert et al., 2005; Llamas et al., 2017).

2.1.1. Ancient DNA extraction

Bones were bathed in UV light for 20 min on each side to remove

Table 1
Archaeological sites with taphonomy and sampling data.

Site	Country	Age (YBP)	N	Skeletal Elements	Context and Taphonomy	Original Citation
Lyminge	UK	1300–1200	9	vertebrae	Anglo-Saxon monastery midden, clay sediment	*Reynolds (2013)*
Janów Pomorski (Truso)	Poland	1200–1150	11	ceratohyale	Sunken cellar, fish salted and barreled, the craft and trade settlement located near the estuary of Vistula; mentioned by Alfred the Great in the Old English Orosius (ca 890 CE or later)	*Makowiecki (2012); Jagodziński (2009)*
Kaupang Tjølling	Norway	1200–1150	16	vertebrae, prootic, unidentified	Larvikite monzonitic bedrock with high phosphate levels, mixture of peat bog and sand layers, elements from this site show signs of burning, site waterlogged, stored in museum storage	*Skre (2007)*
Kołobrzeg-Bodzistowo	Poland	1250–1000	11	dentaries, ceratohyale, unidentified	Stronghold settlement (preurban centre), one of the most important trade centers, among others specialised in herring catches located on the southern Baltic coast	*Leciejewic (2007a), 2007b*
Coppergate	UK	1070–900	10	vertebrae	Urban site near a river with moist, peaty conditions; Stored in an archaeological depot	*Bond and O'Connor, 1999*
Giecz	Poland	1200–700	5	prootica, vertebrae	Stronghold settlement, in the 9th-11th century the residence of the Piast dynasty, founder and ruler of Poland, located in the core of the state on Wielkopolska Lakeland	*Makowiecki et al. (2016); Kurnatowska (2004)*
Schnabelgasse 6	Switzerland	800–700	5	vertebrae	Dryland urban site, craftmen's quarter, cesspit with mineralised preservation and waterlogged condition, dry stored	*Harland et al., 2016*
Kałdus	Poland	800–700	1	unidentified	Stronghold settlement, located on Vistula river, one of the most important centers in the Chełmno Land, a commercial centre, the seat of the Piast state administration (castellany)	*Makowiecki et al. (2016); Chudziak* et al. *(2016)*
Selsø-Vestby	Denmark	1100–620	9	cleithra, dentaries	Coastal fjørd site	*Enghoff (1996)*
Blue Bridge Lane	UK	800–700	9	vertebrae	Urban site near a river with moist, peaty conditions; Stored in an archaeological depot	*Harland* et al. *(2016); Keaveney (2005)*
Vabaduse Sq.	Estonia	700–600	1	prootic	Suburban soil layer near road to larger urban settlement	*Kadakas* et al. *(2010)*
Mała Nieszawka	Poland	700–500	4	dentaries	Teutonic Castle, the seat of the commander and the convent, located on the left bank of the Vistula, the fish assemblage recovered from an area in the near the castle kitchen	*Iwaszkiewicz (1991); Makowiecki (2003); Józwiak (2003)*
Huis den Struys	Netherlands	600–500	5	dentaries, prootics, maxillae	Urban cesspit, cooked fish remains, fish salted and packed in barrels	*Laarman and Lauwerier (1996)*
Kadriorg Wreck	Estonia	700–600	2	dentaries	Underwater shipwreck, fish salted and packed in barrels, excavated on reclaimed land	*Roio* et al. *(2016)*
Museum der Kulturen	Switzerland	600–400	5	vertebrae	Dryland urban site, rich/clerical context, cesspit with mineralised preservation, probably not permanently waterlogged, dry stored	*Häberle, 2019*
Biddinghuizen Colfschip	Netherlands	500–400	18	prootics, dentaries	Underwater shipwreck excavated on reclaimed land, fish salted and packed in barrels; Stored in climate-controlled conditions in an archaeological depot away from UV light; One barrel (M11/58): specimens used for educational purposes, exposed to high temperatures	*Lauwerier and Laarman (2008)*

external contamination, but were too small for further surface cleaning with chemicals or mechanical methods such as sandblasting. After the UV wash, samples were placed in 1.5 ml Eppendorf tubes with 100 μl of digestion buffer (0.5 M EDTA, 0.5 mg/ml proteinase K, and 0.5% N-Lauryl sarcosine) then crushed with single-use, UV-sterilized plastic micro-pestles. An additional 900 μl of extraction buffer was then added to each tube and extraction proceeded following the double-digest protocol from Damgaard et al. (2015). DNA was extracted following overnight digestion using a PB buffer (Qiagen), after which samples were purified through MinElute columns using a QIAvac 24 Plus vacuum manifold system (Qiagen) for a final volume of 65 μl. DNA concentrations after extraction were measured using a Qubit Fluorometer (ThermoFisher Scientific).

