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# Dietary evidence from Central Asian Neanderthals: A combined isotope and plant microremains approach at Chagyrskaya Cave (Altai, Russia)



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## ABSTRACT

Neanderthals are known primarily from their habitation of Western Eurasia, but they also populated large expanses of Northern Asia for thousands of years. Owing to a sparse archaeological record, relatively little is known about these eastern Neanderthal populations. Unlike in their western range, there are limited zooarchaeological and paleobotanical studies that inform us about the nature of their subsistence. Here, we perform a combined analysis of carbon and nitrogen stable isotopes on bone collagen and microbotanical remains in dental calculus to reconstruct the diet of eastern Neanderthals at Chagyrskaya Cave in the Altai Mountains of Southern Siberia, Russia. Stable isotopes identify one individual as possessing a high trophic level due to the hunting of large- and medium-sized ungulates, while the analysis of dental calculus also indicates the presence of plants in the diet of this individual and others from the site. These findings indicate eastern Neanderthals may have had broadly similar subsistence patterns to those elsewhere in their range.

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## 1. Introduction

## 1.1. The eastern Neanderthal range

How Neanderthals, who are thought to have evolved in Europe between 400 and 150 ka (Hublin, 2009), adapted to Eurasian environments has long been discussed. Their distinct biology is commonly thought to have emerged as a result of both genetic drift and as a response to western Eurasian environments (Weaver

et al., 2007). However, since the discovery of a juvenile skeleton at Teshik-Tash in Uzbekistan (Okladnikov, 1939), the Neanderthal range is understood to have stretched far into Central Asia. Further fieldwork has revealed that Neanderthals also occurred thousands of kilometers further East in the Altai Mountains of Siberia (Krause et al., 2007; Viola et al., 2011; Prüfer et al., 2014). It has been proposed that the Neanderthal arrival to the area followed the extended climatic warming of the last Interglacial (Hoffecker, 2002), but the presence of Neanderthal mtDNA in sediments dated to around 190 ka at Denisova cave (Jacobs et al., 2019) indicates that the first appearance of Neanderthals might have happened significantly earlier, during Marine Isotope Stage (MIS) 7. New archaeological and genetic data suggest that in

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Chagyrskaya Cave, there is evidence of a second Neanderthal dispersal into the Altai, at about 60 ka BP. This secondary dispersal is thought to originate in the Micoquian cultures in Central/Eastern Europe, the Crimea, and the Caucasus, and is defined by its own characteristic lithic assemblages and several fossil Neanderthal remains (Kolobova et al., 2020; Mafessoni et al., 2020). Given that Neanderthal ecology and diet has been portrayed as largely stable and conservative (Stiner, 2013), the Neanderthal expansion from Europe and the Caucasus deep into Central Asia raises questions about the adaptability of their ecology to diverse environments.

Little is known about Neanderthal ecology in the eastern part of their range, which is environmentally quite different from western Eurasia, with different resources and food availability. Given the harsher weather and relatively less topographic variation, some expect eastern Neanderthals to have consumed more meat (Chabai et al., 2005). Furthermore, dietary reconstruction in the eastern Neanderthal range is limited to zooarchaeological data from a small number of sites from the East European Plain and Western Asia (Zamyatin, 1961; Tarasov, 1977; Marean and Kim, 1998; Hoffecker and Cleghorn, 2000; Trinkaus, 2014), such as Betovo and Sukhaya Mechetka (Volga region, Russia), Mezmaiskaya Cave (Caucasus, Russia), Shanidar Cave (northern Iraq), and Kobeh Cave (western Iran). However, these sites represent very different habitats and reveal little about the occupation of the vast steppe of the Russian Plains and Siberia. The recovery of Neanderthal remains from the Altai Mountains means that it is now possible to examine the diet and adaptation in a region that is quite distant from their origin through different analytical approaches (Derevianko and Markin, 1992; Wrinn, 2010; Viola et al., 2011; Prüfer et al., 2014; Derevianko et al., 2015, 2018; Rudaya et al., 2017; Kolobova et al., 2019b). Therefore, with this study, we aim to shed new light on dietary adaptations at the less well-known eastern part of the Neanderthal range through direct evidence, by combining stable isotope analysis and studies of plant microremains trapped in dental calculus from the same individuals.

## 1.2. The site of Chagyrskaya

Chagyrskaya Cave is a north-facing cave, 358 m asl, in the piedmont of the Altai Mountains of Siberia (51°26'34.6" N, 83°09'18.0" E). It is situated 19 m above braided sections of the Charysh River, a tributary of Ob' River (Fig. 1A, B). The site lies 75 km southwest of Okladnikov Cave and 105 km west of Denisova Cave. The karstic cave consists of two chambers totaling ca. 130 m<sup>2</sup> (Derevianko et al., 2015). Investigations of the cave started in 2007 by S.V. Markin, and since 2016 the excavations are led by K. Kolobova (Viola et al., 2011; Derevianko et al., 2013, 2015; Rudaya et al., 2017; Kolobova et al., 2020). Excavations unearthed an archaeological sequence containing lithics, numerous bone tools, fauna, and a rich hominin assemblage.

The stratigraphic sequence contains Holocene (1–4) and Pleistocene layers (5–7). The Neanderthal artifacts and anthropological remains are associated with layers 6a, 6b, 6c/1, and 6c/2 (Fig. 1C, D). Recent data indicate that only the lower layer 6c/2 is in situ, and the uppermost layers with Neanderthal lithic, paleontological, and anthropological remains were redeposited from layer 6c/2. Additional confirmation of redeposition has been detected based on sorting of the paleontological remains and bone tools from the bottom to the top layers of the stratigraphic sequence (the largest bones/bone tools came from the lower layers 6c/1 and 6c/2, and the smallest bones came from the upper layers 5, 6a, and 6b; Derevianko et al., 2018; Kolobova et al., 2019b).

The lithic assemblages of Chagyrskaya Cave and Okladnikov Cave together constitute the Sibiriyachikha variant of the Altai

Middle Paleolithic (Kolobova et al., 2020). It is significantly different from all other Altai Middle Paleolithic technocomplexes, such as the Denisova and Kara-Bom variants (Kolobova et al., 2019a). The Sibiriyachikha variant is characterized by radial, orthogonal, and bifacial knapping. A variety of side-scrapers dominate the tool kits: semitrapezoidal and semileaf convergent scrapers were the most numerous. Among the bifacial tools, the Klausennismesser and Bocksteinmesser types were identified. These tool types are characteristic of European Micoquian assemblages. The materials found at Chagyrskaya Cave share many technological and typological similarities with the European Micoquian, a uniquely Neanderthal industry. Therefore, Chagyrskaya Cave is considered to be the easternmost manifestation of the European Micoquian (Jöris, 2002; Chabai et al., 2008; Pinhasi et al., 2011; Derevianko et al., 2018; Stepanchuk et al., 2017; Kolobova et al., 2019a, 2020).