2.1.2. Ancient DNA library prep

Libraries were built following the Santa Cruz Reaction protocol for single-stranded DNA (Kapp et al., 2021) using the Tier 4 dilution modification. A double-indexing scheme was used with a unique index of the P5 and P7 adapters. Only unique indexes were allowed for this study. Each library was amplified with 12–15 cycles of PCR, then purified using the Agencourt AMPure XP PCR purification kit (Bronner et al., 2009) with a 1:1 bead:template ratio for a final volume of 30 μl. Libraries were then assessed for quality using a Fragment Analyzer™ (Advanced Analytical) with the DNF-474 High Sensitivity Fragment Analysis Kit. Libraries with no dimers and library fragment length concentrated between 150 and 250bp, a typical length for aDNA including adapter sequences (Jónsson et al., 2013), were deemed successful and selected for sequencing.

2.1.3. Ancient DNA sequencing

Libraries that were deemed of high enough quality for sequencing were then pooled into one of four total sequencing lanes for screening. These specimens were submitted in pools with samples from other sequencing experiments with between 20 and 50 samples per pool. All pools were balanced so each individual's concentration ratio within the pool was the approximately equal. We aimed for ~8 million reads per individual for screening purposes. Each sample was sequenced using paired-end sequencing on an Illumina NovaSeq 6000 at the Norwegian Sequencing Centre. Reads were demultiplexed allowing for 0 mismatches.

2.1.4. Library success

Bone weights were binned into 10 mg categories, with weights rounded to the nearest 10 mg. All bones weighing less than 10 mg (n = 20) were too small to register on the scale. These samples were coded as weighing "1 mg," although this category contains samples weighing between 0 and 9 mg. We then assessed the relationship between weight and library success using percent failed libraries per bin as a response variable using a Fisher's exact test (Fisher 1934), where a "failed library" refers to those libraries that were not selected for sequencing after assessment with the Fragment Analyzer™. We statistically compared the average weight of all specimens and those successfully sequenced using a student's t-test. The same tests were then run using site, age, and bone element as explanatory variables.

2.1.4.1. Analysis of raw sequencing data. Raw sequence data were aligned to the Atlantic herring reference genome Ch_v2.0.2 (Pettersson et al., 2019) with PALEOMIX (Schubert et al., 2012) using *bwa-aln*. aDNA authenticity based on expected degradation patterns was investigated using mapDamage2.0 (Jónsson et al., 2013). Nuclear sequence quality was assessed from the alignment summary statistics using percent endogenous DNA content (here referring to DNA that belongs to the individual rather than bacterial or other DNA that has contaminated the sample over time) and percent clonal reads as proxies for library quality. As measures of sequence complexity, we further assessed DNA concentration from the extract, total number of reads retained after quality filtering in PALEOMIX, and the total number of reads mapped to the nuclear genome per specimen. We analyzed these proxies as response variables using differential initial bone weight as a categorical explanatory variable.

We further analyzed the relationship between site, age, and bone element using multiple linear regression and sequential regression analysis. Variance inflation factors (VIFs) were used to determine the presence of multicollinearity in the dataset. Chi-squared tests (Pearson 1900) were used to assess interrelatedness between the explanatory variables site, age, element, and weight. All statistical analysis was carried out in RStudio with R version v4.1.2 "Bird Hippie" (R Core Team 2021). Scripts used for statistical analysis can be found on GitHub (https://github.com/laneatmore/small_bones_analysis). A complete dataset can be found in the supplementary materials.