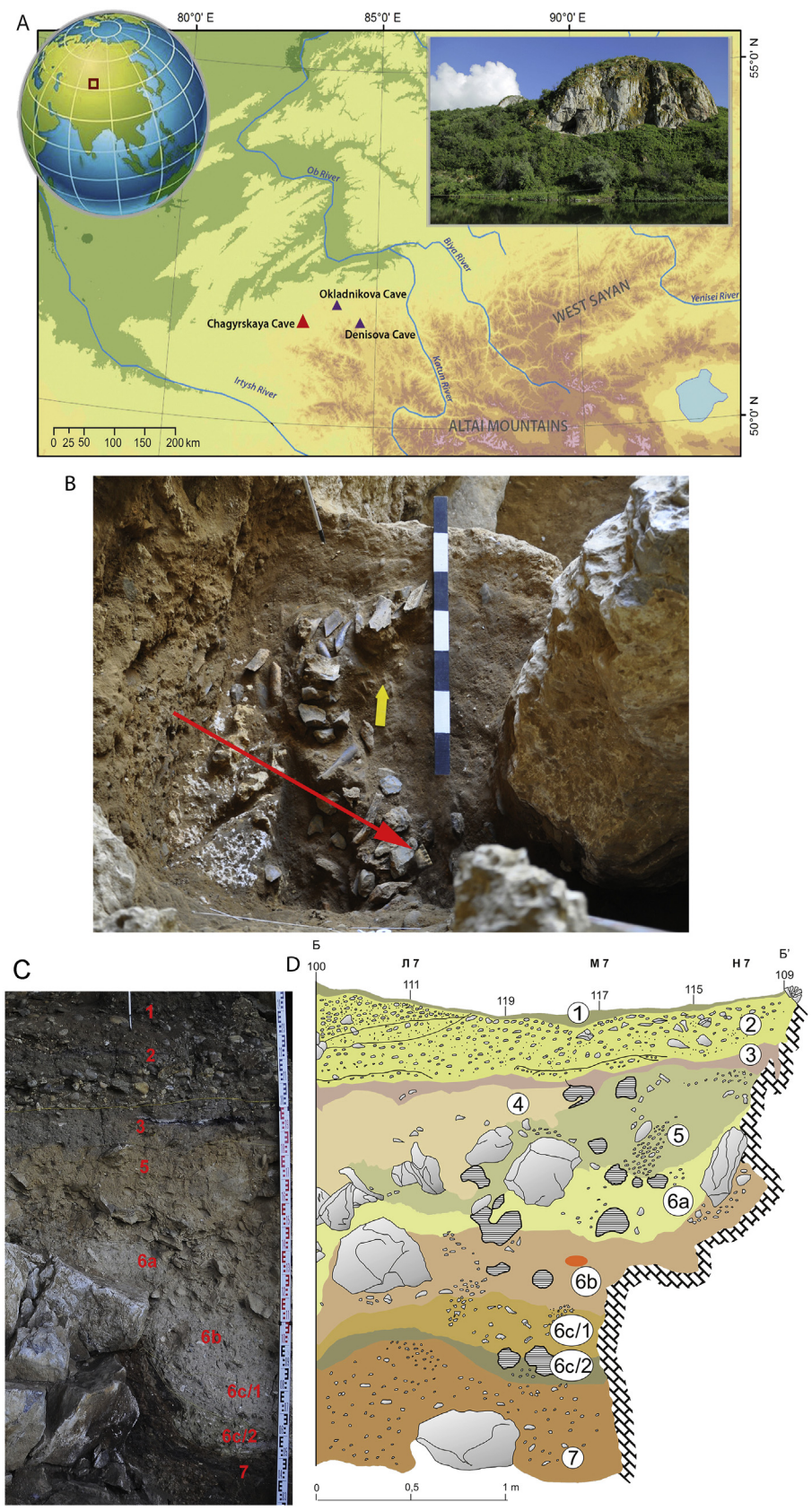
The collection of bone tools from the Chagyrskaya Cave is currently the most substantial and diverse Middle Paleolithic assemblage in Central Asia. Most of the bone tools are retouchers, which were used to retouch and rejuvenate stone tools. There are also formal bone tools such as rounded tip points, retouched tools, and intermediate tools (Baumann et al., 2020). Paleontological data suggest that juvenile, semiadult, and female bison were the main targets for hunting (Kolobova et al., 2019b). Eighteen bison bones were dated from layers 5 and 6. Two samples from layer 5 produced radiocarbon dates of 33 ka BP and >49 ka BP, while radiocarbon dates from layer 6 fall within the interval of ca. 45–51 ka BP (Rudaya et al., 2017). The age of the Chagyrskaya Cave layers with archaeological, anthropological, and paleontological remains has been estimated by post-infrared IRSL to be between 60 and 50 ka BP, corresponding to the end of MIS 4 and beginning of MIS 3 (Derevianko et al., 2018; Kolobova et al., 2020). This period is characterized by steppe and forest-steppe, with pockets of coniferous and mixed birch-coniferous forest in damp valleys (Rudaya et al., 2017).

The discovery of numerous human remains at the site makes Chagyrskaya Cave particularly important for understanding the Middle Paleolithic populations of eastern Eurasia (Viola et al., 2012, 2018). The diagnostic hominin remains from this cave consist solely of Neanderthal bones and teeth. They include a right hemimandible with a C<sub>1</sub> to M<sub>2</sub> tooth series, showing Neanderthal morphology (Viola et al., 2012). This mandibular fragment (Chagyrskaya 6; Fig. 2) and several isolated teeth were selected for paired carbon and nitrogen stable isotope and dental calculus microremains analysis to reconstruct Neanderthal protein and plant consumption.

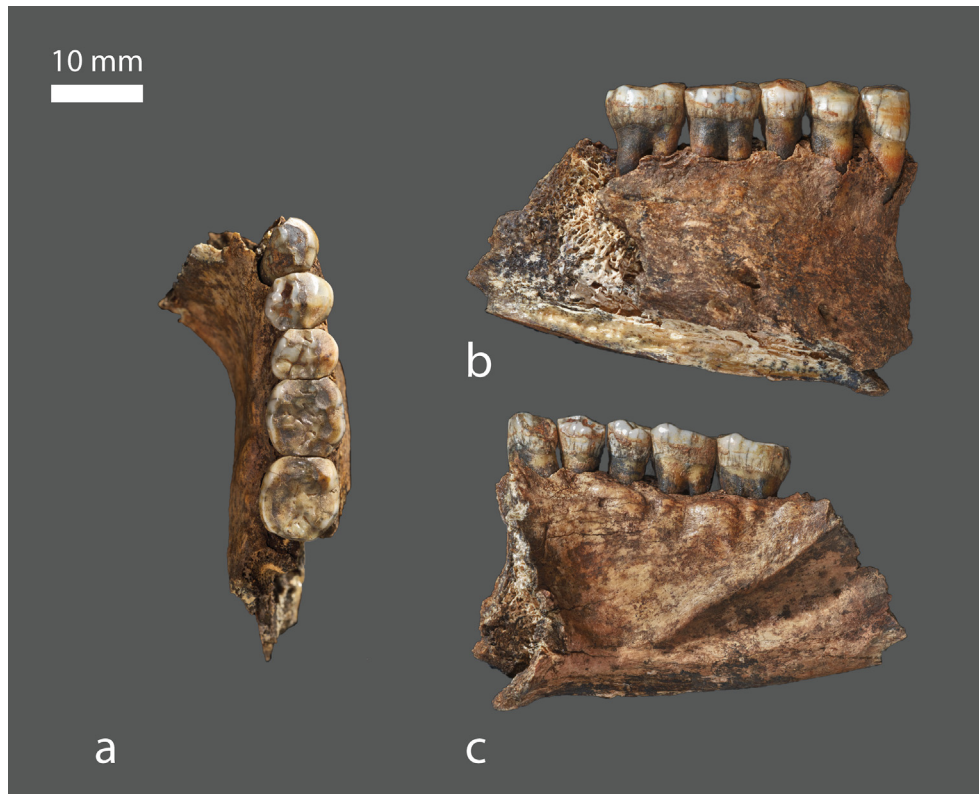
## 1.3. Carbon and nitrogen isotope analysis and Neanderthal dietary reconstructions