3. Results

3.1. Library success

All sites yielded at least one sample with a mappable DNA sequence. Of the 121 samples in the dataset, 90 yielded successful libraries (Table 2). Endogenous DNA content ranged from 0.0028 to 43%, with an average endogenous content of 10.1% (Supplement S1) and a large standard deviation of 12.28%. Clonality was low overall, with a range of 0–15% and an average of 9% (Supplement S1). No significant association was obtained between weight bin and percentage of samples that yielded successful libraries (Fisher's exact test, $p = 1$). Similarly, there was no significant difference in the mean weight of samples contained in the whole dataset versus the mean weight of samples in the dataset containing only successful libraries (Student's t-test, $t = 0.82$, $df = 13.48$, $p = 0.43$).

Given the small sample sizes in the largest weight bins (50, 60, and 70 mg), these three weight classes were further binned into a single group of 50+ mg. The above tests were repeated with minimal change in results (Fisher's exact test for weight, $p = 1$; Student's t-test, $t = 1.49$, $df = 9.97$, $p = 0.17$). Thus, weight does not appear to explain difference in successful DNA recovery. No significant association was found between library success and site (Fisher's exact test, $p = 1$), element (Fisher's exact test, $p = 1$), or age (Fisher's exact test, $p = 1$).

3.2. Determination of sequence quality

The impact of weight on sequence quality showed no significant relationship to percent endogenous DNA content (*lm(nu_end ~ Weight)*, $p = 0.69$, $df = 88$, *adjusted* $r^2 = -0.01$) (Fig. 2a). This pattern was maintained when all larger specimens were grouped into a single 50+ mg bin (*lm(nu_end ~ Weight_grouped)*, $p = 0.81$, $df = 88$, *adjusted* $r^2 = -0.01$). We assessed the ability of all explanatory variables to explain variation in endogenous DNA content using multiple linear regression

Table 2
– High-throughput aDNA library success based on weight of archaeological Atlantic herring bones. Bone weights were binned into 10 mg categories, with weights rounded to the nearest 10 mg. All bones weighing <10 mg were categorized as weighing 1 mg. Libraries were considered a success in absence of dimers and fragment lengths that are typical for aDNA.

Weight (mg)	Total Count	Successful Libraries	Percent Failed
1	20	9	55%
10	26	22	15.4%
20	18	12	33.3%
30	27	22	18.5%
40	19	16	15.8%
50	9	6	33.3%
60	1	1	0%
70	1	1	0%