Carbon and nitrogen stable isotope analysis is a commonly used method for the reconstruction of prehistoric human and animal diets. This technique is based on the principle that the isotopic composition of the food eaten by both animals and humans is recorded by their body tissues after a predictable isotope fractionation (see reviews by Lee-Thorp, 2008; Makarewicz and Sealy, 2015). Well-preserved archaeological remains retain the stable isotope ratios present during life, and therefore provide information about the foods an individual consumed. Bone collagen is the preferred substrate for carbon and nitrogen stable isotope analysis. This is because collagen is the main skeletal substrate with nitrogen isotope ratios linked to diet (Salazar-García et al., 2014a), and because accepted quality indicators can easily indicate the isotopic integrity even from material older than 100 ka (De Niro, 1985; Van Klinken, 1999; Britton et al., 2011). Furthermore, collagen extracted from bone is unlikely to include contaminants from exogenous





**Figure 1.** Location of Chagyrskaya Cave: A) location of the cave relative to other Altai Mountain sites with Neanderthal, Denisovan, and modern human skeletal remains, and photo of the cave overlooking the nearby river from the view of the Charysh River (inset); B) photo of on-site excavation (hominin remains indicated by arrows); C) photo of the stratigraphic profile; D) a section of the archaeological sequence with radiocarbon dates outlined with a red oval marking the spot where the Neanderthal mandible was recovered. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Figure 2.** Occlusal (a), buccal (b), and lingual (c) views of the Chagyrskaya Neanderthal mandible (Chagyrskaya 6).

sources (e.g., the soil environment), unlike analysis of isotopes in carbonate or phosphate components of bone. Collagen also reflects the isotopic signals of the averaged main dietary protein sources consumed over several years preceding the individual's death (Schwarcz and Schoeninger, 1991; Ambrose and Norr, 1993; Hedges et al., 2007).

The consumption of terrestrial ( $^{13}\text{C}$  depleted) and marine ( $^{13}\text{C}$  enriched) foods can be distinguished by  $\delta^{13}\text{C}$ , the relative abundance of the stable carbon isotopes  $^{13}\text{C}$  and  $^{12}\text{C}$  (Chisholm et al., 1982; Peterson and Fry, 1987). This isotopic system also helps to define the relative proportions of  $\text{C}_3$  ( $^{13}\text{C}$  depleted, mainly all trees, shrubs and herbs, as well as temperate or shade-adapted grasses) and  $\text{C}_4$  ( $^{13}\text{C}$  enriched, mainly tropical grasses) plants, or animals that consumed them (Van der Merwe and Vogel, 1978; De Niro and Epstein, 1978; O'Leary, 1981; Bocherens and Drucker, 2003). Since the biomass of edible  $\text{C}_4$  plants reported in Siberia and Western Eurasia during the Late Pleistocene was low (Cerling, 1999; Sage et al., 1999), the main use of  $\delta^{13}\text{C}$  values in Neanderthals is to distinguish between marine and terrestrial  $\text{C}_3$  protein consumption, but the interpretation becomes more complicated if brackish water fish is also eaten (Salazar-García et al., 2014b).

Nitrogen stable isotope ratio analysis indicates the position of an organism in the food chain by increasing  $\delta^{15}\text{N}$  values between 3 and 5‰ with each trophic level (Schoeninger et al., 1983). Therefore, because aquatic food chains tend to contain more trophic levels than terrestrial ones,  $\delta^{15}\text{N}$  values are useful for detecting the consumption of high-trophic-level aquatic resources, either marine or freshwater (Schoeninger and De Niro, 1984), especially when combined with  $\delta^{13}\text{C}$  values. Likewise,  $\delta^{15}\text{N}$  values are also useful for distinguishing animal-rich diets from plant-rich diets (Minagawa and Wada, 1984), because the consumer has higher values than the consumed protein (De Niro and Epstein, 1981; Schoeninger and

De Niro, 1984; Hedges and Reynard, 2007). Theoretically, omnivore  $\delta^{15}\text{N}$  values would fall between herbivore and carnivore values, and it would be possible to determine whether omnivores obtained their protein mainly from plants, animals, or a mix of both. However, because large changes in protein sources may be indicated by only small changes in  $\delta^{15}\text{N}$  values (O'Connell et al., 2012), and the determination can be complicated by special cases in which plants and other nonanimal foods have higher  $\delta^{15}\text{N}$  values than expected (e.g., Warinner et al., 2013), this quantification is less straightforward than previously thought. Furthermore,  $\delta^{15}\text{N}$  values can be influenced by different environmental settings through time and space (Iacumin et al., 2000; Goude and Fontugne, 2016). To ensure these factors are accounted for and that there is detection of differences in the nitrogen environmental baseline (Bocherens et al., 2014), nitrogen isotope ratio values from hominins should be compared with faunal samples, ideally from the same archaeological site and level. In any case,  $\delta^{15}\text{N}$  values are still appropriate to differentiate between Neanderthals who consumed more animal resources from those who consumed less (Bol and Pflieger, 2002; Petzke et al., 2005; Fahy et al., 2013).

Because collagen is not well preserved in warmer climates, analyses on bone collagen like the ones of this study have produced data mainly on Neanderthals that lived in colder climates and at higher latitudes. Neanderthal remains from Atlantic Europe (Bocherens et al., 1991, 1999, 2001, 2005; Fizet et al., 1995; Beauval et al., 2006; Richards et al., 2008; Hublin et al., 2009; Wißing et al., 2016, 2019), Mediterranean Europe (Salazar-García et al., 2013), Central Europe (Richards and Schmitz, 2008), the Balkans (Smith et al., 1999; Richards et al., 2000; Higham et al., 2006), and Siberia (Krause et al., 2007) have already been analyzed for carbon and nitrogen stable isotope analysis. The Neanderthals in most of these



studies had high  $\delta^{15}\text{N}$  values, which has been interpreted as a reliance on animal proteins.

#### 1.4. Plant microremains and Neanderthal dietary reconstructions

Although reconstructing plant consumption is important for understanding Neanderthal ecology, little is known about Neanderthal plant use due in part to the absence of material for traditional macrobotanical analyses. One solution to this problem is to extract information about the diet of prehistoric individuals through the identification of microbotanical remains in dental calculus (mineralized dental plaque). Dental calculus forms through the mineralization of dental plaque and this process traps and preserves dietary and environmental remains from plants, animals, and other sources (Piperno and Dillehay, 2008; Leonard et al., 2015; Power et al., 2015; Boyadjian et al., 2016). Although this approach provides nonquantitative information on diet, it is important for understanding Neanderthal ecology in the absence of other sources of information about plant foods (Jones, 2009; Hardy et al., 2012; Henry et al., 2014; Power et al., 2018).

Some recent analyses have identified aDNA and proteins from archaeological dental calculus (Warinner et al., 2014; Hendy et al., 2018). However, these methods are of limited use for reconstructing diet from small, ancient calculus samples, because aDNA analyses are not always suited for identifying food sources (Mann et al., 2020), while protein analyses require large (>5 mg) and well-preserved calculus samples, and have not yet been applied to material older than the Holocene. Analysis of microremains in calculus, while not providing complete dietary information, is a well-established method for the reconstruction of Neanderthal diets (Henry et al., 2011, 2014; Hardy et al., 2012; Salazar-García et al., 2013; Power et al., 2018). These previous analyses suggest that European and Near Eastern Neanderthals consumed a large variety of plants, including seeds of grasses, leafy greens, and plant storage organs. There was little indication of variation in the use of plant foods across that geographic range (Power et al., 2018).