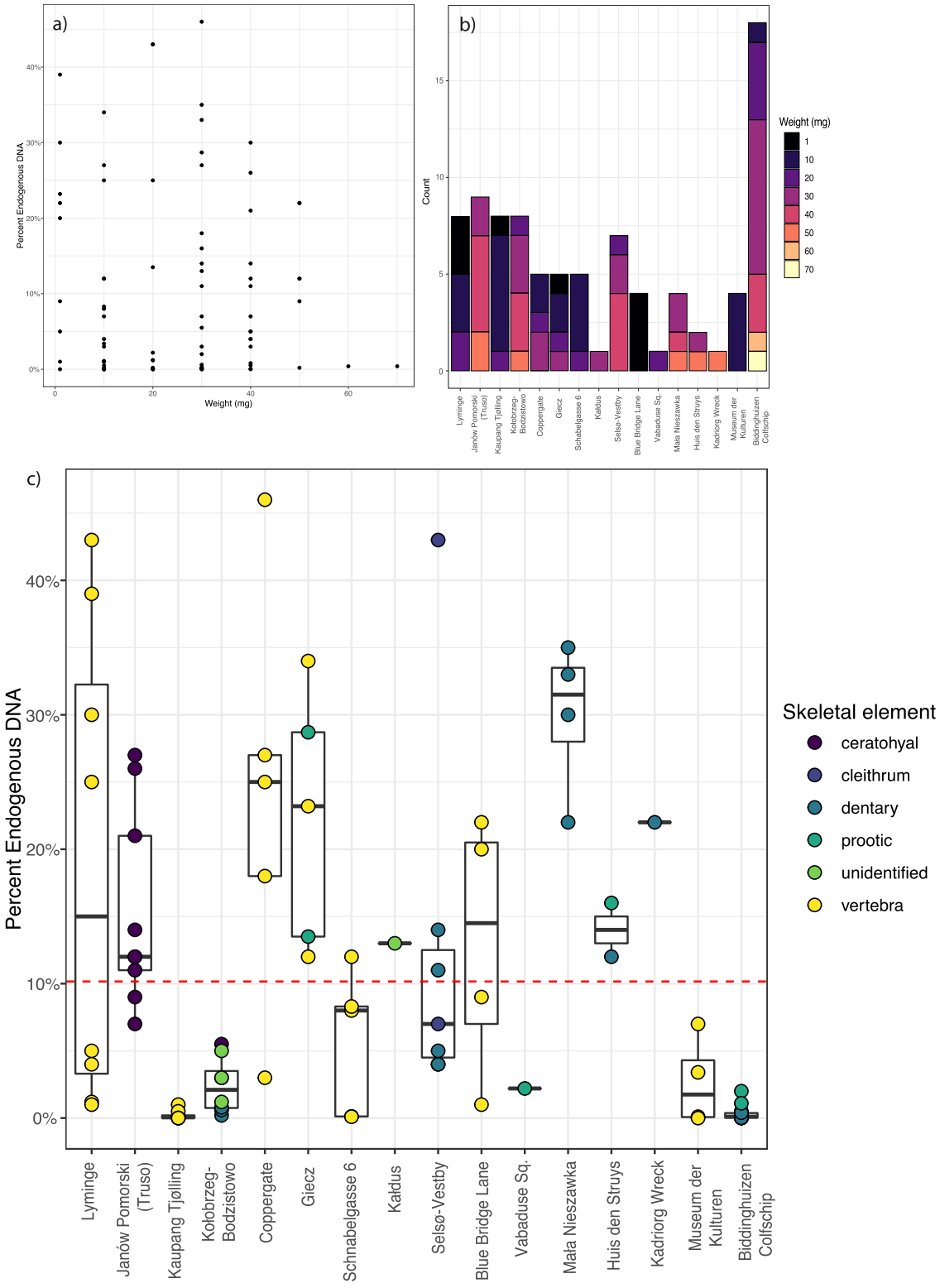

Fig. 2. – Relationship between archaeological context and percent endogenous DNA. a) Distribution of percent endogenous DNA content by bone weight. Each dot represents a DNA sequence extracted from a single archaeological herring bone element. The x axis denotes the different weight class bins in 10 mg increments. The weight class <10 mg is here classified as "1 mg". There appears to be no relationship between weight and endogenous DNA content, which is supported by linear regression analysis ($p = 0.69$, adjusted $r^2 = -0.01$). Only those samples that were suitable for sequencing are represented here. b) Distribution of bone weight bins by site. c) Distribution of percent endogenous DNA content per archaeological site. Each dot represents a DNA sequence extracted from a single archaeological herring bone element. Sites are arranged according to age from oldest (Lyminge, 1250 YBP) to youngest (Biddinghuizen Colfschip, 500 YBP). Linear regression showed a significant relationship between archaeological site and endogenous DNA content ($p = 0.88e\text{-}9$, adjusted $r^2 = 0.488$). The red dashed line indicates the average percent endogenous DNA across all sites and samples. Color represents skeletal element. Only those samples that were suitable for sequencing are represented here. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(*nu_end* ~ *Site* + *element* + *age_ybp* + *Weight_mg*). This model significantly explained 49.1% of the data (*adjusted* $r^2 = 0.49$, $df = 67$, p = 8.8e-9). However, the only independent variables that had p-values within the range of significance were three of the archaeological sites (Coppergate, Giecz, and Mała Nieszawka). Nearly identical results were obtained when grouping the larger weight classes (*adjusted* $r^2 = 0.49$, $df = 67$, $p = 2.3e\text{-}7$). To ensure the result of this model was not artifactual, further analysis was required.

We assessed if multicollinearity affected the efficiency of this model, given the likely interrelatedness between some of the variables (e.g., the relationship between weight and element or site and age). The vif() function from the *car* package (Fox et al., 2022) showed a variance inflation factor (VIF) of 1.9 for weight, 1.4 for site, 2.18 for element, and 11.2 for age. The dataset with grouped 50+ mg weight class showed similar or higher VIFs: grouped weight, 2.13; site, 1.4; element, 2.19; and age, 11.2. Given the similarity between the non-grouped and grouped weight datasets and the higher VIF for grouped weight, this dataset was discarded and subsequent analysis was conducted only on the dataset containing the original weight classes in 10 mg increments. Age was the only explanatory variable with a VIF over the accepted threshold of 5, indicating it is not independent from one or more of the other variables.