#### 1.5. Specific aims and goals

The Neanderthals from Chagyrskaya display many technological similarities to Neanderthal groups living further west, specifically with the European Micoquian, yet the habitats in which they lived were remarkably different. We asked: What can be said about the diets of these eastern Neanderthals? Can we see differences between the diets of the Chagyrskaya Neanderthals and those of western European Neanderthal groups? The answers to these questions could shed light on how Neanderthals survived in the far eastern steppes. We therefore performed a combined stable isotopic and plant microremain analyses on several individuals from the site.

## 2. Materials and methods

### 2.1. Carbon and nitrogen isotope ratios

**Materials studied** Bone samples from one Neanderthal and 22 animals of six different species, all from layers 6a, 6b, and 6c of Chagyrskaya Cave were sampled for C and N stable isotope analysis on bone collagen (Table 1). Whole bone pieces of 200–400 mg from each of the animal remains were sampled. From the Neanderthal mandible, Chagyrskaya 6 (SP 2923), ca. 100 mg of bone powder was sampled by microdrilling. The faunal and human remains are stored in Novosibirsk (Russia) at the Institute of Archaeology and Ethnography of the Siberian Branch of the Russian Academy of Sciences and its museum.

**Collagen extraction and isotope ratio analysis** Before analysis, visible contaminants were removed with aluminum oxide powder abrasion. Collagen extraction proceeded following Richards and Hedges (1999), with the addition of an ultrafiltration step (Brown et al., 1988). To summarize, samples were demineralized in 0.5 M HCl solution at 5 °C over the course of 1 week (except the Neanderthal powder sample, which was demineralized for ca. 24 h), and were then rinsed three times with deionized water until the pH became neutral. This was followed by gelatinization over 48 h at 70 °C, and later by filtering with a 5  $\mu\text{m}$  EZEE® filter and ultrafiltering with >30 kDa Amicon® ultrafilters. The purified solution was finally frozen and lyophilized before being weighed into tin capsules and loaded into the mass spectrometers.

The carbon and nitrogen isotope ratios in collagen were measured using a Delta XP continuous-flow isotope ratio mass spectrometer after being combusted in an elemental analyzer Flash EA 2112 that was interfaced with it (Thermo-Finnigan®, Bremen, Germany) at the Department of Human Evolution of the Max-Planck Institute for Evolutionary Anthropology (Leipzig, Germany). All samples were measured in duplicates, except faunal samples S-EVA 24482 to 24489, from which only one analytical run was carried out. Stable carbon isotope ratios were expressed relative to the VPDB scale (Vienna PeeDee Belemnite) and stable nitrogen isotope ratios were measured relative to the AIR scale (atmospheric  $\text{N}_2$ ), using the delta notation ( $\delta$ ) in parts per thousand (‰). Repeated analysis of internal and international standards determined an analytical error better than 0.1‰ (1 $\sigma$ ) for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

### 2.2. Microremains from dental calculus

**Materials studied** To complement the isotope study, we retrieved and analyzed plant microremains (starch grains and phytoliths) trapped in dental calculus isolated from the teeth of the Chagyrskaya 6 mandible, as well as from several Neanderthal isolated teeth and associated faunal teeth (Supplementary Online Material [SOM] Table S1). One of the isolated teeth, the left  $\text{I}_2$  Chagyrskaya 14, derives from the same individual as the mandible, based on its find position and morphology, whereas the other teeth, Chagyrskaya 12, 17, and 20, represent three different individuals based on their estimated ages.

**Sample preparation and microscopic analysis** The sampled Neanderthal teeth were encrusted with a visible band of supragingival calculus situated on the enamel surface. A dental scalar was used to remove small areas of calculus at the field laboratory, which were transferred into microcentrifuge tubes. The samples were then transported for analysis to the Department of Human Evolution at the Max-Planck Institute for Evolutionary Anthropology (Leipzig, Germany). Two different workflows (mechanical extraction and chemical extraction) were implemented. Initially, a mechanical extraction method was used. This involved gently grinding the sample with a micropestle for 1–5 strokes in a solution of 25% glycerine in water. To gain as much information as possible, a different chemical extraction method on remaining samples using an EDTA-decalcification approach was tried (Chag 12, 14, 17, and 20). Each sample was prepared by decalcifying the sample in 1 ml of 0.5 M EDTA for approximately 48 h. The supernatant was then pipetted away, and 200  $\mu\text{L}$  of 25% glycerine water solution was added to the tube. The suspension was then mounted on glass slides with 18  $\times$  18 and 20  $\times$  20 mm coverslips.

*Bison priscus* and *Ovis ammon* teeth were additionally examined and sampled as a control to test for potential museum or site microremain contamination. Calculus deposits were sparse on these teeth so sediment traces adhering to the tooth surface and

**Table 1**Chagyrskaya  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, collagen control indicators (yield, %C, %N, C:N), sampled bone, archaeological context and S-EVA number.

S-EVA	Species	Context	Bone	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	%C	%N	C:N	% collagen
24482	<i>Bison</i> sp.	Layer 6a, horizon 1	Phalanx	-19.2	5.4	52.5	19.2	3.2	1.2
24483	<i>Bison</i> sp.	Layer 6b, horizon 3	Rib	-19.3	8.8	36.2	13.3	3.2	0.9
24484	<i>Bison</i> sp.	Layer 6b, horizon 4	Metatarsal	-19.5	9.1	43.6	15.9	3.2	0.9
24485	<i>Bison</i> sp.	Layer 6b/1, horizon 1	Long bone	-19.0	6.3	43.6	15.9	3.2	1.5
24486	<i>Bison</i> sp.	Layer 6b/1, horizon 4	Indeterminate bone	-19.5	6.4	36.4	12.9	3.3	3.0
24487	<i>Bison</i> sp.	Layer 6b/1, horizon 5	Rib	-21.2	7.3	42.3	13.6	3.6	0.8
24488	<i>Bison</i> sp.	Layer 6b/1, horizon 5	Long bone	-19.2	8.0	43.8	15.7	3.3	1.1
24489	<i>Bison</i> sp.	Layer 6b/2	Phalanx	-19.1	7.5	41.7	15.1	3.2	1.3
27413	<i>Equus ovodovi</i>	Layer 6b(b) level 2	Tarsal	-20.8	4.8	40.1	14.5	3.2	2.0
27414	<i>Equus ovodovi</i>	Layer 6a level 1	Tarsal	-20.2	6.8	40.9	14.7	3.2	3.6
27415	<i>Equus ovodovi</i>	Layer 6b(b) level 1	Metacarpal	-20.0	6.7	39.3	14.3	3.2	2.4
27416	<i>Equus ovodovi</i>	Layer 6b(c)1 level 5	Sesamoid	-19.5	8.2	40.0	14.4	3.2	3.5
27417	<i>Capra sibirica</i>	Layer 6a level 3	Metapodial	-19.1	6.5	39.9	14.4	3.2	3.5
27418	<i>Capra sibirica</i>	Layer 6a level 3	Metatarsal	-17.8	6.0	37.1	13.3	3.3	4.8
27419	<i>Capra sibirica</i>	Layer 6b(c)1 level 2	Intermediate phalanx	-18.7	5.1	39.5	14.3	3.2	4.4
27420	<i>Capra sibirica</i>	Layer 6a level 1	Metacarpal	-19.0	6.0	40.9	14.6	3.3	2.6
27421	<i>Crocota spelaea</i>	Layer 6b(c)1 level 1	Tarsal	-18.6	10.9	39.1	14.2	3.2	2.2
27422	<i>Crocota spelaea</i>	Layer 6a level 2	Mandible	-19.6	5.6	35.5	12.7	3.3	1.6
27423	<i>Canis lupus</i>	Layer 6b(c)2 level 1	Metatarsal	-18.6	11.2	38.6	14.0	3.2	2.8
27424	<i>Canis lupus</i>	Layer 6b(b) level 1	Ulna	-18.6	9.9	36.2	13.2	3.2	2.7
27425	<i>Vulpes vulpes</i>	Layer 6b(b) level 3	Ulna	-19.5	6.2	37.8	13.8	3.2	2.8
27426	<i>Vulpes vulpes</i>	Layer 6b(c) level 2	Tibia	-20.9	7.8	40.4	14.5	3.2	3.4
27427	Neanderthal (Chagyrskaya 6)	Layer 6b(b) level 3	Mandible	-19.2	13.8	32.6	11.7	3.3	1.0