To explore further the relationships between the variables, chi-squared tests were used. Weight was found to be significantly correlated with site (Chi-squared test, $p = 0.047$) and bone element (Chi-squared test, $p = 0.003$). Element and site were also correlated (p = 0.0005) (Figure S3) as were site and age ($p = 0.0005$). Despite their interrelatedness, the VIF analysis did not show strong multicollinearity between these variables. Therefore, to determine the individual contribution of these variables to endogenous DNA content, sequential regression analysis was used with each explanatory variable isolated. Age was not significantly correlated with endogenous DNA content (*adjusted* $r^2 = 0.032$, $df = 88$, $p = 0.051$), neither was element (*adjusted* $r^2 = 0.055$, $df = 84$, $p = 0.083$). In contrast, a linear regression of the relationship between archaeological site and percent endogenous DNA explained 49% (*adjusted* $r^2 = 0.49$) of the overall variation with a p-value of 0.88e-9 (Fig. 2c; $df = 74$, residuals reported in Fig S4). Site is therefore likely driving the majority of variation in percent endogenous DNA content.

Given the significant interaction between weight, site, and element, we investigated if non-random distributions of weight throughout each site (Fig. 2b) confounded the impact of site on percent endogenous DNA. To determine if there is a significant interaction between weight and site impacting the regression analysis results, we allowed for an interaction between the two variables (*nu_end* ~ *Site* * *weight*). This model was then compared to an additive regression model (*nu_end* ~ *Site* + *weight*) using a chi-squared test (*anova(model2, method = 'Chisq')*). We obtained no significant relationship between site and weight (p = 0.079), indicating it is not necessary to stratify the weight regression by archaeological site.

Finally, we determined whether there was an interaction between site and skeletal element, which also revealed that only site was significantly associated with percent endogenous DNA (significance of interaction model: $p = 0.95$). These results suggest the model using site only is the most appropriate model for understanding the variance in percent endogenous DNA content per sample. Percent clonality was then assessed in a similar fashion. There was no significant relationship between weight and clonality (*adjusted* $r^2 = -0.011$, $df = 88$, $p = 0.91$). Further, no variables showed significant association with clonality except site, which explained 19.9% of the variation in clonality (*adjusted* $r^2 = 0.199$, $df = 74$, $p = 0.0052$; residuals reported in Figure S5). This result strengthens the conclusion that sequence quality variation depends on differences in site preservation.

3.2.1. Assessing library complexity

To better assess the question of library quality and complexity, we then evaluated the relationship between bone weight and site and several additional factors, including: DNA concentration as measured by the Qubit Fluorometer (ng/μl), total number of reads (after quality and duplicate filtering) and total number of reads mapped to the nuclear genome per specimen (see Supplementary Data S1). These last two values were obtained from the summary output files from PALEOMIX after sequence alignment.

DNA concentration as measured by the Qubit Fluorometer (ng/μl) were significantly correlated with bone weight ($p = 0.0006$), yet this explained only 11.7% of the variation in DNA concentration (*adjusted* $r^2 = 0.117$, $df = 88$). While raw concentration was associated with weight, total retained reads per sample was not (*adjusted* $r^2 = 0.016$, $df = 87$, $p = 0.12$), nor was the number of mapped hits on the nuclear genome (*adjusted* $r^2 = -0.0009$, $df = 88$, $p = 0.34$). Quality and complexity factors are plotted against weight class in Figure S6. Given the shown interaction between skeletal element and weight, element was used as an explanatory variable, which gave similar results (DNA concentration: *adjusted* $r^2 = 0.15$, $df = 84$, $p = 0.002$; Total reads: *adjusted* $r^2 = -0.007$, $df = 83$, $p = 0.51$; Mapped nuclear reads: *adjusted* $r^2 = 0.006$, $df = 84$, $p = 0.36$). Again, DNA concentration was the only significantly-correlated variable. Further analysis of the relationship between DNA concentration and complexity (here only using mapped nuclear reads) showed no significant result (*adjusted* $r^2 = 0.004$, $df = 88$, $p = 0.24$). This indicates that bone weight may have something to do with DNA concentration, but not ultimate sequence quality or complexity.