Abbreviation: S-EVA = ArchSci lab sample code from the Department of Human Evolution of the Max-Planck Institute for Evolutionary Anthropology.

calculus was both sampled from these teeth in comparable volumes to the Neanderthal calculus samples.

Both groups of samples were then centrifuged at  $1691 \times g$  using a Heraeus MEGAFUGE 16 with a TX-400 Swinging Bucket Rotors for 10 min. These samples were mounted in a 25% glycerine solution water on a slide in a Bio Air Aura Mini laminar flow and examined under brightfield and cross-polarized light on a Zeiss Axioscope microscope at  $400\times$  magnification. The whole slide was examined and the encountered microremains were photographed, described, and documented using the International Code for Starch Nomenclature (ICSN, 2011) and International Code of Phytolith Nomenclature (Madella et al., 2005).

To control the risk of unwanted airborne contamination from modern plant material and laboratory supplies (Langejans, 2011; Crowther et al., 2014; Henry, 2014), a regime of weekly laboratory cleaning was conducted. All laboratory work surfaces were cleaned with hot water, washed with starch-free soap and with 5% sodium hydroxide (NaOH). To document what contamination was present, additional wipe tests were performed before and after weekly cleaning to quantify contaminating starch and other contaminants. Wipe tests retrieved settled particles of the surface area ( $74 \times 43 \text{ cm}^2$ ) of the laboratory positive-pressure laminar flow hood used for mounting. The results of these contamination control tests are found in SOM Table S2.

### 3. Results

#### 3.1. Carbon and nitrogen isotope ratios

The stable isotope results for the humans and animals from Chagyrskaya are presented in Table 1 and Figure 3. All human and animal remains yielded enough collagen at the  $>30 \text{ kDa}$  fraction and met published quality criteria (Ambrose, 1993; Van Klinken, 1999).

Samples from 16 herbivores and 6 carnivores produce the isotopic background for the Neanderthal. The herbivore  $\delta^{13}\text{C}$  values ( $m = -19.4 \pm 0.8 [1\sigma] \text{‰}$ ,  $\text{min} = -21.2\text{‰}$ ,  $\text{max} = -17.8\text{‰}$ ) are typical values for a terrestrial  $\text{C}_3$  European ecosystem. The carnivore  $\delta^{13}\text{C}$  values ( $m = -19.3 \pm 0.9 [1\sigma] \text{‰}$ ,  $\text{min} = -20.9\text{‰}$ ,  $\text{max} = -18.6\text{‰}$ ) are consistent with most herbivore values and a terrestrial  $\text{C}_3$  food web.

The herbivore  $\delta^{15}\text{N}$  values ( $m = 6.8 \pm 1.3 [1\sigma] \text{‰}$ ,  $\text{min} = 4.8\text{‰}$ ,  $\text{max} = 9.1\text{‰}$ ) define the trophic baseline of the local mammalian food web. The carnivore  $\delta^{15}\text{N}$  mean value is  $8.6 \pm 2.4 (1\sigma) \text{‰}$  ( $\text{min} = 5.6\text{‰}$ ,  $\text{max} = 11.2\text{‰}$ ), which is almost  $2\text{‰}$  higher than the herbivore mean value ( $6.8\text{‰}$ ), consistent with these species being almost a trophic level above the herbivores at the site, although one of the hyena specimens (S-EVA 27422) show a surprisingly low value ( $5.6\text{‰}$ ) that lowers the  $\delta^{15}\text{N}$  carnivore mean value.

The Neanderthal  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values are  $-19.2\text{‰}$  and  $13.8\text{‰}$ , respectively. Its carbon isotope value describes a diet where the protein was based on terrestrial  $\text{C}_3$  resources. Its nitrogen isotope value is higher than those of the herbivores ( $7\text{‰}$  higher), as well as those of the carnivores (about  $5\text{‰}$  higher). This pattern is similar to those found in other European Neanderthals (Richards and Trinkaus, 2009), showing a high protein input in the diet. By using standard isotopic offsets between predators and prey (ca.  $0.5\text{--}1\text{‰}$  in  $\delta^{13}\text{C}$ , and  $3\text{--}5\text{‰}$  in  $\delta^{15}\text{N}$  values), we can compare the Neanderthal's isotopic values to those of the fauna to predict which animals were the most likely sources of protein. Of the fauna for which we have isotopic data, the closest likely prey species were horse and bison (Fig. 3).

#### 3.2. Dental calculus microremains

Phytoliths dominate the faunal dental calculus samples. We found just one starch in these samples, but 32 phytoliths. Nearly all of the phytoliths represent grass types; however, a small number may represent eudicots or monocots. Control samples of selected *Bison priscus* teeth from layers 6a and 6b produced a microremain assemblage composed of solely grass phytoliths, which is consistent with diets associated with this taxon (Guthrie, 1990; Merceron and Madelaine, 2008). Although grass phytoliths are sometimes found in Neanderthal samples, including at Chagyrskaya, they are not dominant. Therefore, we consider the fauna assemblage to be dissimilar from the hominin assemblage (see below; Table 2). These bison results support the integrity of the microremain assemblage as a representation of diets and are an indication against contamination. Furthermore, many of the microremains in the faunal calculus were visibly encrusted within the mineral matrix. Although these in situ microremains are obscured and therefore not readily

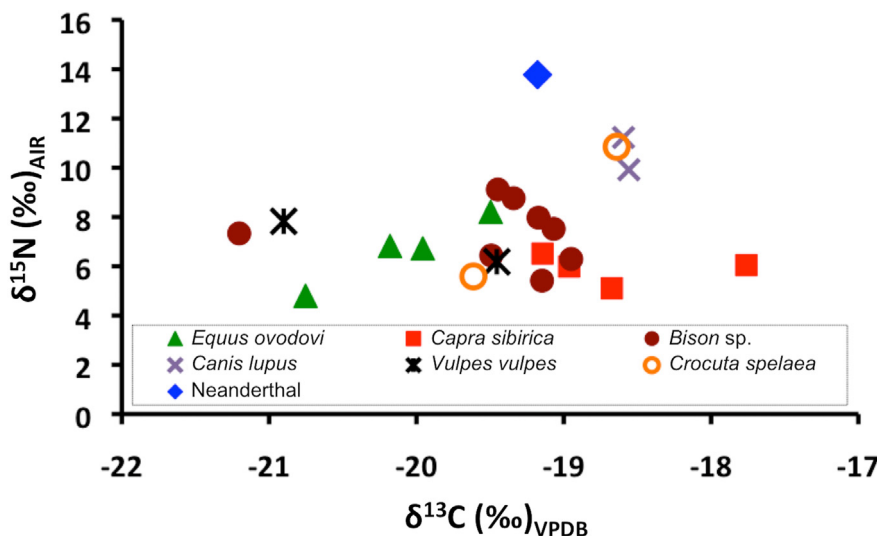


Figure 3. Plot of human and animal bone collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values from Chagyrskaya.

identifiable to the highest possible taxonomic level, their placement within calculus deposits is a further indication that they do not derive from environmental contamination.

We detected 33 starches in the Neanderthal samples representing seven starch types (Table 2). Represented types include a variety of simple starch forms, such as ovoid, subspherical and lenticular. Twenty-one phytoliths were recovered from the Neanderthals including monocot plants such as multicell examples of grass and dicots, as well as epidermal jigsaw cells (Fig. 4). However, forms that can be from monocots or dicots, including hairs and blocks, were also abundant. Notably, seven starch grains and three phytoliths were observed trapped in situ in a piece of calculus. In addition to starches and phytoliths, we found abundant charcoal particles, spores and related fungal fragments, a variety of fibers, a single calcium oxalate crystal, an annular tracheid, a hair fragment, and several unknown particles (Table 2).

#### 4. Discussion

Little is known about the behaviors of the Neanderthals who lived in the eastern part of their range. By studying the material from Chagyrskaya in a multidisciplinary context, we are able to explore if, and to what degree, their lifeways and subsistence patterns varied from those in western Eurasia. Characterizations of Neanderthal diet more generally have been of recent interest because some have proposed that dietary differences may have contributed to Neanderthals being replaced by modern humans (Fabre et al., 2011; Power and Williams, 2018).

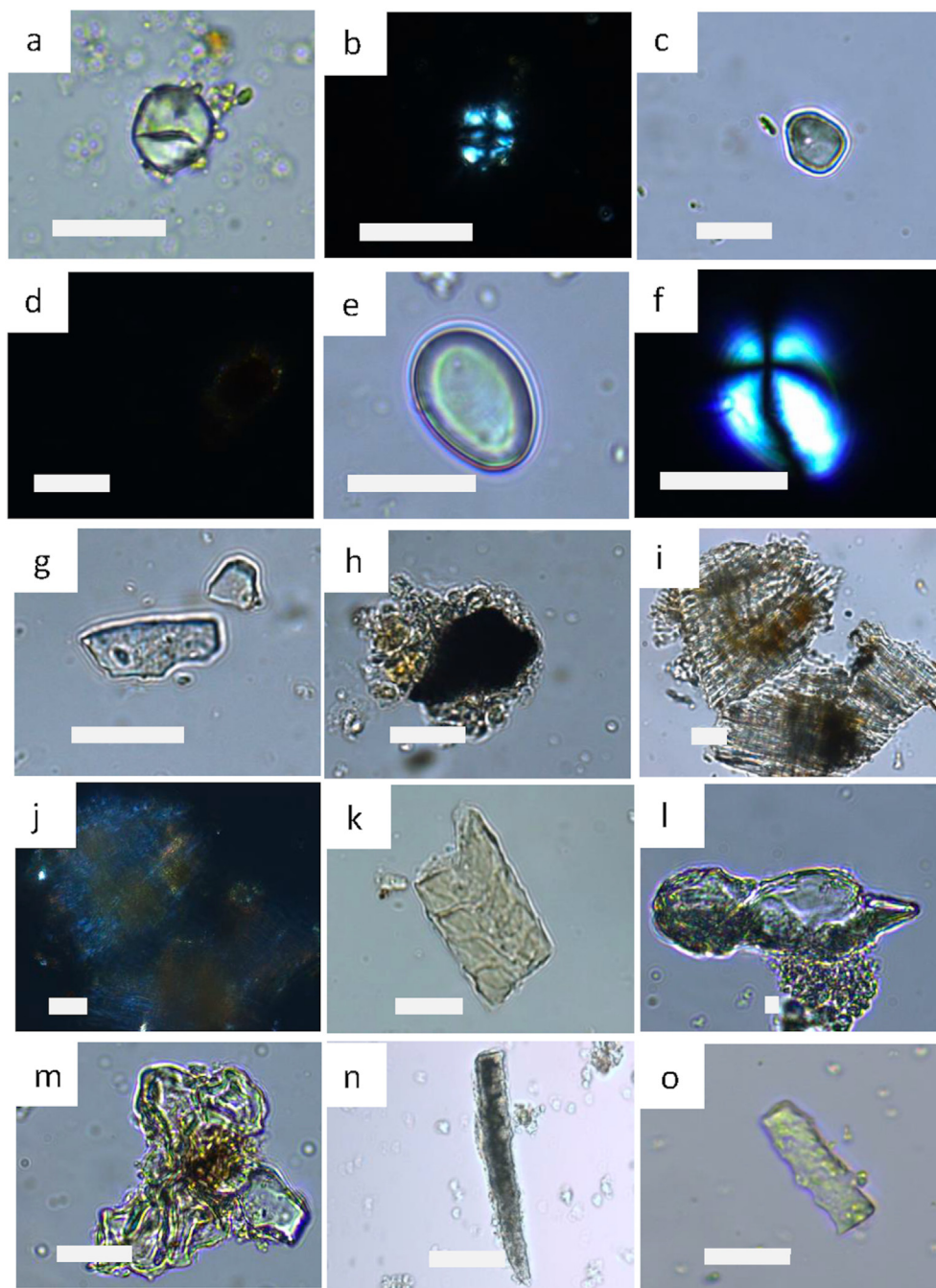
The occupation period of the late Neanderthals in the Altai corresponds to the end of MIS 4 and the beginning of MIS 3. Locally, these climatic phases are known as the Zyryanskiy (Ermakovo) stadial and the Karginiyskiy interstadial. In accordance with palynological data, the climate of this period in the West Siberian Plain, located northward of the Altai Mountains, is characterized as harsh with tundra-steppe vegetation (Volkova and Kulkova, 1984). However, the most complete paleoecological data from this region comes from Denisova Cave (Fig. 1A) in a forest mountain zone. There, the data show instability during this period, with changes from a cool humid climate with significant forest cover toward a more open landscape (Derevianko et al., 2013; Rudaya et al., 2017). Paleontological and palynological data of Chagyrskaya Cave indicate that the late Neanderthals lived in milder climatic conditions.

In the process of sedimentation of layer 6c2 from the site, which contains Neanderthal material culture, the in situ geological context suggests dry steppe communities were widespread. This is accompanied by the absence of tundra components in the palynological and paleontological complexes of layer 6c2 (Rudaya et al., 2017; Kolobova et al., 2020a).

Recent studies of lithic assemblages and bone tools in the Chagyrskaya Cave based on attribute, statistical, and geometric morphometric analyses show that the late Neanderthals of the Altai produced exactly the same tools, and had a similar composition of the industry, as the Micoquian cultures of Central/Eastern Europe and Crimea (Baumann et al., 2020; Kolobova et al., 2020a). Despite several thousand kilometers separating these regions, the similarity in tool technologies suggests that the Altai Neanderthals derived from the central Micoquian region, with little innovation or change to their traditions (Chabai et al., 2008; Delagnes and Rendu, 2011; Kozłowski, 2014). This idea is also supported by the fact that during the late Neanderthal occupation in the Altai, there is no evidence for the adoption of new technological or adaptive ideas from the Denisovans. Instead, there are significant differences between the Denisovan and Neanderthal complexes in the Altai (Kolobova et al., 2020a). The aforementioned evidence suggests that the Altai Neanderthals were conservative, having brought and continued using technologies and subsistence strategies already known to them. Given this tendency for similarity in technologies, we explored the dietary evidence from the Chagyrskaya individuals and compared them with what is known about Neanderthal populations that lived in Europe.