In contrast, site was significantly associated with DNA concentration (*adjusted* $r^2 = 0.28$, $df = 74$, $p = 0.0003$) and total retained reads (*adjusted* $r^2 = 0.21$, $df = 73$, $p = 0.004$), although not mapped nuclear reads (*adjusted* $r^2 = 0.1$, $df = 74$, $p = 0.08$). However, site does not appear to explain all the variation in library complexity, given that a minimum of 70% of the variation in these measures is unexplained by site differences. The models were marginally improved by includimg age as an explanatory variable (DNA concentration: *adjusted* $r^2 = 0.3$, $df = 73$, $p = 0.00.0002$; Total reads: *adjusted* $r^2 = 0.22$, $df = 72$, $p = 0.004$; Mapped nuclear reads: *adjusted* $r^2 = 0.12$, $df = 73$, $p = 0.06$).

4. Discussion

Here, we investigated the potential of whole genome, aDNA retrieval from small, archaeological herring bones. We assessed the relationship between various contextual variables including archaeological site, bone weight, skeletal element, and age. We make several observations.

First, 90 of 121 samples (73%) yielded successful sequence libraries, with a wide range of percent endogenous DNA content and percent clonality. Given the rough scale of our weight measurements, it is possible that there is some impact on library success from extremely small samples that was not observed due to a lack of resolution in our analysis. Although less than half of the samples in the smallest bin (<10 mg) yielded libraries, we found no significant relationship between bone weight and library success, neither was site or any of the other explanatory variables. It must be noted, however, that we were successful with 45% of the 20 bones in the smallest size bin, indicating that even the tiniest bones can yield useable DNA sequence data.

Further, we sampled entire bones with minimally-destructive cleaning beforehand; no bleach or surface removal was used to clean the surface of the bone, techniques which have been shown to increase endogenous DNA content (Boessenkool et al., 2017; Pinhasi et al., 2015). These techniques are typical components of the aDNA lab pipeline, but are too destructive for such small bones. Therefore, we expect that the endogenous DNA content of our samples was likely lower than it could have been, yet we were able to achieve an average endogenous DNA content of 10.1%. These results therefore show the viability of using archaeological small fish bones for providing historical aDNA data that can be applied in historical ecology studies (e.g., Oosting et al., 2019; Atmore et al., 2021).

Second, we found that the level of endogenous DNA retrieved from our small samples is comparable to studies using larger quantities of

bone powder (Ferrari et al., 2021; Martínez-García et al., 2022). Previous studies have shown that fish bone may be particularly suited to ancient DNA analysis given the lack of bone remodeling (Ferrari et al., 2022), a developmental process that is shown to have an impact on endogenous DNA preservation (Kontopoulos et al., 2019; Sirak et al., 2020). However, no studies have thus far explored the impact of bone size on DNA library success, instead largely focusing on differences in laboratory protocols, variables that are here held constant (Ferrari et al., 2021; Sirak et al., 2017; Sandoval-Velasco et al., 2017).

Moreover, we found no relationship between bone weight and endogenous DNA content or library complexity, and only a weak relationship between complexity and archaeological site. While the DNA concentration of each extract was weakly correlated with skeletal element and weight, neither of these were correlated with quality or complexity measures in the end, indicating that the increase in DNA concentration is likely from exogenous sources. Library complexity does not have to be particularly high to sequence a genome to low coverage, and even ultra-low-coverage genomes can yield meaningful biological results (Ferrari et al., 2022; Atmore et al., 2022). However, for some analyses, such as demographic inference, a greater amount of coverage and complexity is required (Schiffels and Wang 2020). We here found that weight is not related to complexity when sequencing to low effort. To further support this result, future analysis should be conducted on samples that have been exhaustively sequenced, allowing better assessment of the library complexity that can be attained from each specimen.

One limitation this analysis faces is the lack of ability to sample a single bone multiple times with different volumes. Previous analysis has shown multiple extractions can dramatically increase library complexity (e.g., Boessenkool et al., 2017), and by sampling multiple times we would have been able to control for individual variation and/or within-site preservation differences. Unfortunately, given the small size of herring bones (see inset photo on Fig. 1), this approach was impractical. We therefore rely on the distribution of weights across the sample dataset to serve as an imperfect proxy. Future research efforts should be focused on using larger bones that can be sequentially sampled in decreasing quantities to further expand upon our results.