Carbon and nitrogen stable isotope analysis on bone collagen extracted from Chagyrskaya 6 allows us to quantify the average long-term protein diet of this individual. Its diet was based on  $\text{C}_3$  terrestrial resources, mainly animal protein, suggesting this Neanderthal was a successful predator and that meat was an important component of this individual's diet. This analysis relies on the comparison of its isotope values to those of the fauna from the site. If the individual came from another environment, the sampled animals from the site would not provide the correct baseline. There is no archaeological evidence for this interpretation, however. The most parsimonious explanation is that the Neanderthal isotopic values represent its trophic relationship with the fauna in the site. The animals most likely consumed were horse and bison, which is in agreement with the results of zooarchaeological analysis





**Figure 4.** Recovered plant microremains: a) type 2 starch in brightfield from Chagyrskaya 6; b) the same starch in cross polarization; c) type 2 starch in brightfield from Chagyrskaya 14; d) the same starch in cross polarization; e) type 7 starch in brightfield from Chag 14; f) the same starch in cross polarization; g) broken phytolith in Chag 14 brightfield; h) charcoal particle in brightfield from Chag 4; i) unidentified fibrous material, possibly fused raphides in brightfield in Chag 14; j) same particle in cross polarization; k) mammalian hair fragment with brightfield in Chag 14; l) unspecified insect fragment with brightfield in Chag 14; m) unidentified fragment with brightfield in Chag 14; n) curved sinuate elongate phytolith with brightfield from *Bison priscus* (N9) in brightfield; o) elongate thick parallelepipedal phytolith with brightfield from *Bison priscus* (N9). Scale bars = 20  $\mu\text{m}$ .

(Kolobova et al., 2020), and there is no isotopic evidence for any aquatic protein consumption. However, while the low  $\delta^{13}\text{C}$  values (and the very large distance from any marine protein sources) exclude the consumption of marine protein, it is harder to assess the potential consumption of freshwater protein sources. Freshwater foods have a very wide range of isotopic values, which may overlap with other types of resources, and thus freshwater protein consumption cannot be clearly excluded using stable isotope analyses and thus could partly explain the high  $\delta^{15}\text{N}$  values observed. However, there is no archaeological evidence for the consumption

of freshwater resources at Chagyrskaya. The only other Siberian Neanderthal with published isotopic values, from Okladnikov Cave (Krause et al., 2007), has almost identical  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values to Chagyrskaya 6 but, unfortunately, there was no analyzed associated fauna.

A comparison of the carbon and nitrogen isotopic values from Chagyrskaya and Okladnikov to the ‘European’ Neanderthals suggests an overall degree of similarity (Fig. 5). Care must be taken in the interpretation of such data, however. Given that Neanderthals spread widely across Eurasia, comparing Neanderthal

**Table 2**

List of all microremain results from each dental calculus sample.

Microremain type	Chag 12 (Nea)	Chag 14 (Nea)	Chag 17 (Nea)	Chag 20 (Nea)	N.16 (Nea)	N.17 (Nea)	N.18 (Nea)	N.19 (Nea)	N.20 (Nea)	N.6 (Bpr)	N.8 (Bpr)	N.9 (Bpr)	N.11 (Bpr)	N.12 (Bpr)	N.13 (Bpr)	N.14 (Bpr)	N.15 (Oam)
Starches <sup>a</sup>																	
Type 1				7				1									
Type 2						1											
Type 3								1									
Type 4		1		2			1										
Type 5	1																
Type 6		1															
Type 7		1															
Dmg/indeterminate						1	4	2				1					
Possible starch				6			1										
Phytoliths																	
Short-cell rondel						2					1					2	
Short-cell indeterminate	1																
Long-cell elongate				1													
parallelepiped multi-cell																	
Long-cell elongate				2				1				9	1		9	2	1
parallelepiped																	
Hair		1		4								2			1		
Curved elongate				1													
Oblong elongate			1														
Oblong trapezoid			1														
Bulliform blocky				1													
Prickle		1															
Verrucate												1					
Parallelepipedal													1		1		
Sulcate tracheid												1					
Thick blocky			1														
Thin blocky				1													
Jigsaw				1													
Indeterminate				1	1												
Other:																	
Calcium oxalate		1															
Indet. spore/pollen		1															
Mite or other invertebrate				1													
Annular tracheid		1															
Clear chain spore	14	6	5	13													
Birefringence bundle		3															
Spicule																	1
Indeterminate brown sphere	1																
Mammal hair				1													
Blue lump	3																
Mycelium		1															
Human scale	1		1	1													
Charcoal	22	22	26	1													
Cellulose type fiber	17	1	5	14													
Synthetic fiber			1														
Possible blue textile fiber		2															
Possible red textile fiber	1																
Possible textile fiber	1	1															
Other	6	5	1	3													

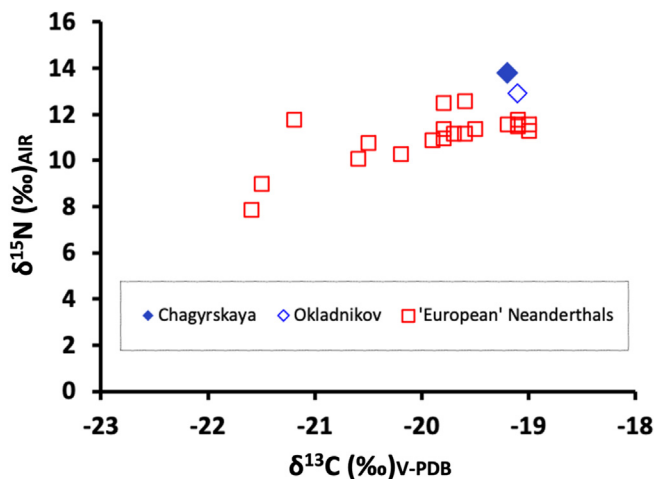
Abbreviations: Nea = Neanderthal; Bpr = *Bison priscus*; Oam = *Ovis ammon*.

<sup>a</sup> Starch types: 1 = small, circular to oval with no clear lamellae visible, simple, cross arms are clear and straight; diameter is 2–14  $\mu\text{m}$ ; 2 = concave-convex, simple, triangular/D-shape, with no clear lamellae; it has a faint cross with straight arms, hilum is marked by a depression but this area is damaged, starch diameter is about 20  $\mu\text{m}$ ; 3 = similar to type 1 but has distinct and fine lamellae, 2–14  $\mu\text{m}$ ; 4 = small simple starch polyhedral indicative of hard endosperm starch of seeds or nuts or grains; some small fissures; starch diameter is about 7–27  $\mu\text{m}$ ; 5 = small simple starch with central cavity, no fissures, weak birefringence cross; starch diameter is about 4–6  $\mu\text{m}$ ; 6 = small, lenticular, pronounced lamellae, no central cavity, no fissures, strong birefringence cross; starch diameter is about 20–28  $\mu\text{m}$ ; 7 = ovoid, faint lamellae, no central cavity, no fissures, strong birefringence cross; starch diameter is about 17–24  $\mu\text{m}$ ; Dmg/indeterminate = forms which were classified as indeterminate types and damaged starch.

absolute values from specimens of different environments and chronologies can be misleading, as background isotopic baselines might differ through time and between areas of study. What must be compared is the individual interpretation inferred from each site's isotopic background. Based on these interpretations, this and most previous isotope studies on Neanderthal specimens portray a quite similar diet, regardless of geographic area and chronology (Fiorenza et al., 2015). Neanderthals usually fall, as is the case for the Chagyrskaya 6 specimen, at the highest level of the food chain when compared with associated fauna from the same site (e.g., Richards et al., 2000, 2008; Bocherens et al., 2005) or to

contemporary Eurasian fauna (e.g., Iacumin et al., 2000; Richards and Hedges, 2003).