We found that the most significant relationship in determining DNA sequence quality was archaeological site rather than bone weight, bone element, or age. Archaeological sites can have different taphonomic histories, therefore preservation of molecules between different sites can be drastically different (Ferrari et al., 2021). For example, colder and drier climates with stable temperatures lend themselves to DNA preservation, whereas ancient DNA recovery from tropical climates is possible but much more time-restricted (Reed et al., 2003; Bollongino et al. 2008; Willerslev et al. 2004; Kistler et al., 2017; Dommain et al., 2020; Woods et al., 2018). While general site conditions are known for these specimens (see Table 1), regression analysis of specific site characteristics is not possible here, as measurements on important variables (e.g., soil pH, humidity, temperature, storage temperature and UV exposure) were not available for all sites under consideration.

Each site also had a different history relating to sample storage. Exact storage conditions and full storage history are not available for all specimens in this study therefore the impact of storage temperature and UV exposure could not be explored systematically for this dataset. Where available, storage conditions are listed under “Taphonomy” in Table 1. These factors indicate that explanatory variables beyond the scope of this study likely have some relationship with sequencing success. Given the controlled variables of laboratory protocols and sequencing effort, which were the same for all samples, it is likely something not included in the dataset. This could be an as-of-yet unknown component of preservation or methodological bias, such as within-site variation (Pálsdóttir et al., 2019; Massilani et al., 2022), storage history and taphonomy, and/or manual variation in laboratory processing.

While this study is focused on the question of bone size, future research should focus on issues such as soil condition and pH, storage history, and within-site variation in determining the impact of site and taphonomy on molecular preservation. Further, our samples stem from relatively young sites in northern Europe, which typically results in better DNA preservation. In order to determine whether such microsampling is feasible in various contexts, future research efforts should attempt to replicate our results for sites in regions with poorer preservation and/or sites that are older than 1500 years.

Previous studies have indicated the potential for archaeological herring bone weighing >10 mg in ancient DNA research (Speller et al., 2012; Moss et al., 2016). These studies successfully amplified mitochondrial DNA, as well as generated microsatellites and SNP assay data from Pacific herring (*Clupea pallasii*) bones dating up to 10,000 YBP. However, whole-genome sequencing of ancient herring bones has not been successful until very recently (Ferrari et al., 2022). Combined with our results that DNA library quality is not determined by bone size, but by archaeological context, these studies illustrate the high potential of small bones in ancient DNA analysis.

Due to the high potential for fish bone to yield successful ancient DNA libraries despite its brittle, porous nature and lack of petrous bone (Ferrari et al., 2021), these results may not hold true for other types of bone, such as mammal remains. Prior to 2017, the majority of micromammal ancient DNA studies focused on sequencing segments of the mitogenome, such as the *cytB* sequence (Woods and Melissa, 2017). However, recent studies have shown that micromammal remains can also yield whole-genome sequences (Cucchi et al., 2020; Yu et al., 2022). Thus, smaller quantities of bone powder and small-boned specimens should be considered viable options for ancient DNA sequence analysis rather than discarded or immediately pooled into bulk-bone sequencing.

5. Conclusion

We have here shown that successful ancient DNA whole-genome sequence recovery is possible from individual archaeological fishbone weighing less than 10 mg. This supports the growing consensus that fish bone is an excellent material for DNA preservation. We show that there is no significant relationship between bone weight and DNA sequence quality or suitability for sequencing. Instead, our results support that the most important factor to consider in destructive analysis for ancient DNA extraction is taphonomy and site preservation rather than the quantity of retrievable bone powder.

Our results therefore provide novel evidence that site preservation and taphonomic history are also the crucial determining factors in DNA sequence recovery for small fishbones. Importantly they show that archaeological context is more important in determining whether or not to sequence a site rather than the amount of bone powder that can be recovered. This, in combination with bioinformatic approaches specially designed for low-coverage and poor-quality sequences have also reduced the DNA quantity and quality required for meaningful analysis (Ferrari et al., 2021), thereby further increasing the ability to use those samples that may have otherwise been discarded.

Author contributions

LMA, GF, and LMG conducted the lab work. LMA and GF designed the laboratory modifications for small bone samples. IJ, RB, JG, SH, KD, LQ, LL, DM, AKH, and JHB provided archaeological material and contextual information. LMA and BS designed the study. LMA conducted the statistical analysis and wrote the manuscript with input from BS and GF. All authors revised and agreed to the final version of the manuscript.

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Data availability

Sequences used for analysis are available on the European Nucleotide Archive at the accession PRJEB54658.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2022.105703.

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