This high position in the food chain implies a high intake of animal proteins that is usually interpreted as indicative of high activity levels among Neanderthals, and supporting the view that they were sophisticated hunters and successful predators (Richards, 2002; Richards and Schmitz, 2008; Richards and Trinkaus, 2009). While other alternative hypotheses, such as intense nutritional stress (Doi et al., 2017) or the consumption of fermented resources (Speth, 2017), have been put forward to explain the high  $\delta^{15}\text{N}$  values observed among Neanderthals,



**Figure 5.** Plot of Neanderthal bone collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values from all noninfantile individuals available to date (values only from clear non-duplicate individuals, data from bibliography cited in the text).

recently published exceptionally high  $\delta^{15}\text{N}$  values in Neanderthal collagen single amino acids confirm the most plausible interpretation for these values is an animal protein-rich diet (Jaouen et al., 2019). This reliance on animal protein is compatible with what is known of modern-day and recent-historical high-latitude foraging societies, who also rely heavily on animal foods, particularly for protein and fat (Binford, 2001; Kirby et al., 2016). This suggests that the Chagyrskaya Neanderthals, who lived in the harsh climatic conditions of Siberia but used a technology similar to European Neanderthals, likely also had similar adaptive strategies that resulted in a similar trophic dietary ecology.

Our study of the microremains indicates that several individuals from Chagyrskaya, including the one for which C and N stable isotope analysis were performed, consumed a number of different types of plant foods. The phytoliths present in the Neanderthal calculus samples partly derive from grasses and include a multicell grass phytolith, which is rare in dental calculus and prone to breakdown in reworked sediments (Power et al., 2015; Hardy et al., 2018). Grass phytoliths could enter the mouth through the consumption of stomach contents of prey (Buck and Stringer, 2014), or be linked to the use of grass bedding by Neanderthals (Cabanes et al., 2010), or result from grass being held in the mouth during craftwork or food processing (Radini et al., 2017). They could also have entered the mouth on other food items such as tree bark, which is known to be a dietary staple among high-latitude foragers (Albert and Weiner, 2001; Sandgathe and Hayden, 2003).

The microremains also provide evidence for the consumption of several different kinds of starchy plants. Even in relatively open and cold environments, nutritious plants were seasonally available, such as the starch-rich, lipid- and protein-dense legume Siberian peashrub, *Caragana arborescens* (Shortt and Vamossi 2012). The nutritional contribution of plants in the Neanderthal diet at Chagyrskaya is unclear, in part because many plant foods do not produce microremains, but the use of plants may have assisted with optimal nutrition for human development (Eaton et al., 2010; Butterworth et al., 2016). As only a few starch types were recovered, a limited range of plant species may have been consumed. This might relate to the fact that the landscapes around Chagyrskaya Cave were dominated by steppe vegetation even though the cave itself lays at a mountain-plain-forest intersect. Although steppe environments

support a range of energy-rich plants, they tend to be highly dispersed (Khasbagan, 2000). Furthermore, the steppe environments' extreme seasonality would have resulted in limited plant food availability compared with more temperate regions. This pattern of plant use is compatible with what is known of cold-dwelling foraging societies, which ubiquitously used plants as sources of nutrition when available (Binford, 2001; Kirby et al., 2016).

Analysis of dental wear and dental calculus has already verified that some Neanderthals consumed plants in the more temperate western and southern areas of their range (Henry et al., 2011; El Zaatari et al., 2011, 2016; Salazar-García et al., 2013; Fiorenza, 2015; Estalrich et al., 2017; Power et al., 2018). The identified microremains at Chagyrskaya indicate that, even at the easternmost limit of their range, and in the most continental environment occupied, Neanderthals may have retained elements of a diverse dietary strategy that incorporated plant foods into their diet.

Our results from isotopic and microremain analyses may at first seem contradictory, as the former indicates a high reliance on animal proteins and the latter records the use of a variety of plant foods. Given the kinds of data each kind of method produces, however, there is no conflict. Evidence that points to the animal origin of most of the protein consumed does not preclude the consumption of plants, even on a regular basis, as plant foods have a much lower proportion of protein than animal meat. It has been recognized that even moderate quantities of meat in the diet could mask significant plant consumption, since the quantity of plant versus meat reflected in the  $\delta^{15}\text{N}$  values is nonlinear—some calculations even suggesting that the difference in  $\delta^{15}\text{N}$  values between a 100% meat diet and a diet with a 50:50 meat to plant ratio would be roughly 0.5‰, making it difficult to distinguish the proportion of meat consumed over that threshold (Bocherens, 2009). Isotopic and microremains analyses are complementary but cannot provide information about the exact proportions of plant and animal foods consumed, nor can they identify all of the sources of foods. Instead of contradictory, these methods are in fact complementary. Isotope analysis on bulk collagen provides a long-term average signal of the protein sources, while microremains on dental calculus can record the presence of particular plant food items. Using both of these methods on the same individual provides a more comprehensive view of the diet than either does alone.

Our results do allow us to state that the Chagyrskaya Neanderthals ate a variety of plant and animal foods, and were neither vegetarians nor strict carnivores. This mixed subsistence with a high reliance on animal protein likely represents a compromise between biology and environment. It is likely that Neanderthals had a similar physiological protein ceiling to that of humans, so that no more than 35–40% of their calories could come from protein. However, the steppic environments in which the Chagyrskaya Neanderthals lived would have been, at least seasonally, depauperate in plant foods. Neither of our methods provides information about the fat component of diet, but given analogies to modern and recent-historical northern foraging groups, animal fats (e.g., brains, viscera, subcutaneous fat, marrow) likely made up a large proportion of their diet, especially in winter.

What is most remarkable about our findings, however, is that despite the Chagyrskaya individuals living on the far eastern edge of the known Neanderthal range, their dietary suite is largely indistinguishable from those of western Neanderthals when considering broad categories of food types and macronutrients. While the specific plants and animals that were consumed likely varied among habitats and potentially through time, the



Neanderthal niche seems to have involved a high reliance on animal proteins and the consumption of a variety of plant foods to meet their nutritional requirements.

## 5. Conclusions

Isotopic data from bone collagen indicate that the studied Neanderthal from the Altai region relied on animal sources for most of its dietary protein intake. Plant microremains retrieved from dental calculus indicate that plants were also exploited, probably either for micronutrients or as a seasonal minor source of energy. The ecological niche that is apparent at Chagyrskaya is shared with all previously studied Neanderthals, most of them living thousands of kilometers away in western Eurasia. This reoccurring picture of Neanderthal subsistence perhaps broadly reflects the many ecological similarities in the regions they colonized in western Eurasia and the Altai in eastern Eurasia.

## Declaration of competing interest

The authors report no conflict of interest.

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## Supplementary Online Material

Supplementary Online Material to this article can be found online at <https://doi.org/10.1016/j.jhevol.2021.102985>.

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